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Preparation of Low Density Lipoprotein-9–Methoxy-ellipticin Complex and its Cytotoxic Effect Against L1210 and P 388 Leukemic Cells *In Vitro*

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Abstract—*Previous studies have suggested that low density lipoprotein (LDL) may be used as a drug targeting carrier for chemotherapeutic agents to neoplastic cells. In this study the cytotoxic agent 9-methoxy-ellipticin (MeOE) was incorporated into dimirystoyl phosphatidylcholine, cholesteryl oleate stabilized microemulsion and the latter fused with human LDL. Both agarose electrophoresis migration and the electron microscopic shape of the drug–LDL complexes were similar to those of native LDL. The* in vitro *cytotoxic tests on L1210 and P388 leukemic cells demonstrated that the complex was able to kill cells and was more effective than the free drug. This cytotoxic activity of the drug–LDL complex depends on the LDL high affinity receptor: the native LDL reduces the killing power. In contrast, methylated LDL, which does not bind to the LDL receptor, has no effect on it. On the other hand, heparin, which prevents binding on the cell surface receptors, partially reduced the cytotoxic activity of the drug–lipoprotein complex. These results suggest that it is possible to incorporate lipophilic cytotoxic drugs into LDL, using a technique of fusion with the microemulsion which contains the drug. This technique allows us to obtain a drug–LDL complex which is able to kill cells via the LDL receptor pathway.*

INTRODUCTION

THE MAIN PROBLEM in cancer chemotherapy is the inability of the cytotoxic drug to discriminate between normal and neoplastic cells. Therefore, toxic effects on normal cells remain a major problem in the treatment of neoplastic disease. A possible way of reducing the undesired effects of antincoplastic drugs would be to link a potent anticancer agent to a carrier with a high affinity for the malignant cells.

There is evidence to show that LDL could be used as a carrier of cytotoxic drugs [1, 2]. The LDL particles consist of an apolar core of neutral lipids (cholesteryl esters, triglycerides) surrounded by phospholipid monolayers containing free cholesterol and apolipoprotein B (Apo B) [3]. Its structure allows substantial quantities of a lipophilic drug to be stored inside it. Several authors have shown that it was possible to incorporate cytotoxic drug derivatives in LDL with different techniques [4–7]. The complexes formed in this way were able to interact with the LDL high affinity receptors [5] and had an *in vivo* fate similar to that of native LDL [8]. Several neoplastic cells, like normal human cells, may take up the LDL by a specific high affinity receptor for Apo B on the cell membrane [9, 10]. After binding itself to a receptor, LDL is internalized and degraded in the lysosomes with the subsequent release of cholesterol and amino acids [11]. In the case of the drug–LDL complex, the drug would be released through the same pathway.

However, the principal reason for using LDL as a cytotoxic drug targeting carrier is that tumor cells could have a higher receptor activity than normal cells. This is supported by studies done by Gal *et al.* [9] on human uterine tumor cells and by Ho *et al.* [10] on freshly isolated human leukemic cells. This property seems to be linked to cellular kinetics and proliferation. Moreover, these *in vitro* tests, showing an elevated receptor activity of tumor cells, have been supported by studies on the *in vivo* behavior of LDL in mice with soft tissue tumors [12, 13].

In the present study, we report a technique to prepare a LDL–ellipticin derivative complex and to study its efficiency on the cytotoxicity of cancer cell lines (L1210 and P388) *in vitro* compared to that of the free drug. The antitumor action of

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ellipticin and its derivatives in animals has been reported by several authors [14, 15]. In human oncology, methoxy-ellipticin has been used successfully in the treatment of acute myeloplastic leukemia [16] and hydroxy-9-methyl-2-ellipticinium in advanced cancer, especially in breast cancer [17].

MATERIALS AND METHODS

Materials

Cholesteryl-oleate and dimirystoyl phosphatidylcholine (DMPC) were purchased from Sigma and were judged 99% pure and used without further purification; 9-methoxy-ellipticin (MeOE) was kindly provided by Sanofi Group (France).

Millex 0.45 nm filters were from Millipore S.A., methanol was chromatographic grade (Merck), Ammonium hexafluorophosphate, ammonium acetate and glacial acetic acid were obtained from Merck. [^{3}H]Leucine (63 Ci/nmol) was obtained from Amersham France (Les Ulis, France).

Fetal calf serum (FCS), RPMI 1640, phosphate buffer saline (PBS), penicillin, streptomycin and glutamine were obtained from Intermed (Strasbourg, Francc). T75 flasks, 1.9 cm^2 multiwell dishes and culture tubes were from Nunc Laboratories, U.S.A.

L1210 and P388 murine leukemic cells were obtained from the National Cancer Institute (Bethesda, MD, U.S.A.) and kept by Laboratoire de Pharmacologie et Toxicologie Fondamentale (Toulouse, France).

LDL and LPDS preparation

Lipoproteins were obtained from the plasma of healthy fasted normolipidemic volunteers by ultracentrifugation flotation [18]. The LDL were isolated over the density range of 1.030 g/ml. Lipoprotein purity was assayed by 1% agarose gel electrophoresis and by immunochemical analysis. LDL migrated as a single band on agarose and did not react with antibodies raised against albumin or with anti-Apo AI and anti-Apo AII. Positive reactions were obtained with anti-Apo B. After extensive dialysis against LDL buffer (Tris–HCl 5 mM, EDTA 0.3 mM, NaCl 9 g/l, pH = 7.4) LDL were sterilized by filtration through 0.45 nm filter and then stored at 4°C for no more than 3 weeks. LDL was quantitated as protein by the assay of Lowry *et al.* [19] using bovine serum albumin as standard. LPDS of $d > 1.25$ g/ml was prepared by ultracentrifugation flotation. LDL were methylated as described by Weisgraber *et al.* [20].

Fusion of LDL and synthetic microemulsion containing 9-methoxy-ellipticin

The preparation of the microemulsion containing MeOE–cholesteryl oleate–DMPC was according to Parks *et al.* [22] and Craig *et al.* [23] with a few modifications concerning the molar proportion of each component: 2 mg of both DMPC and MeOE were added to 1 mg cholesteryl oleate in chloroform. The solvent was evaporated and the residue was dissolved in 200 µl of dry isopropanol and injected into 3 ml of rapidly vortexing phosphate buffer with the entire system kept at 46°C throughout the procedure. In order to incorporate MeOE into LDL, 5–6 mg LDL (2 mg/ml) were incubated with equal volume of MeOE containing microemulsion in LDL buffer for 5 h at 37°C according to Parks *et al.* [22]. The density of mixture was adjusted to 1.019 g/ml by the addition of solid KBr. LDL were then separated from microemulsion by overnight ultracentrifugation at 35,000 rpm at 4°C. Microemulsion and density solution were aspirated and the infranatant was then subjected to extensive dialysis for 48 h against LDL buffer to remove KBr and sterilized by filtration through a 0.45 nm Millipore filter.

High-performance liquid chromatographic studies of the mixture before and after the incubation showed that 9-MeOE was stable under these conditions.

HPLC assay of MeOE

An aliquot (200 µl) of LDL-drug complex and native LDL were spiked with 25 µl of N^2-propyl-9-hydroxy-ellipticinium (9-OH-NPE) as internal standard (final concentration 1.25 µg/ml). All the drug and internal standard were extracted twice with ethylacetate (2 × 1 ml) after the addition of 100 µl of ammonium hexafluorophosphate (final concentration 250 mM) as counter-ions and (2 × 3 ml) acetate buffer 0.5 M, pH = 5.5.

The mixture was centrifuged twice at 2000 rpm for 10 min. The organic phase was aspirated and dried under a stream of nitrogen. The residue was redissolved in 200 µl of mobile phase (methanol–water 70:30 with 25 mmol/l, ammonium acetate adjusted to pH = 5 with glacial acetic acid) and chromatographed according to Bellon *et al.* [21].

Electron microscopy

Negative stain electron microscopy was performed with 2% potassium phosphate tungstate pH = 6.5. LDL, microemulsion and drug–LDL complexes were dialyzed against 0.01% EDTA, 0.02% NaN_3, pH = 7.4, before negative staining. Diluted samples were concentrated directly onto the grill by evaporation under a stream of nitrogen.

Cells

L1210 and P388 murine leukemic cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM glutamine, 10 IU/ml streptomycin and 100 IU/ml penicillin.

The cells were maintained in a humidified incubator (5% CO_2) at 37°C. The cells were usually grown in T75 flasks and the experiments were performed in culture tubes or in 1.9 cm multiwell dishes.

ID_{50} calculation assay

Experiments were carried out by harvesting cells in the exponential growth phase, plating out into the multiwell dishes at a cell density of 5×10^4 cells/ml. The drug or the drug–LDL complex was added at each concentration in triplicate dishes. Cells were sampled after 48 h and counted (three counts/well), after dilution, in a coulter counter (Coultronic Model Z.M). Six wells in each experiment were left free of drug as a control. The mean of the cell count value from the control well was taken to represent 100% of cell growth and the counts of the other wells were expressed relative to this result. A plot of percentage cell growth against log (concentration M) was constructed for each of the drug and drug–LDL complexes studied. From these curves, the dose which corresponds to 50% inhibition of cell growth was calculated for each drug.

Receptor dependent cytotoxicity assay

Cells were incubated for 48 h in LPDS (5 mg/ml) supplemented by RPMI medium prior to the addition of the drug or the drug–LDl complexes. The cells were washed once and seeded at a final cell density of 5×10^5 cells/ml in LPDS supplemented medium in culture tubes. Dilutions of the drug or the drug–LDL complex (whether native or methylated LDL had been added or not) were administered to the cells for the period indicated at 37 or 4°C. Following the exposure, the cells were washed twice with warm (37°C) or cold (4°C) phosphate buffered saline (PBS) and resuspended at a cell density of 5×10^4 cells/ml in triplicate in 24 multiwell dishes in 10% FCS supplemented RPMI medium. They were then allowed to recover for 48 h prior to cell counting or addition of [^{3}H]leucine. Six culture tubes in each experiment were left free of the drug as a control and were taken to represent 100% of cell growth or 100% of [^{3}H]leucine cellular uptake. The values for the other cells were expressed relative to these results.

[^{3}H]*Leucine cellular uptake assay*

The cells were exposed to the tracer for 6 h then harvested and washed on a glass fiber filtering and harvesting device (Nunc Laboratories). The filter radioactivity was measured by scintillation counting (Tricarb 360 CD, Packard). An extra plate of cells was seeded on day 0 for use in monitoring cell growth. At intervals during the experiment several cells were counted with the aim of verifying that the cells were in an exponential growth phase during the assay, an essential prerequisite for valid protein synthesis measurement.

RESULTS

MeOE–LDL characterization

MeOE–LDL complexes are obtained by incubation of LDL with a microemulsion containing MeOE. The proportion of drug and LDL protein in several preparations of MeOE–LDL by this method is variable. One to 2.5 μg of the drug are incorporated per mg of LDL protein corresponding to five to 10 molecules per LDL particle. After precipitation of MeOE–LDL by $MnCl_2$, more than 95% of MeOE is precipitated with LDL (precipitation is performed according to Pattnaik *et al.* [24] using [^{125}I] LDL as a control of precipitation). The recovery of the drug in the final preparation is about 2.5%.

MeOE–LDL is characterized by agarose gel electrophoresis. The results of a typical experiment are shown in Fig. 1. Native LDL and MeOE–LDL complexes have a typical mobility (lanes A and B). In contrast, incubation of microemulsion with LDL (5 h at 37°C) results in a diffusion band which is intermediate in mobility between native LDL and microemulsion (lane D). Microemulsion (lane C) has a slight migration out of the well towards the cathode.

An electron microscopic study was undertaken to obtain further information about size, shape and homogeneity of the microemulsion population and also the LDL and MeOE–LDL complexes. Figure 2 is a composite of the micrographs obtained for the different fractions. When incubation of microemulsion and LDL mixture (panel C) was viewed by electron microscopy, particles showed excess surface in the form of a flap. However, when LDL–MeOE complexes were viewed (panel B), after separation of unreacted microemulsion by ultracentrifugation, the fusion product was spherical with some angular shaped particles similar to native LDL (panel A).

Cytotoxic activity of MeOE–LDL complexes

The ability of the MeOE–LDL complexes, prepared by the microemulsion method described above, to kill cells was studied by a cell growth assay with L1210 and P388 murine leukemic cells. The results are summarized in Fig. 3. LDL–MeOE complexes retain biological activity and are more effective on the cell growth inhibition than the free drug. The ID_{50} of the drug falls from 7×10^{-7} to 8×10^{-8} M and 4×10^{-7} M to 2.5×10^{-8} M for L1210 and P388 cells respectively, when transported within LDL, illustrating the ability of the lipoprotein to potentiate the action of this cytotoxic drug. Control experiments which included native

Table 1. *L1210 cytotoxic assay with LDL–9MeOE complexes compared to free 9MeOE tested by* [^{3}H]*leucine uptake on cultured cells grown in* 5% *LDPS supplemented medium during drug infusion*

Preparation	Concentration LDL (μg/ml)	Concentration MeOE (M)	Percentage [^{3}H]leucine cellular uptake 5 h	Percentage [^{3}H]leucine cellular uptake 24 h
Native LDL	75	0	100	98
Native LDL + MeOE	75	10^{-8}	100	95
	75	5×10^{-8}	100	80
MeOE	0	10^{-8}	104	97
	0	5×10^{-8}	102	85
LDL–MeOE	15	10^{-8}	65	30
	75	5×10^{-8}	10	0
MeOE + hep (3 mg/ml)	0	10^{-8}	102	100
	0	5×10^{-8}	100	83
LDL–MeOE + hep (3 mg/ml)	15	10^{-8}	98	78
	75	5×10^{-8}	30	15

Cells were preincubated in 5% LPDS supplemented medium prior addition of the cytotoxic compounds. Experiments and cytotoxicity assay, determined by [^{3}H]leucine uptake, were performed as described under Materials and Methods. Each value is the mean of triplicate incubations in two experiments. The results are expressed as indicated in Materials and Methods.

Table 2. *L1210 cytotoxic assay with LDL–9MeOE complexes compared to free MeOE tested on cultured cells preincubated or not for* 48 h *in LPDS supplemented medium*

	MeOE (M)	LDL (μg)	Preincubation medium FCS	LPDS	LPDS + native LDL (200 μg)
			Percentage cell growth		
MeOE	10^{-7}	—	100	100	102
LDL–MeOE	10^{-7}	20	76	46	57

Experiments were performed as described under Materials and Methods. The drug or the drug–LDL complexes were incubated for 4 h at 37°C. Each value is the mean of triplicate incubations. The results are expressed as described under Materials and Methods.

LDL, in the same LDL protein concentration range, had no effect on the cell growth.

The MeOE–LDL complex, after cell incubation for 4 h at 37°C, has a greater cytotoxic effect on L1210 cells when these cells are preincubated for 48 h in LPDS medium than when they are not preincubated in LPDS medium. However, in both of these conditions, the complex is more efficient than the free drug. The addition of native LDL during preincubation does not lead to a large reduction in the complex killing power (Table 2).

In order to prove that native LDL reduces the cytotoxic effect of the drug–LDL complex, experiments were carried out for 2 h at 4°C, a temperature which only allows the binding of LDL. When we add increasing quantities of LDL to LPDS supplemented medium which contains the MeOE–LDL complex (20 μg of LDL protein) (Fig. 4), the cytotoxic effect is continuously reduced and reaches about 100% level of cell growth. In the same condition, increasing quantities of methylated LDL, which are unable to bind themselves to the LDL receptor, have no effect on the drug–LDL complex cytotoxicity. Likewise, the free drug does not show, at the same drug concentration as the drug LDL complex, any cytotoxicity.

Finally (Table 1), we tested the action of the drug–LDL complex, by a [^{3}H]leucine cellular uptake assay on L1210 previously incubated for 48 h in LPDS supplemented medium.

When a concentration of 10^{-8} M MeOE–LDL complex is incubated for 5 h, only 65% [^{3}H]leucine was taken up, while for 24 h this uptake is 30%. When the concentration of cytotoxic MeOE–LDL complex is increased, the toxic effect is enhanced; with an incubation for 24 h at a concentration of 5×10^{-8} M, no proteosynthesis is observed. In contrast, the same concentration of free drug presents a high percentage of [^{3}H]leucine uptake (85% with MeOE 5×10^{-8} M during 24 h of incubation). Moreover, LDL coincubated with the cells in the presence of free MeOE exhibits a similar

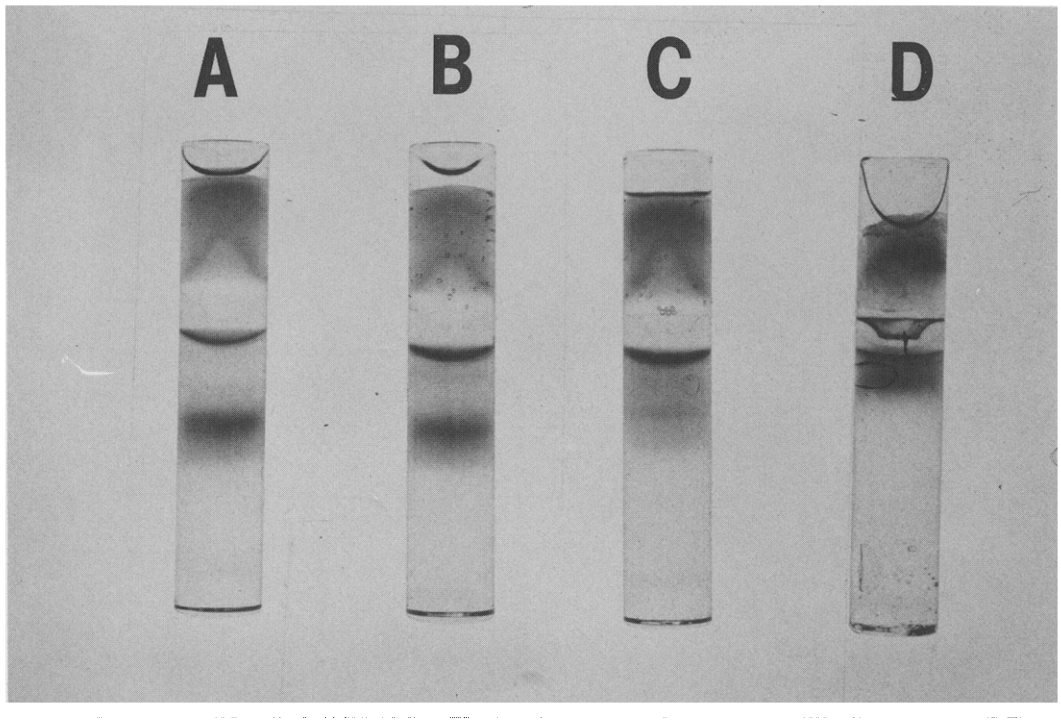

Fig. 1. Agarose gel electrophoresis of native LDL (A), LDL–MeOE complexes (B), MeOE containing microemulsion (C), LDL–MeOE containing microemulsion mixture (D).

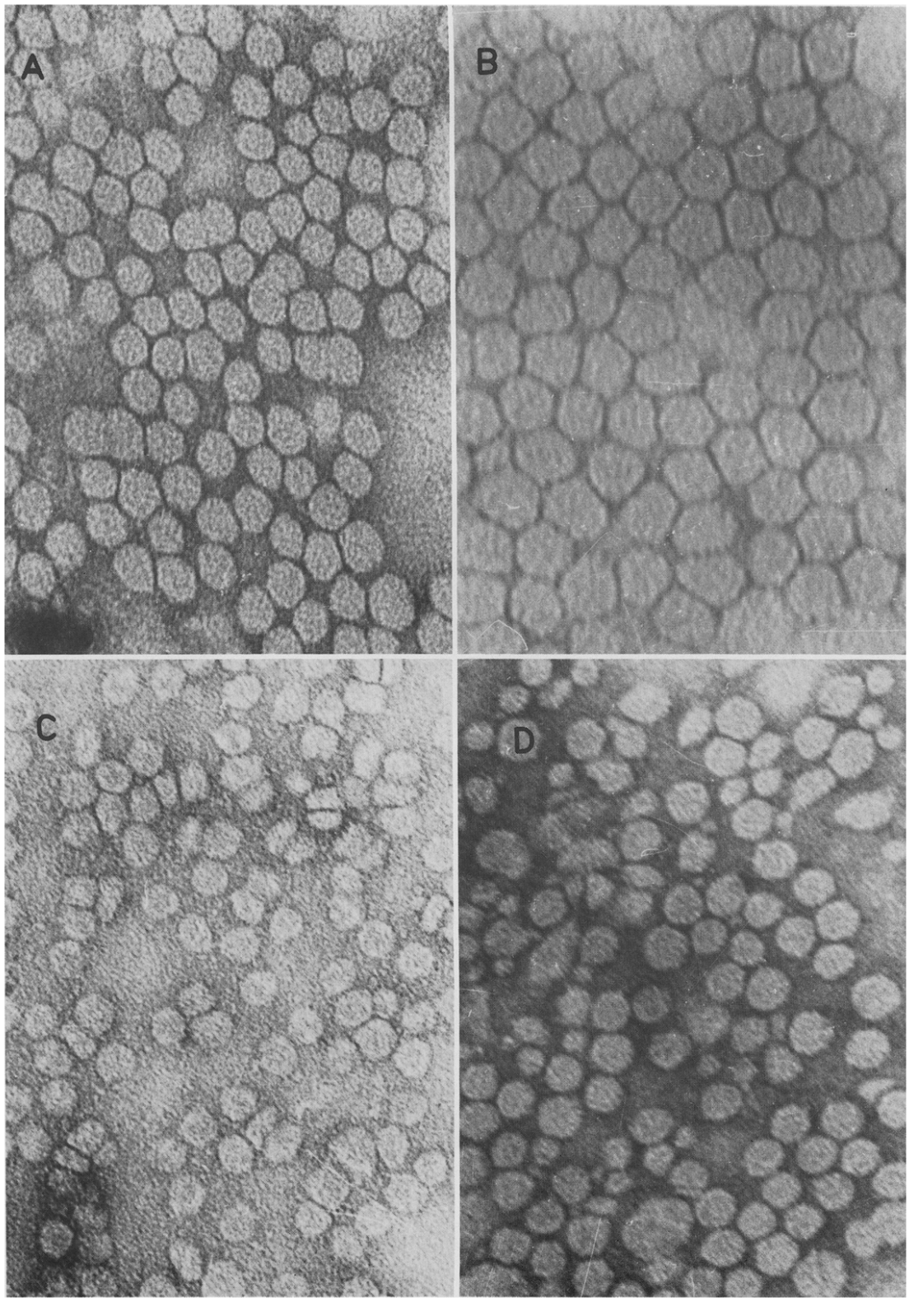

Fig. 2. Negative stain electron micrographs of native LDL (A), LDL–9MeOE complexes (B), MeOE containing microemulsion (C), LDL–9MeOE containing microemulsion mixture (D). Electron microscopy was performed as indicated in Materials and Methods.

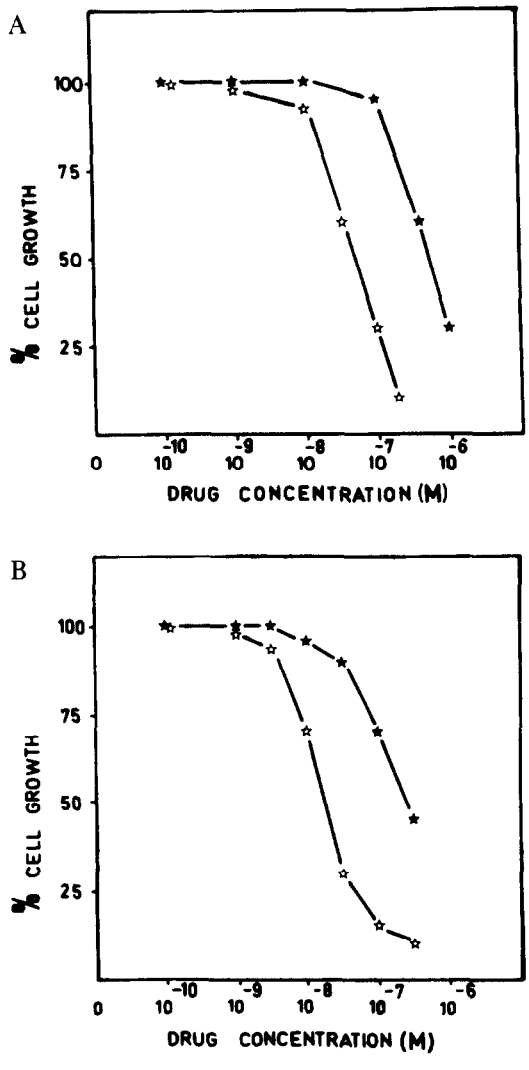

Fig. 3. L1210 (A) and P388 (B) cell growth inhibition assay with LDL–9MeOE complexes compared to free 9MeOE tested on cultured cells grown in 10% FCS supplemented medium. L1210 and P388 cells (5 × 10⁴ cells/ml) were incubated with various concentration of LDL–9MeOE complexes (open stars) and 9MeOE free cytotoxic drug (shaded stars). Cells were sampled after 48 h of drug infusion and counted with a coulter counter. Each point is the mean of triplicate dishes in three experiments. The results are expressed as indicated in Materials and Methods.

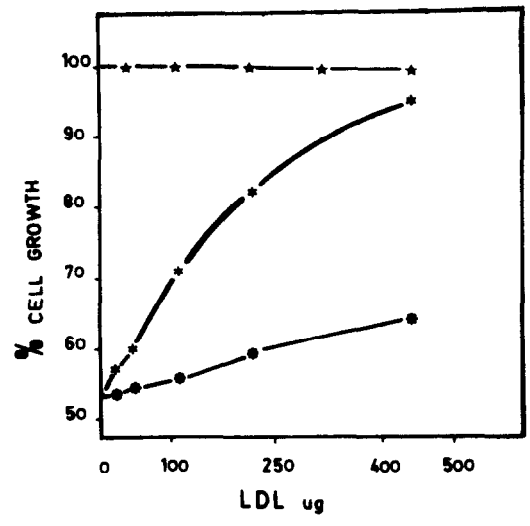

Fig. 4. Cytotoxic effect of the drug–LDL complex with addition of increasing quantities of native LDL (asterisks) or methylated LDL (bold asterisks) on L1210. LDL + MeOE alone, with no drug–LDL complex (stars). Experiments were carried out for 2 h at 4°C, see Materials and Methods.

percentage of [³H]leucine uptake to that of the free drug. When heparin (3 mg/ml) is added to the incubation medium, the toxic effect of MeOE–LDL complex is greatly reduced for a MeOE concentration of 10^{-8} M. However, for higher MeOE–LDL concentration (5×10^{-8} M), a toxic effect is observed.

DISCUSSION

The present study describes the successful incorporation of the cytotoxic compound MeOE into LDL and the evaluation of the biological activity of the resulting complex on L1210 and P388 murine leukemic cells *in vitro*.

MeOE was incorporated into the lipoprotein by fusion of LDL with drug–lipid microemulsion. The critical step in this drug incorporation procedure was the preparation of the MeOE containing microemulsion. MeOE strongly interacts with negatively charged phospholipids [25] so it is necessary to use DMPC (a neutral phospholipid) for the synthesis of the microemulsion. In this condition, MeOE penetrates deeper into the lipid core of microemulsion as indicated by Terce *et al.* [26] and can even be fused with the LDL lipophilic core.

This procedure for preparing LDL–MeOE complexes should be taken as a general method which could be applied to other ellipticin derivatives (or other cytotoxic drugs) providing that they are apolar compounds. In contrast, hydroxy-9-methyl-2-ellipticinium (9 NMHE) (a dipolar ellipticin derivative) leads to immediate aggregation with phospholipid and cholesterol ester in the LDL buffer.

This procedure for partitioning drugs within LDL was successfully used by Hynds *et al.* [27]. They incorporated cholesterol into LDL and presented the biological activity of the resulting complex against glioma cells *in vitro* and Walker's tumor implanted in rats *in vivo*.

Other reconstitution methods have been described:

1. The simple fusion of a drug with LDL [5]; however, 35–45% of the drug is accessible to the LDL–drug complexes and may be able to stimulate the immune system to remove drug–LDL complexes from the circulation.
2. Covalent attachment to the apoprotein moiety of the LDL particles [6]; however, there are immediately obvious drawbacks, particularly

because drug attachment to Apo B may modify the protein structure and reduce its binding to the receptor. In fact, the methotrexate–LDL covalent complexes, synthesized by Halbert *et al.* [6], when tested against L1210 cells *in vitro*, were less active than free methotrexate.

3. The Krieger method [28] has been shown to insert significant amounts of exogenous drug into lipoproteins. But 'Krieger particles' are rapidly removed from the circulation by cells of the reticuloendothelial system *in vivo*. Masquelier *et al.* [8] have still managed, by modifying the procedure, to obtain LDL–anthracycline (AD32) complexes which are removed from mouse plasma at a rate similar to that of native human LDL.
4. The reconstitution of LDL by the addition of an Apo B preparation to a sonicated microemulsion containing cytotoxic agents [7]. The *in vivo* fate of this reconstituted LDL remains unknown and as with 'Krieger' particles, they may be removed from the circulation by the reticuloendothelial system.

The LDL–MeOE complexes prepared by a fusion technique, when tested against L1210 and P388 murine leukemic cells *in vitro*, are more active than the free drug. The ID_{50} of the drug tested by cell growth inhibition was lowered by a factor of 10 and 16 for L1210 and P388 cells respectively. The ID_{50} of the LDL–MeOE complexes reached that obtained with the 9-NMHE (Celiptium), the drug used for human therapy.

There is some evidence to show that the drug–LDL complex kills the L1210 cells exclusively via the LDL receptor mechanism.

When the LDL receptor activity is high, regulated by preincubating the cells with lipoprotein deficient serum, the cytotoxicity obtained is higher than that observed when cells are preincubated in fetal calf serum containing lipoproteins. However, a 10-fold excess of native LDL reduces the killing power of the drug–LDL complex. In these experiments, carried out at 37°C, a drug transfer between the drug–LDL complex and the native LDL could occur, involving the lipid transfer protein contained in the LPDS in the same way as physiological lipids. This mechanism leads to an increase in the drug–LDL complex quantities and limits the research competition between LDL and the drug–LDL complex. On the other hand, the interiorization of LDL could give sufficient quantities of drug–LDL complex for killing the cells.

For the experiments carried out at 4°C, LDL uptake and degradation are inhibited. The cytotoxic effect is dependent on the drug–LDL complexes bound to the cell surface receptor. In this case, the addition of an excess of native LDL but not an excess of methylated LDL, which cannot bound to the LDL receptor, counteracts the killing power of the complex.

The incorporation of drug–LDL complex into cultured L1210 cells is reduced by heparin which is known to selectively release the LDL bound to the cell surface receptor. For a high protein LDL concentration (75 g/ml), the drug–LDL complex seems to be resistant to heparin release. As indicated by Goldstein *et al.* [29], slight interiorization of LDL occurs at 37°C in the presence of heparin. This mechanism could explain the low percentage of [^{3}H]leucine uptake obtained with high MeOE–LDL complex protein concentrations in the presence of heparin.

These data show that it is possible to incorporate cytotoxic drugs into LDL by a fusion technique with a cytotoxic drug containing microemulsion. This drug complex has the advantage being more active than the free drug *in vivo*, this activity is associated with the high affinity receptor.

Studies are in progress to investigate the incorporation of other hydrophobic ellipticin derivative compounds to increase incorporation yields, and the *in vivo* therapeutic effects of the drug LDL complex in a murine leukemia model.

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