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Failure of Liposomal Encapsulation of Doxorubicin to Circumvent Multidrug Resistance in an *In Vitro* Model of Rat Glioblastoma Cells

Y.-P. Hu, N. Henry-Toulmé and J. Robert

We studied the capacity of doxorubicin encapsulation in liposomes of various lipid compositions to circumvent multidrug resistance in several variants of the C6 rat glioblastoma cell line in culture, and to inhibit azidopine binding to membranes isolated from these cells. Three formulations of liposomes were prepared: (a) phosphatidylcholine (PC)/phosphatidylserine (PS)/cholesterol (cho) at a 9/2/4 ratio; (b) PC/cardiolipin (CL)/cho at 10/1/4 ratio; (c) dipalmitoylphosphatidylcholine (DPPC)/cho at 11/4 ratio. Unloaded liposomes presented no cytotoxicity against sensitive or resistant cells. Doxorubicin encapsulated in PC/PS/cho and PC/CL/cho liposomes had a cytotoxic activity close to that of free doxorubicin, whereas doxorubicin encapsulated in DPPC/cho liposomes was significantly less active than free doxorubicin in sensitive as well as in two of the three multidrug resistant cell lines, and as active as free doxorubicin in the third one. Free doxorubicin was able to decrease 50% of [^{3}H]azidopine photolabelling to P-glycoprotein at a concentration of 40 μM; doxorubicin encapsulated in PC/CL/cho or PC/PS/cho liposomes was able to inhibit [^{3}H]azidopine binding similarly as free drug, whereas doxorubicin encapsulated in DPPC/cho liposomes had no significant effect on this parameter. Unloaded liposomes of either lipid composition had no effect on [^{3}H]azidopine binding. Together with physical studies performed in parallel on doxorubicin trapping in liposomes (*J Liposome Res* 1993, 3, 753–766), these results suggest that doxorubicin leaked out of fluid liposomes (PC/PS/cho or PC/CL/cho), whereas rigid liposomes (DPPC/cho) were able to sequester the drug more efficiently. In that case, however, no availability of the drug to the cells was possible and only a weak cytotoxicity was exhibited, especially without any favourable effect on multidrug resistance. In conclusion, no reversal of doxorubicin resistance was found to occur through liposomal encapsulation of the drug.

Key words: doxorubicin, anthracyclines, liposomes, drug carriers, multidrug resistance
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INTRODUCTION

SEVERAL WAYS are currently being explored to restore anticancer drug activity in resistant tumours. Among numerous potential mechanisms of resistance to natural products, multidrug resistance (MDR) has proven to be of clinical importance, especially in haematological malignancies [1], and its reversal by circumvention or inhibition of P-glycoprotein appears feasible in clinics after extensive studies in *in vitro* and *in vivo* models. A number of modulators have been described, which inhibit the active efflux of anticancer drugs from the tumour cells and restore both cellular accumulation of drugs and sensitivity to their action (for review, see [2]). These compounds only share some very general physical–chemical features, such as lipophilicity and positive charge at neutral pH, and they are believed to interfere directly with P-glycoprotein, as has been shown by the inhibition they exert on the photoaffinity labelling of P-glycoprotein by several ligands [3]. In addition to the development of these simple organic chemicals, nanoparticulate carriers have been successfully used *in vitro* for the circumvention of MDR. It has been hypothesised that drug encapsulation may enable the drug to escape P-glycoprotein-mediated drug efflux by avoiding a step of dissolution within the membrane, which is believed to be required for this efflux [4]. In this respect, we and others have already shown that doxorubicin loaded in polyisohexylcyanoacrylate (PIHCA) nanospheres retains an important activity in MDR models [5, 6]. We have examined, in the present study, the potential of liposomal encapsulation of doxorubicin to circumvent MDR. Encouraging data in the literature have stimulated interest in such liposomal formulations [7–10]. The mechanism by which such doxorubicin formulations remain active against MDR is however, unclear, since in several investigations, the addition of unloaded liposomes to free doxorubicin also resulted in circumvention of multidrug resistance. With three different liposomal formulations of doxorubicin, we have been unable, in contrast, to detect any reversal of multidrug resistance on the C6 rat glioblastoma model; no modulating activity of unloaded liposomal formulations was observed.

Correspondence to J. Robert.
Y.-P. Hu and J. Robert are at the Fondation Bergonié and Université de Bordeaux II, 180, rue de Saint-Genès, 33076 Bordeaux Cedex; N. Henry-Toulmé is at the Centre de Recherche Paul Pascal, CNRS, Avenue des Facultés, 33405 Talence Cedex, France.
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MATERIALS AND METHODS

Cell culture

In this study we used the C6 rat glioblastoma cell lines which we have already characterised [11, 12] and used in the study of the reversal of MDR by various chemicals [13] and by doxorub-

icin encapsulation in PIHCA nanospheres [6]. The C6 0.001 variant grows continuously in medium containing 0.001 μg/ml doxorubicin, and presents a pure typical MDR phenotype and a resistance factor to doxorubicin of 6-fold. The C6 0.5 variant currently grows with 0.5 μg/ml doxorubicin and presents MDR features clearly associated with other doxorubicin-specific resistance mechanisms; its resistance factor to doxorubicin is around 600-fold. The C61V cells grow with 1 μg/ml vincristine and present essentially an MDR phenotype mediated by the *mdr 1b* gene product, in contrast to the C6 0.5 cells which overexpress the *mdr 1a* gene product [14]. The resistance of this line to doxorubicin is approximately 30-fold.

Cells were grown in Dulbecco's modified minimal Eagle Medium (Seromed, Berlin, Germany) supplemented with 10% fetal calf serum (Seromed) in a humidified atmosphere containing 5% CO_2. They were replicated each week and the medium was changed every 2 days. The drug was removed 4 days before experiments.

Liposomal preparations

Pure doxorubicin was obtained from Farmitalia (Milan, Italy). Cholesterol (cho) and dipalmitoylphosphatidylcholine (DPPC) were obtained from Sigma. Egg yolk phosphatidylcholine (PC) was prepared in the laboratory and purified according to Singleton and associates [15], phosphatidylserine (PS) and cardiolipin (CL) were purchased from Lipid Products (Nutfiel Nurseries, Scotland). Three different formulations of liposomes were studied; they contained various proportions of phosphatidylcholine or pure DPPC, PS or CL and cho in the following proportions: (a) PC/CL/cho (10/1/4), (b) PC/PS/cho (9/2/4), (c) DPPC/cho (11/4).

Liposomes were prepared as described by Mayer and associates [16]. Briefly, a dry lipid film of the desired composition was hydrated with 300 mM citric acid adjusted at pH 4.0 to achieve a total lipid concentration of 25 mM. The preparation was frozen and thawed several times and extruded through polycarbonate filters (200 nm pore size) using a Lipex Biomembranes extruder (Vancouver, Canada). A transmembrane pH gradient was established by passing the vesicles over an AcA 54 Ultrogel column, equilibrated at pH 7.4 with HEPES saline buffer. Doxorubicin was then added at a final concentration of 1 mM for a total lipid concentration of 4 mM. The samples were then settled for 30 min at 37°C (PC/CL/cho and PC/PS/cho liposomes) or at 60°C (DPPC/cho liposomes) to ensure doxorubicin loading. Loaded liposomes were passed again over the gel column to control the trapping efficiency and eventually eliminate free doxorubicin. Trapping efficiency was always found higher than 0.95, as already mentioned [17]. Unloaded liposomes were prepared similarly, except that no doxorubicin was added.

Cytotoxicity

Growth inhibition was measured with the MTT assay performed during the exponential phase of growth of the cells. Since the cells had different growth doubling times, it was necessary to adapt the experimental procedures to the growth conditions in order to optimise the MTT assay, as already described [11]. Briefly, an adequate number of cells were seeded in 96-well microplates; one cell cycle later, doxorubicin was added, either in free soluble form, or in liposomal form, and incubated with the cells for 2 h. Cell monolayers were rinsed and cells were allowed to further grow for 2 cell cycles, after which the MTT assay was performed. The concentrations of doxorubicin ranged between 0.01 and 30 μg/ml; unloaded liposomes were used in parallel at the same concentrations as doxorubicin-loaded liposomes.

Azidopine binding

Azidopine labelling of P-glycoprotein and its inhibition by free and liposomal doxorubicin and by unloaded liposomes was evaluated on membrane preparations of C6 0.5 cells. Membranes were obtained from 1×10^8 cells, homogenised with a Dounce homogeniser in 25 ml of 0.01 M Tris–HCl buffer, pH 7.5, containing 0.25 M sucrose and 0.2 mM $CaCl_2$. After addition of EDTA to a final concentration of 1 mM and of 100 ml of 0.01 M Tris–HCl buffer, pH 7.5, containing 0.025 M sucrose, the homogenates were centrifuged at 1000 ***g*** for 10 min. The supernatant was then distributed in ultracentrifuge tubes, upon a layer made of 0.01 M Tris–HCl buffer, pH 7.5, containing 1 M sucrose and 1 mM EDTA, and centrifuged at 11 000 ***g*** for 30 min. The interfacial cloudy layer was recovered in 0.01 M Tris–HCl buffer containing 0.25 M sucrose and pelleted at 76 000 ***g*** for 1.25 h. This pellet was recovered in 4 ml of the last buffer, distributed into aliquots containing 1–2 mg of protein and kept frozen at −70°C.

To aliquots of these membrane preparations containing 20 μg of protein, free or liposomal doxorubicin or unloaded liposomes were added at various final concentrations (12.5–100 μM in doxorubicin); 5 μCi of [^{3}H]azidopine (specific activity: 50 Ci/mmol; Amersham France) and 0.01 M Tris–HCl buffer, pH 7.5, containing 0.25 M sucrose, were then added to a final volume of 100 μl. This mixture was first incubated in the dark for 30 min at room temperature, then irradiated under UV light (350 nm) for 30 min. The reaction was stopped with the electrophoresis buffer [18]. Each preparation was then submitted to SDS-PAGE as described by Laemmli [18]. The gels were incubated in an Amplify solution (Amersham) and dried under vacuum for 1 h. Autoradiography was performed by 11-day exposures at −70°C to MP-hyperfilm (Amersham). Spots were quantified on the autoradiograms using a Hoefer densitometer (Bioblock, Strasbourg, France).

RESULTS

Figures 1–3 show the cytotoxicity of free and liposomal doxorubicin and of unloaded liposomes. In no case were the unloaded liposomes toxic to the cells at the doses used, up to 0.1 mM lipids. In no case did unloaded liposomes incubated simultaneously with free doxorubicin exhibit an effect on the cytotoxicity of doxorubicin.

Doxorubicin encapsulated in PC/CL/cho liposomes had no increased cytotoxicity in comparison to free doxorubicin in any of the cell lines used (Figure 1); there was no reversal of doxorubicin resistance and even a trend to a lower activity than free doxorubicin, which was at the limit of statistical significance in C6 0.001 cells. Similarly, doxorubicin encapsulated in PC/PS/cho liposomes presented no higher cytotoxicity than free doxorubicin (Figure 2); it was even significantly less active than free doxorubicin in C6 1V cells.

Doxorubicin encapsulated in DPPC/cho liposomes was much less active than free doxorubicin in C6 0.001 and C6 1V cells (Figure 3). No activity was detected at 1 μM doxorubicin concentration, a concentration which provides 70–90% growth inhibition with free drug. In the C6 0.5 line, this liposomal doxorubicin presented the same activity as free doxorubicin (60% growth inhibition at 50 μM concentration).

Figure 4 shows the inhibition, by doxorubicin and liposomal preparations, of [^{3}H]azidopine binding to P-glycoprotein from

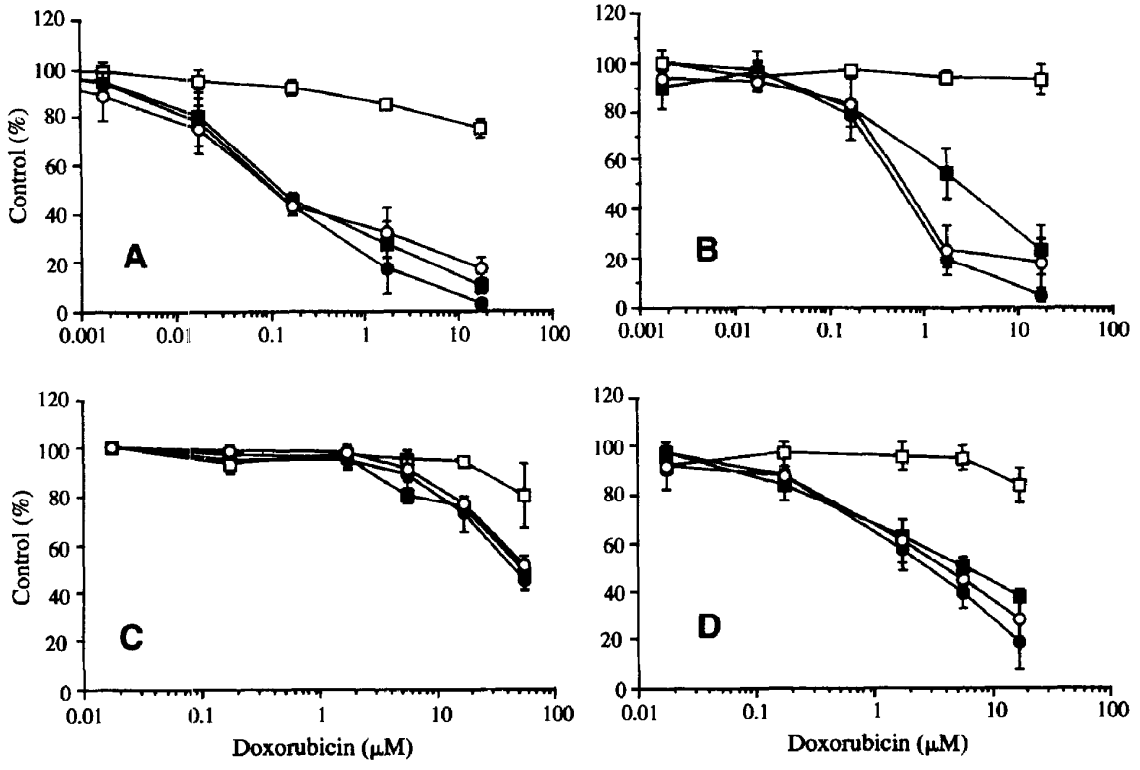

Figure 1. Cytotoxicity of free doxorubicin and doxorubicin-loaded PC/CL/cho liposomes in C6 variants. A, C6 sensitive cells; B, C6 0.001 cells; C, C6 0.5 cells; D, C6 1V cells. (-□-): unloaded liposomes; (-■-): liposomal doxorubicin; (-●-): free doxorubicin; (-○-): free doxorubicin added to unloaded liposomes. Results are means ± SD of two independent experiments performed in triplicate. Cell numbers were estimated by the MTT assay and plotted as a function of controls without drug.

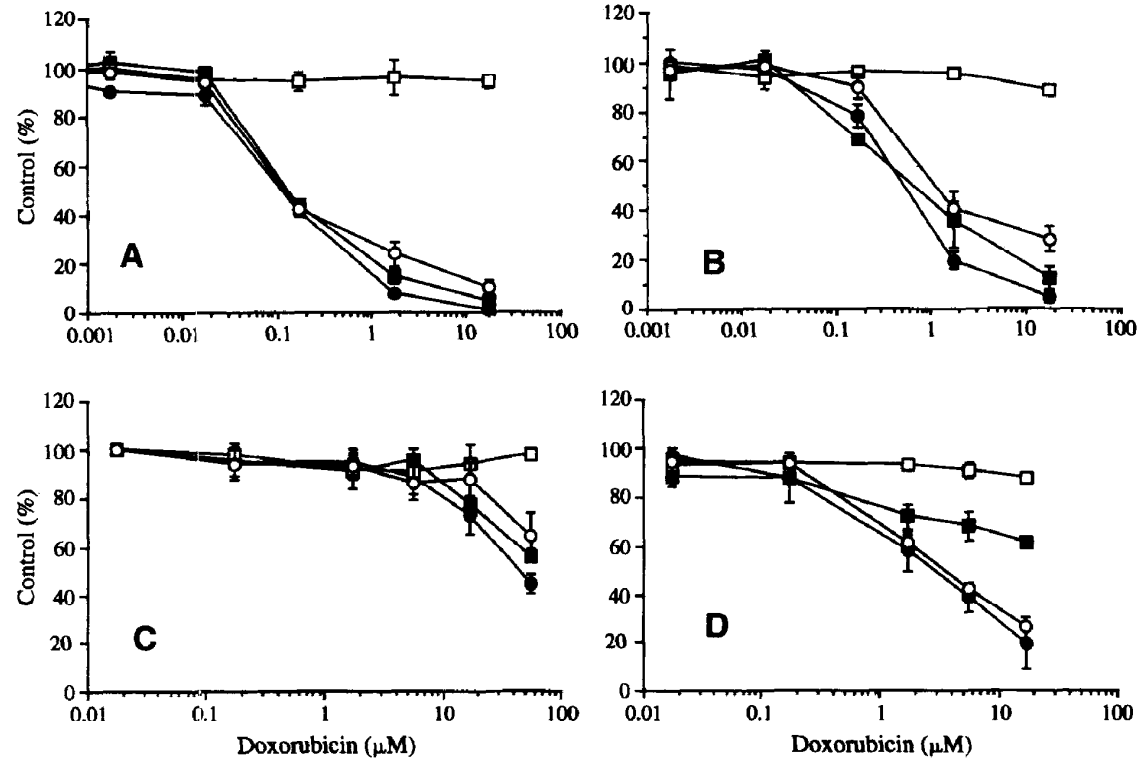

Figure 2. Cytotoxicity of free doxorubicin and of doxorubicin-loaded PC/PS/cho liposomes in C6 cell variants. See Figure 1 for legend.

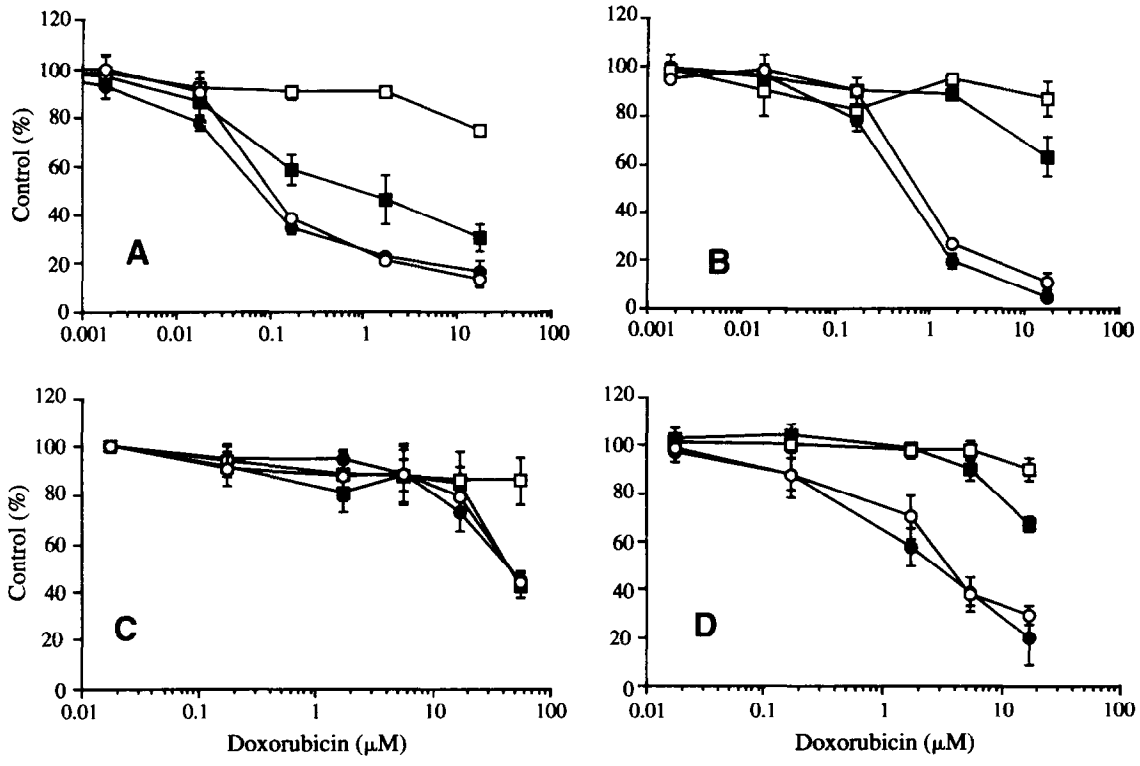

Figure 3. Cytotoxicity of free doxorubicin and of doxorubicin-loaded DPPC/cho liposomes in C6 cell variants. See Figure 1 for legend.

C6 0.5 cell membranes and Figure 5 presents the quantitative data obtained from two independent experiments. Neither type of unloaded liposomes was able to modify significantly [^{3}H]azidopine labelling to P-glycoprotein of C6 0.5 cells. Free doxorubicin was able to decrease azidopine binding by 50% at a mean concentration of 40 μM. Doxorubicin encapsulated in PC/CL/cho or PC/PS/cho liposomes similarly inhibited [^{3}H]azidopine photolabelling of P-glycoprotein with a 50% effect at 40–50 μM doxorubicin concentration. In contrast, doxorubicin encapsulated in DPPC/cho liposomes only slightly inhibited azidopine binding, with only 20% inhibition at 100 μM doxorubicin.

DISCUSSION

Encapsulation of doxorubicin in liposomes did not provide any increase of its toxicity to sensitive or multidrug resistant cells. With doxorubicin encapsulated in PC/PS/cho or PC/CL/cho liposomes, the IC_{50} observed were generally close to those obtained with free doxorubicin, whereas doxorubicin encapsulated in DPPC/cho liposomes was much less cytotoxic than free doxorubicin. In addition, doxorubicin-containing PC/PS/cho or PC/CL/cho liposomes inhibited azidopine photolabelling of P-glycoprotein just as free doxorubicin did, whereas doxorubicin-containing DPPC/cho liposomes had a minor effect. These results show that, in this cell system, the activity of doxorubicin-containing liposomes is mediated by the release of free doxorubicin in the medium. Indeed, we have shown that liposomes made of lipids providing a fluid phase membrane, such as PC/PS/cho or PC/CL/cho liposomes, rapidly release their drug content upon dilution [17]; only these liposomes were able to display a cytotoxic activity and to inhibit azidopine binding to an extent close to that of free doxorubicin. In contrast, DPPC/cho liposomes have a gel phase membrane and retain doxorubicin over a much longer period of time [17]; they show practically no cytotoxic activity and no ability to inhibit azidopine binding. This free doxorubicin-mediated cytotoxicity of doxorubicin-loaded liposomes had already been noted by Horowitz and associates [19] on an ovarian carcinoma cell line, and is likely to be the prevailing mechanism of action of doxorubicin-containing liposomes on tumour cells.

Our results strongly differ from those already presented in the literature [7–10] in two major respects: (i) doxorubicin-loaded liposomes were unable to circumvent doxorubicin resistance and were even sometimes less cytotoxic than free doxorubicin; (ii) unloaded liposomes were unable to inhibit [^{3}H]azidopine binding to P-glycoprotein. An initial explanation could lie in the model we used for the evaluation of doxorubicin encapsulation in liposomes. This model was, however, well characterised from molecular and pharmacological points of view [6, 11–14]; it overexpresses P-glycoprotein and its resistance to doxorubicin could be reversed by various modulators of MDR, especially verapamil [13], as well as by doxorubicin encapsulation in PIHCA nanospheres [6]. We rather think that the discrepancy we observed lies in the liposomal formulation we have used compared with that by other authors. The fact that unloaded liposomes were able to inhibit azidopine binding in the study of Rahman and colleagues [7] clearly shows that they directly interfere with P-glycoprotein just as chemical modulators do. This is corroborated by the fact that the mixture of unloaded liposomes and free doxorubicin also circumvented MDR. This was not the case in our hands, since we always observed similar cytotoxicity of free doxorubicin alone and of free doxorubicin added to unloaded liposomes. Our liposomes differ from those used by Rahman and associates [7] in several respects. We have used a method of liposomal encapsulation of doxorubicin which requires relatively low concentrations of lipids and allows high doxorubicin/lipid molar ratios, which might not be the case for other liposomal preparations. The lower amount of exogenous lipids added to the cells might avoid an effect of the liposomal

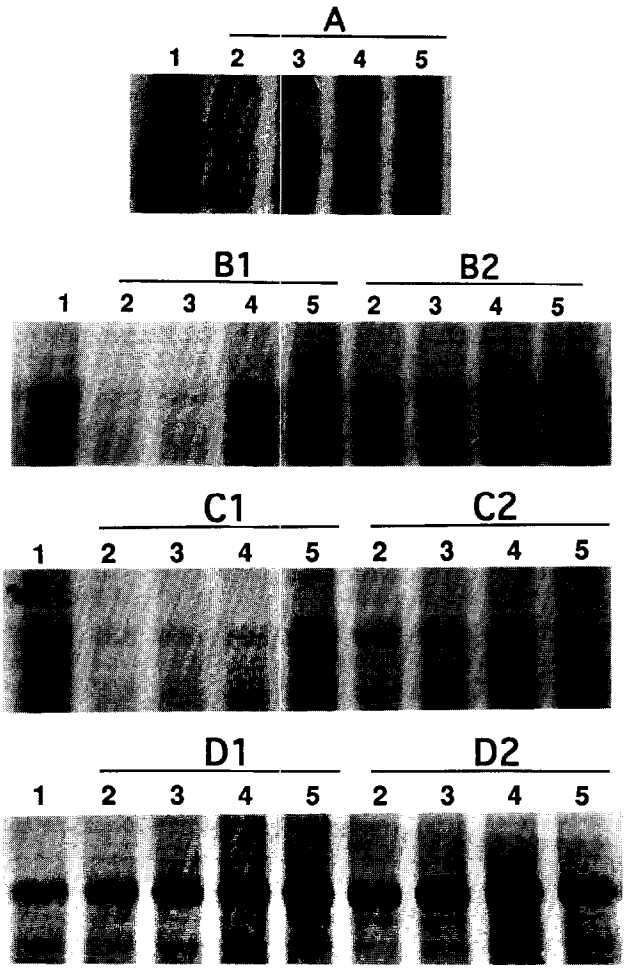

Figure 4. Autoradiograms of SDS-PAGE electrophoreses of membrane preparations of C6 0.5 cells after incubation with 5 μCi [^{3}H]azidopine and photolabelling in the absence (lane 1) or presence (lanes 2–5) of free or liposomal doxorubicin or of unloaded liposomes at the following concentrations: lane 2 100 μM; lane 3 50 μM; lane 4 25 μM; lane 5 12.5 μM. A, free doxorubicin; B, PC/CL/cho liposomes (B1, loaded with doxorubicin; B2, unloaded); C, PC/PS/cho liposomes (C1, loaded with doxorubicin; C2, unloaded); D, DPPC/cho liposomes (D1, loaded with doxorubicin; D2, unloaded).

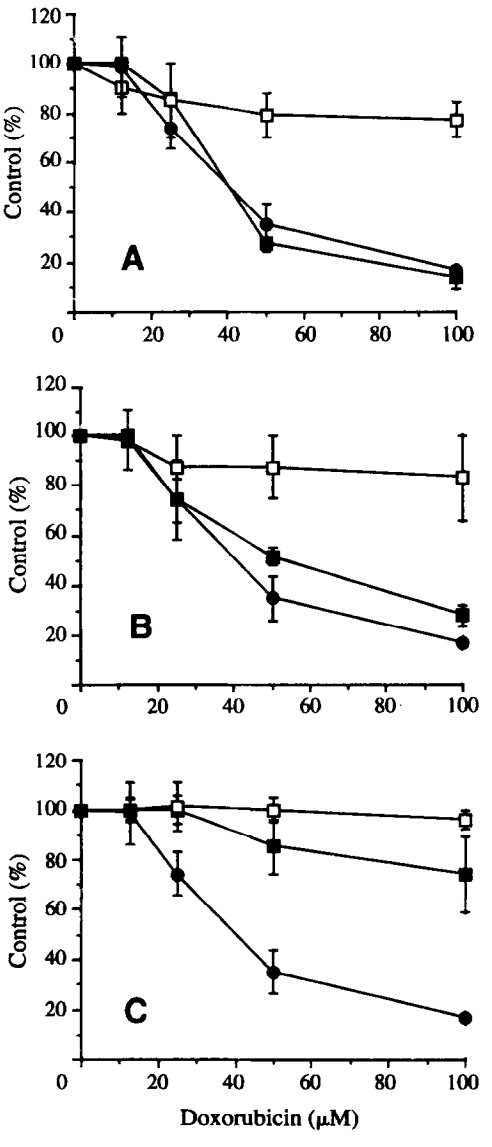

Figure 5. Densitometric records of the spots obtained in Figure 4, expressed as a percentage of control (no drug) and as a function of doxorubicin concentration (or equivalent concentration in the case of unloaded liposomes). A, PC/CL/cho liposomes; B, PC/PS/cho liposomes; C, DPPC/cho liposomes. (–□–): unloaded liposomes; (–■–): liposomal doxorubicin; (–●–): free doxorubicin.

lipids themselves on cell membrane structure and activity. One can also hypothesise that there is a component in the liposomal formulations of Rahman and colleagues [7] or of Warren and associates [9] which directly acts on P-glycoprotein and inhibits its action; because the origin of the lipids used for our liposomal preparation was different, this component would be absent from our formulations. It must be emphasised that the reversal of MDR observed by these authors differs from the circumvention we had observed with another particulate formulation of doxorubicin, i.e. encapsulation in PIHCA nanospheres [5, 6]. With these nanospheres, MDR was reversed only when doxorubicin was encapsulated and not when it was extemporaneously added to the unloaded vehicle; in addition, no inhibition of azidopine photolabelling of P-glycoprotein occurred with unloaded particles, in contrast to the unloaded liposomes of Rahman and colleagues [7], also active on MDR reversal. This shows that the mechanisms of MDR circumvention observed by Rahman and associates [7] with liposomes and by us with nanospheres [6] are quite different and cannot be assigned to a general property of nanoparticulate carriers of doxorubicin.

We would like, in conclusion, to emphasise the possibly crucial importance of the origin of the lipids used for liposomal preparations of doxorubicin. In view of the interest of pharmaceutical companies in the development of liposomal anthracyclines, the assumption of their potential effect in MDR tumours should only be made if specific experiments on the formulation they propose have been conducted. It is clear that resistance reversal is not a general feature obtained by the use of particulate formulations of doxorubicin, and that the reversal obtained by liposomes [7–10] or PIHCA nanospheres [5, 6] cannot be attributed to encapsulation itself, but to other interfering factors.

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Clonal Diversity, Measured by Heterogeneity of Ig and TCR Gene Rearrangements, in Some Acute Leukaemias of Childhood is Associated With a More Aggressive Disease

T. Stankovic, J.R. Mann, P.J. Darbyshire and A.M.R. Taylor

The pattern of immune system gene rearrangements in acute leukaemias of childhood is heterogeneous. The biological significance of this heterogeneity in childhood acute leukaemia is still poorly understood. In this study, we analysed 49 children with acute leukaemia (29 B-precursor acute lymphoblastic leukaemia (ALL), 5 relapsed cALL, 6 T-ALL, 7 acute non-lymphocytic (ANLL) and 2 mixed lineage leukaemias), for the presence of different immune system gene rearrangements (Ig JH, Cκ, Cλ, TCR Jγ, Cβ, Jδ and Jα) by Southern blot hybridisation. The most prominent heterogeneity of immune system gene rearrangements was observed in the group of B-precursor ALL. The results from our study suggest that the heterogeneity of immune system gene rearrangement reflects clonal diversity in approximately one-third of patients with B-precursor ALL at presentation and in most patients in relapse. The observed association of clonal diversity with high white blood cell count, pre-B immunophenotype and age under 1 year in B-precursor ALL may have clinical significance. There was a significantly shorter disease-free survival in the group of B-precursor ALL patients with clonal diversity compared with those without clonal diversity. Clonal diversity may, therefore, be a mechanism of disease progression common to different types of aggressive B-precursor ALL.

Key words: Childhood leukaemias, immune system, gene rearrangements, clonal diversity
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