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Cell membrane fluidity and adriamycin retention in a tumor progression model of AKR lymphoma

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Abstract

Counteraction of drug resistance is a major challenge in cancer therapy, particularly in advanced stages. The main mechanism of multidrug resistance is related to an increased drug efflux. In the present study we examined the effect of modifying cell membrane lipid fluidity on uptake of adriamycin (ADR) in cells of AKR lymphoma malignancy variants. Modification of cell membrane fluidity, either by lecithin or by lecithin-cholesterol mixtures, induced in a high proportion of cells of all variants a higher capacity to accumulate ADR. The chemosensitizing effect, for lecithin in particular, was proportional to the degree of malignancy of the lymphoma variants. The increased ADR uptake was up to 1.4-fold in the variant of lowest malignancy and up to 5-fold in the one of highest aggressiveness. This tendency correlates with our previous studies and is of particular value since highly-malignant tumors are often drug resistant. The cholesterol-lecithin mixture, induced, however, in part of the variants the appearance of a small subpopulation with very low ADR permeability. Cell membrane rigidification is of value for exposing tumor cell cryptic antigens but may be deleterious when used in conjunction with chemotherapy.

Keywords: Drug resistance counteraction; Cell membrane fluidity; Metastatic potential

1. Introduction

Drug resistance is probably the major mechanism responsible for failure of therapy in advanced cancer. One of the cell properties which can undergo modifications during tumor progression is sensitivity to drugs [1]. The cellular alterations occurring during tumor evolution, including the emergence of drug resistant clones, impose the necessity to test the efficacy of therapeutic means on tumor progression models rather than on static ones [2].

Tumor cells may lose their sensitivity to several drugs simultaneously. The main mechanism responsible for the phenomenon of multidrug resistance (MDR) is an increased drug efflux due to an augmented expression of a membrane protein, the P-glycoprotein (Pgp, a 170 kDa protein) [3] or the more recently described multidrug resistance protein, MRP (a 190 kDa protein) [4]. Other non-Pgp-related mechanisms have been described during the last years [5–7].

Possibilities to counteract drug resistance have been explored, particularly with relation to the Pgp-related mechanism. Hyperthermia [3,8–10], or membrane active agents such as ethanol [11] or tween-80 [12] have been shown to reverse drug resistance by augmenting tumor cell permeability to drugs. Calcium channel blockers, such as verapamil, acting by a different mechanism [13,14], have also been found to be effective in counteracting drug resistance.

Cell surface properties may also undergo changes during tumor progression [15,16]. Presuming that the cell membrane of highly-malignant tumor cells may be more fluid [17], we examined the possibility of affecting cell permeability to drugs by membrane active agents, more efficiently in high- rather than in low-malignancy cells. It is in fact a challenge, to find more efficient therapies against the more problematic highly-malignant cells, which are often resistant to drugs. Along this strategy, we have

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found that this was the case in several tumor progression models, in both murine [10,12] and human systems [18,19] using hyperthermia [10,20], Tween-80 [12] or verapamil [21] as modulators.

In the present study we examined the effect of increasing or decreasing cell membrane fluidity, by modifying its content in lecithin or cholesterol, on the retention of adriamycin in AKR lymphoma cells derived from variants differing in their degree of malignancy. This model of T-cell malignancy is of relevance to human T-cell lymphomas which in most cases are resistant to chemotherapy.

Increasing rigidity of the cell membranes has been used in order to enhance immunogenicity of tumor cells by exposing cryptic antigens [22]. In the present study our aim was the converse, to augment membrane fluidity, in order to facilitate permeability to drugs.

2. Materials and methods

2.1. AKR lymphoma malignancy variants

In the present study, four AKR lymphomas differing in biological behavior (derived from different spontaneous tumors) were used: TAU-39, of low malignancy, TAU-33 and TAU-44, of intermediate malignancy and TAU-42 of high virulence. The TAU-44 variant has a particular predilection for metastasis to certain organs. All four variants were originally derived from different spontaneous lymphomas. The TAU-39 and TAU-33 have previously been isolated and characterized by us [23], while the TAU-44 and TAU-42 have been recently isolated [24].

The lymphoma variants were maintained by subcutaneous inoculation in the back of AKR/J mice. The mice were obtained and bred at the Animal Breeding Center of the Tel-Aviv University.

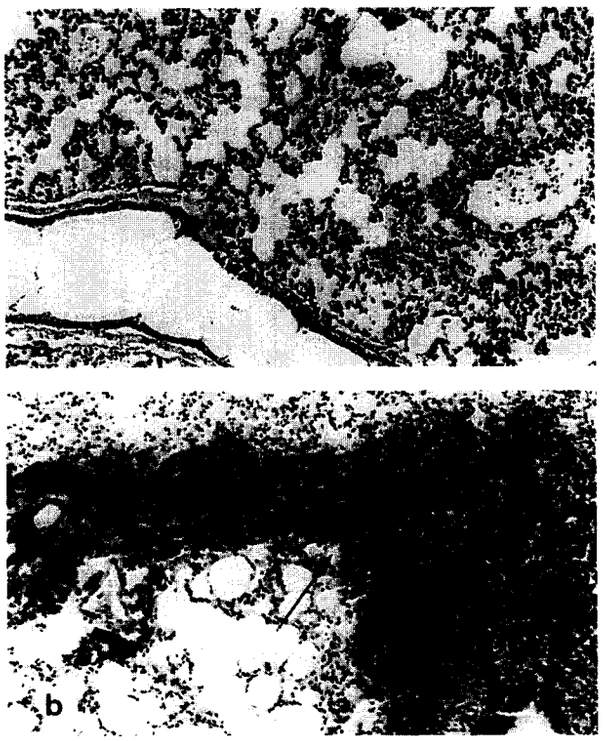

Fig. 1. Comparison of dissemination to lungs of the two AKR lymphoma variants TAU-39 (a) and TAU-42 (b). Identical inocula ($2 \cdot 10^5$ cells) were injected s.c. to AKR/J mice. Metastatic spread to lungs and other (non-presented) organs was followed by histopathological examination after sacrificing mice at the same day after tumor inoculation (day 15). Samples were fixed in formalin, embedded in paraffin and stained by H and E. Magnification: ×100. Note the dense hyperchromatic area representing prominent metastatic growth of the lymphoma seen only in the lungs of the TAU-42 variant-bearing mouse (arrow).

Table 1
Incidence of enlarged inguinal lymph nodes following i.v. inoculation of the AKR lymphoma malignancy variants

Variant	Incidence of metastasis	
	Day 9	Day 14
TAU-39	0/5	1/5
TAU-33	2/5	4/5
TAU-44	4/5	5/5
TAU-42	5/5	5/5

Incidence of experimental metastasis was evaluated in vivo as number of mice having tumoral enlarged lymph nodes per total number of animals.

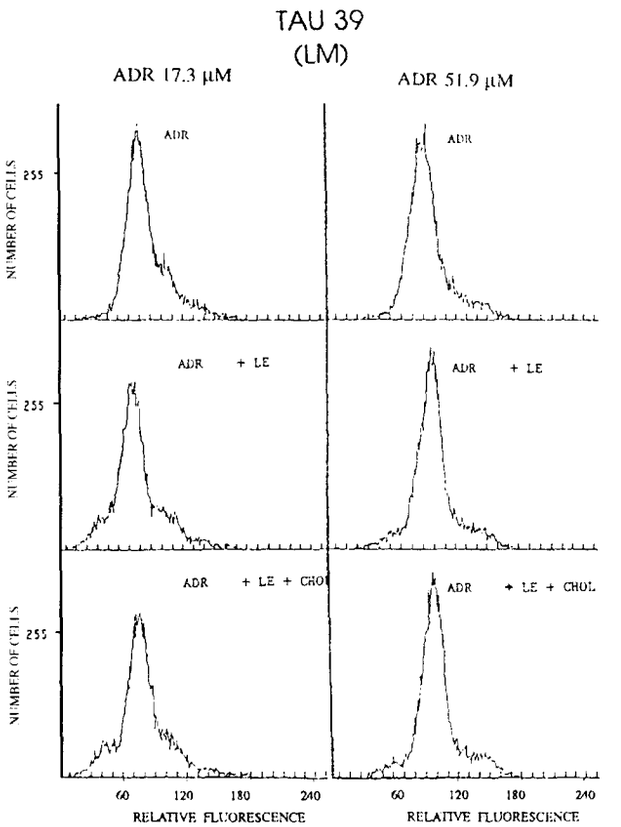

Fig. 2. Effect of treatment with lecithin or lecithin-cholesterol liposomes on ADR uptake of cells derived from the TAU-39 AKR lymphoma variant (low malignancy): FACS analysis profiles.

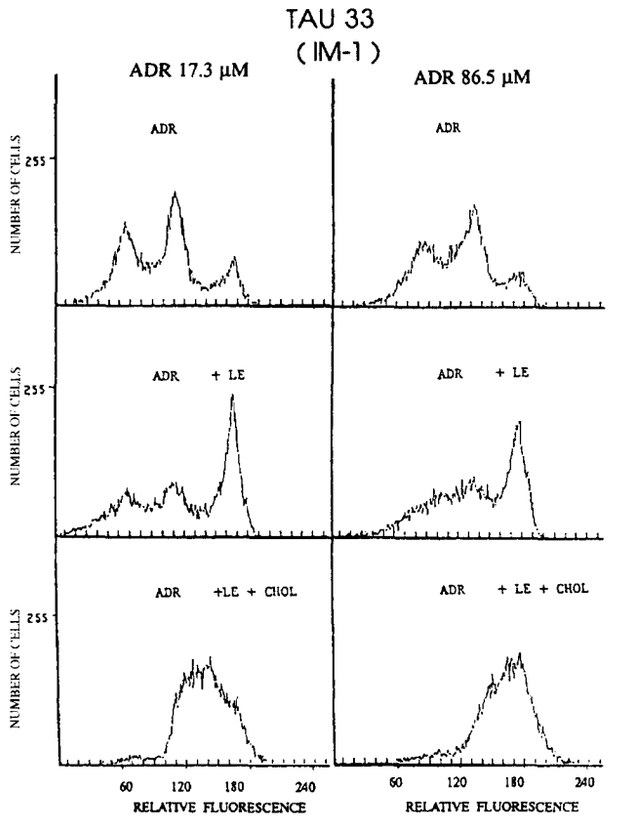

Fig. 3. Effect of treatment with lecithin or lecithin-cholesterol liposomes on ADR uptake of cells derived from the TAU-33 AKR lymphoma variant (intermediate malignancy): FACS analysis profiles.

For the experiments, single cell suspensions were prepared as previously described [2]. In short, tumors were excised and placed in RPMI medium. After mincing, the cell suspension was filtered through several layers of gauze to discard cell aggregates.

2.2. Adriamycin

Adriamycin (ADR) was purchased from Farmitalia, Italy and dissolved in double distilled water before use.

2.3. Liposome preparation

Liposomes of lecithin or of lecithin-cholesterol (1:1, mol/mol) were prepared as follows. Solutions of 80 mg of egg lecithin or 80 mg of egg lecithin mixed with 40 mg cholesterol in chloroform-methanol (2:1, v/v) were evaporated to dryness under nitrogen and dispersed in 5 ml PBS. The dispersions were then subjected to an ultrasonic irradiation with a Braun-Sonic 300 sonicator (B. Braun, Melsungen, Germany). Sonication was carried out with ice cooling using a 9-mm diameter tip at maximum energy output for 10 min for liposomes of lecithin, and for 40 min for liposomes of lecithin-cholesterol. Insoluble material and big liposomes, amounting to less than 10% of the total lipid, were discarded from the sonicated solutions by centrifugation at $30\,000 \times g$. The obtained liposomes are of a small diameter and are suitable for manipulation of membrane cholesterol [25].

2.4. Treatment of tumor cells with adriamycin and lecithin or lecithin-cholesterol liposomes

Lecithin and lecithin-cholesterol were used at 1:50 dilution in PBS. Cells of the different AKR lymphoma variants ($1 \cdot 10^6$/ml) were incubated for 1 h at 37°C as follows: (1) without treatment; (2) with ADR (8.6–86 μM) alone; (3) with lecithin liposomes (LE) alone; (4) with lecithin-cholesterol liposomes (LE-CHOL) alone; (5) with ADR + LE; (6) with ADR + LE-CHOL.

2.5. Fluorescence Associated Cell Sorter (FACS) analysis

Based on the fluorescence of ADR, cytofluorimetric analysis of the AKR lymphoma-treated cells was performed in a FACS IV Beckton-Dickinson (Mountain View, CA, USA) combined with Consort 40 data processing. ADR fluorescence was detected through an LP 570 filter.

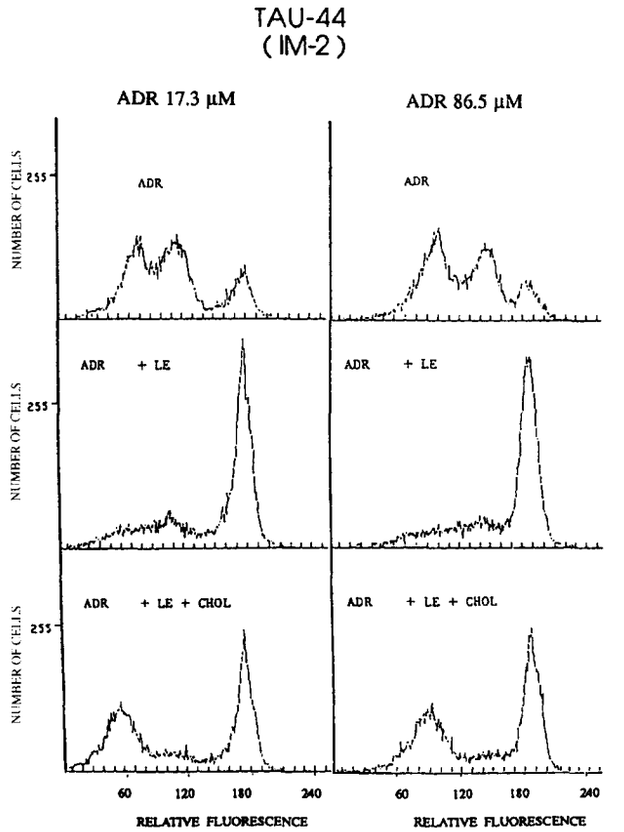

Fig. 4. Effect of treatment with lecithin or lecithin-cholesterol liposomes on ADR uptake of cells derived from the TAU-44 AKR lymphoma variant (intermediate malignancy): FACS analysis profiles.

3. Results

3.1. Comparison of the malignant behavior of the AKR lymphoma variants

A comparison between the rate of tumor growth of the four AKR lymphoma variants, as assessed in vivo by the cumulative incidence of tumoral enlarged inguinal lymph nodes, is presented in Table 1. The observed order of metastatic capacity was TAU42 > TAU-44 > TAU-33 > TAU-39. Fig. 1 compares the microscopic dissemination to lungs of the two extreme variants, TAU-39, of low-malignancy (LM) and TAU-42 of high-malignancy (HM). While practically no lung metastases were seen in the LM variant, HM displayed massive invasion of the organ.

3.2. Effect of lecithin and lecithin-cholesterol liposomes on intracellular accumulation of ADR in the AKR lymphoma variants

Adriamycin retention in cells derived from the four AKR lymphoma variants in single treatment or in combination with lecithin or lecithin-cholesterol mixture is presented in Figs. 2–5, for the TAU-39, TAU-33, TAU-44 and TAU-42, respectively. Quantitative evaluations of these FACS data are given in Table 2 and Fig. 6.

Very marked differences among the lymphoma variants were recorded with regard to the chemosensitizing effect of lecithin or lecithin-cholesterol.

The cells derived from the variant of the lowest malignancy, TAU-39, were not affected by the membrane active agents when used in conjunction with ADR at 17.3 μM/1 $\cdot 10^6$ cells/ml. At the higher ADR concentration, slight increases in ADR uptake were observed, particularly with lecithin treatment. Very pronounced shifts of cell populations towards higher peaks were observed with all the

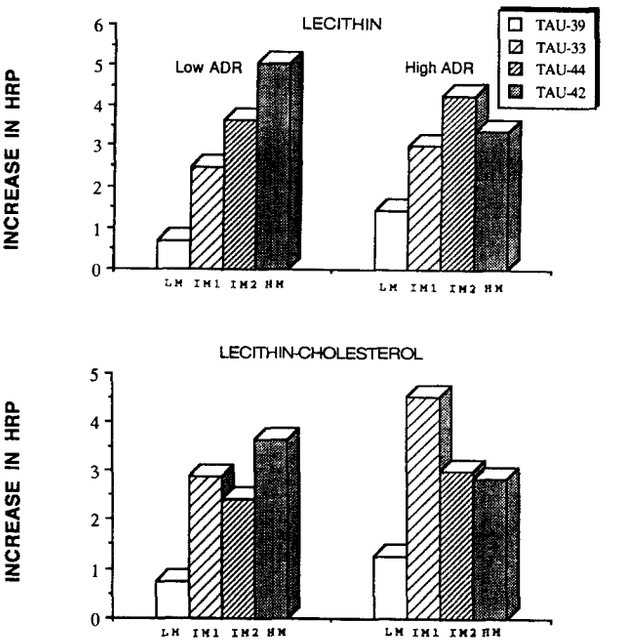

Fig. 6. Increase in the high retention population (HRP) induced by the membrane fluidity modifiers in the four AKR lymphoma variants. Increase in HRP = HRP of cells treated with ADR + Lec or Lec-Chol/HRP of cells treated with ADR alone. The data are those presented in the last column of Table 2.

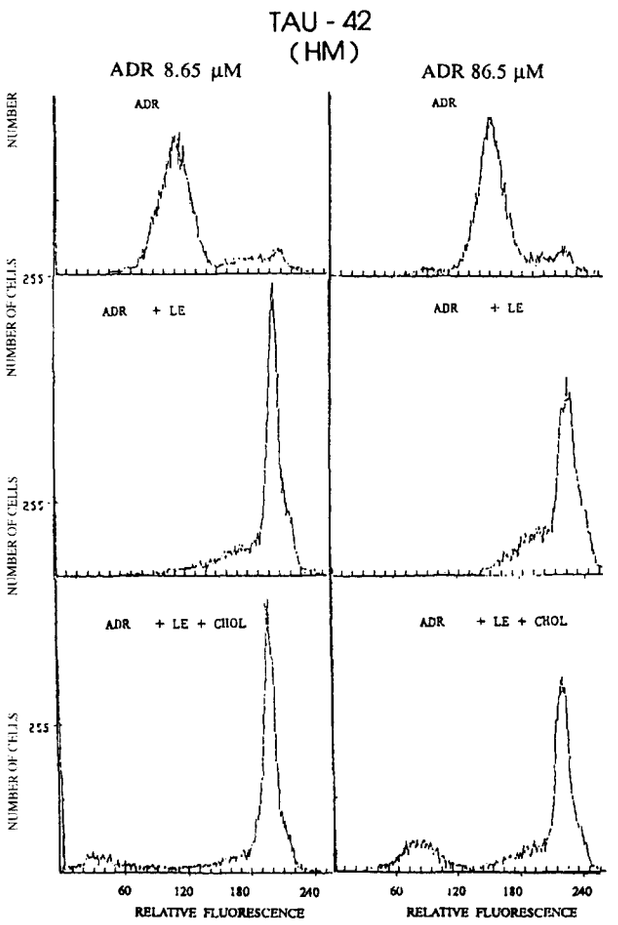

Fig. 5. Effect of treatment with lecithin or lecithin-cholesterol liposomes on ADR uptake of cells derived from the TAU-42 AKR lymphoma variant (high malignancy): FACS analysis profiles.

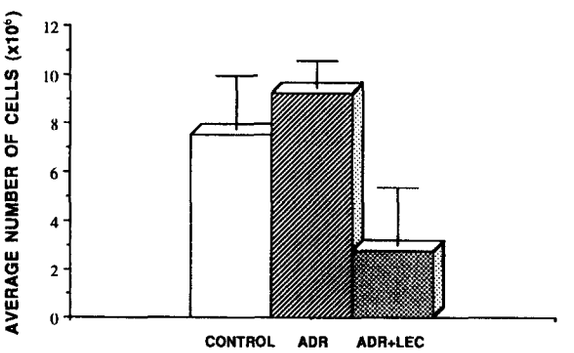

Fig. 7. Effect of adriamycin (86.5 μM) as single treatment or in combination with lecithin on viability of the TAU-42 AKR lymphoma variant.

Table 2
Effect of lecithin and lecithin-cholesterol on adriamycin retention in cells of AKR lymphoma malignancy variants – FACS analysis according to content in cellular subpopulations

Variant	ADR concentration (μM)	Chemosensitizer	Subpopulations (%)				Ratio of HRP with Le or Le/Ch per control HRP
			VLRP	LRP	IRP	HRP	
TAU-39	17.3	–		64		36	
	17.3	Le		75		25	0.69
	17.3	Le/Ch		73		27	0.75
	51.9	–		81		19	
	51.9	Le		73		27	1.42
	51.9	Le/Ch		76		24	1.26
TAU-33	17.3	–		39	44	17	
	17.3	Le		29	29	42	2.47
	17.3	Le/Ch		5	46	49	2.88
	86.5	–		37	50	13	
	86.5	Le		22	39	39	3.00
	86.5	Le/Ch		5	36	59	4.54
TAU-44	17.3	–		41	42	17	
	17.3	Le		19	19	62	3.65
	17.3	Le/Ch		45	14	41	2.41
	86.5	–		48	37	15	
	86.5	Le		18	18	64	4.27
	86.5	Le/Ch		42	13	45	3.00
TAU-42	8.65	–		83		17	
	8.65	Le	25	14		86	5.06
	8.65	Le/Ch		13		62	3.65
	86.5	–		74		26	
	86.5	Le		12		88	3.38
	86.5	Le/Ch	10	16		74	2.85

VLRP, Very low retention population; LRP, low retention population; IRP, intermediate retention population; HRP, high retention population. Increase in HRP = HRP of cells treated with ADR + Lec or Lec-Chol/HRP of cells treated with ADR alone.

other more malignant variants, TAU-33, TAU-44 and TAU-42. Moreover, there was a tendency of increase of the chemosensitizing effect of both agents, proportional to the malignancy of the variant. Generally, the effect of lecithin alone was greater than that of the lecithin-cholesterol mixture (see Fig. 6). Interestingly, small peaks of very low retention populations (VLRP) appeared in the presence of cholesterol-lecithin. This was particularly evident in the TAU-44 and TAU-42 variants. These VLRP cells were not observed among the cells treated with ADR alone.

As for lecithin, at the low ADR concentration, there was no increase in the high retention population (HRP) in the low-malignancy variant, TAU-39 (see Table 2). However, a 2–3-fold increase in the two variants of intermediate-malignancy, TAU-33 and TAU-44, and a 5-fold increase in the variant of highest malignancy, TAU-42, were observed. A similar trend of a more efficient chemosensitizing effect in the highly malignant variants was also induced by the lecithin-cholesterol treatment, although to a lower extent. At the high ADR concentration, slight augmentations in HRP was seen in the low-malignancy variant, with the two membrane modifiers. High increases in HRP (3–4-fold) were observed in the other three variants upon treatment with either lecithin or lecithin-cholesterol. At the high ADR concentration, the correlation between the degree of malignancy and efficacy of chemosensitizing effect of the two membrane modifiers was less evident.

The effect of ADR as single treatment or in combination with lecithin on cell viability, as assessed by Trypan blue exclusion is shown in Fig. 7. While ADR alone did not affect cell viability in the conditions used, the combined ADR-lecithin treatment reduced viability of the tumor cells by 64%.

4. Discussion

During the process of tumor progression, modifications in morphology [26], karyotype [27], cell surface structure [28,29], antigenicity [30] and sensitivity to drugs [31,32] take place. The latter is often reflected in multidrug resistance (MDR), which is a serious problem in clinical oncology. Membrane active agents may increase cell permeability to drugs, thereby counteracting the MDR phenotype. Since cell membranes of highly-malignant (HM) cells have been found to be more fluid than those of low-malignancy (LM) ones [17], we have suggested that HM cells might be more susceptible than LM cells to the chemosensitizing effect of membrane active agents [20]. We have indeed found that this was the case in most [10,12,18–21] although not all [20] models used by us. It should be noted

that the partitioning of anthracycline aminoglycosides, the compound family of ADR, into cell membranes is determined by the membrane lipid fluidity [33]. Therefore, the latter has been assumed to be a critical factor in the potency of anthracycline drugs [33].

In the present study we have found that by modifying the fluidity of the cell membrane, either by lecithin or treatment with liposomes of lecithin-cholesterol mixture, a high proportion of the treated lymphoma cells acquire a higher capacity of accumulating ADR. This increase in ADR uptake was low in the low-malignancy variants and considerably higher in the highly-malignant ones. On the whole, the susceptibility to chemosensitizing by lecithin and to a lesser extent by lecithin-cholesterol, was directly proportional to the degree of malignancy of the AKR lymphoma variants. This was particularly evident with lecithin at the low concentration of ADR used here (see Fig. 4). This tendency is in accordance with our previous findings with various membrane active agents in different tumor progression models [10,12,18–21]. Our results therefore indicate that the effect of our treatments are multi-functional and probably involved simultaneous changes in lipid fluidity and in protein exposure.

The high augmentation in adriamycin intracellular retention induced by lecithin could be due to the high affinity binding of the drug to DNA. The bound ADR then does not contribute any more to the equilibrium between influx and efflux of the drug. This drives the balance towards a higher influx of the cytotoxic agent. In addition, if adriamycin attaches to a membrane receptor, not only diffusion will act, and higher influx than efflux is expected.

One of the tools previously used by us as a chemosensitizer is hyperthermia. It has been found by other laboratories [8,9] and by us [10,19,20] that hyperthermia can counteract drug resistance via an increase of cell permeability to the drug. Moreover, the effect of hyperthermia was more pronounced on high- than on low-malignancy cells. One of the main targets of supranormal temperatures is the cell membrane. Hyperthermia increases cell membrane fluidity [34] and its effect depends on the lipid content, in particular the cholesterol level [35–37].

Structural cell membrane changes induced by modification in cell fluidity may cause functional alterations due to changes in protein position, like exposure of antigens by vertical displacement or growth factor receptors or lateral mobility of receptors [38,39]. These have indeed been found to occur both following cell treatment by hyperthermia [34,40,41] and by modifying the cholesterol content of cells [39,42]. In this context, it is possible to presume that modifying the cholesterol content of the cell membrane could lead, in addition to modification in membrane fluidity, which is in itself relevant to drug resistance, to an alteration in exposure or function of the Pgp, modifying thereby drug resistance.

Marked decrease in membrane phospholipids accompanies irreversible ischemic injury, demonstrating their importance in the membrane structure. Lecithin (phosphatidylcholine) is the major membrane phospholipid in all eukaryotes. In virtually all cell membranes, its level exceeds by far the other phospholipids.

Cholesterol content of the cell membrane can determine the molecular structure of the lipid bilayer. Cholesterol is nonrandomly organized in the plasma membrane. The asymmetry of the cell membrane is manifested in an uneven transbilayer cholesterol distribution, the inner leaflet being more rich in cholesterol than the outer one [43]. In the lateral plane of the cell membrane, rich and poor cholesterol domains were described [44]. These cholesterol domains are of importance for the location, structure and function of transmembrane proteins. Some proteins are located in rich [45], others in poor [46] cholesterol domains. Cholesterol appears to be required for the function of membranal enzymes [47], transport proteins [48], ion channels [49] or receptors [50], part of which may be of importance in both tumor progression and resistance to drugs.

Interaction of drugs with the membrane lipid bilayer may also be affected by the content in cholesterol. Plasma membrane lipid composition may influence binding and partitioning of the drugs in the cell membrane and thereby their bioavailability and pharmacological activity [51].

The cell membrane represents with regard to adriamycin, not only a physical barrier: it can serve by itself a target for its cytotoxic activity [52]. Acquisition of drug resistance may therefore be caused by cell membrane alterations of different types [53]. Resistance to ADR can be acquired following modifications in membrane cholesterol via alteration of membrane fluidity [54], changing thereby diffusion of the lipophilic ADR through the lipid bilayer [53]. Resistance to ADR has indeed been found to be related to increased cholesterol content of the cell membrane [55–57], although reduced sterol content has also been reported [53].

While the effect of lecithin treatment could be related to membrane fluidization, the analogous effect with lecithin-cholesterol liposomes seems to be a net of a series of simultaneous changes.

The cholesterol-lecithin mixture caused an increase in cell permeability to ADR, although to a lesser extent than lecithin. However, in part of the variants, a small subpopulation of cells became less permeable to the chemotherapeutic agent. The rigidification of the cell membrane upon incorporation of cholesterol might bear a value for the exposure of cryptic sites [38]. It could, however, induce deleterious consequences in combination with chemotherapy. In this respect, treatment with lecithin is expected to be the most beneficial.

The levels of ADR exceed those applied in the clinics. Adjustment to a pharmacological therapeutic range evidently still necessitates a quantitative evaluation of the efficiency and toxicity of the drugs in an in vivo system.

Yet this in vitro stimulation can still possibly contribute to a new approach in the counteraction of drug resistance, particularly in the late stages of tumor progression, when this problem is critical.

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