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Membrane transport changes in an adriamycin-resistant murine leukemia cell line and in its sensitive parental cell line*

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Summary. Multidrug resistance in cancer chemotherapy occurs when cells develop resistance towards structurally and functionally unrelated drugs. It is speculated that alteration of some fundamental process(es) in the cells leads to the development of multidrug resistance. The sodium pump activity of murine leukemia cell lines P388/S (sensitive) and P388/ADR (resistant) was measured and found to be different in the two cell lines. The rate of sodium pumping, i.e., the ouabain-sensitive rubidium uptake, was consistently lower in the resistant cells compared to their parental controls. Uptake of adriamycin was lower in the resistant cells. Depolarizing the cells with potassium chloride or by inhibiting the pump with ouabain increased the adriamycin uptake in the sensitive cells but not in the resistant cells. Adriamycin did not have any acute effects on the sodium pump activity. It is concluded that the development of drug resistance in cell line P388 is associated with a decrease in sodium pump activity and a lack of depolarization-induced adriamycin uptake; these processes may be causally linked via alterations in cytosolic calcium concentration.

Introduction

One of the major problems in cancer chemotherapy is the development of drug resistance. The phenomenon of multidrug resistance has been well described in vitro and occurs when cells develop resistance toward a specific agent; other agents with different structures and modes of action also appear to be ineffective [12]. This would lead to the speculation that some fundamental process common to many drugs is at least partly responsible for multidrug resistance. Many anthracycline-resistant cancer cells have been known to be cross-resistant to vinca alkaloids. A higher drug efflux and hence a lower drug accumulation in the resistant cells as compared to the sensitive cells is generally considered an important underlying cause of this resistance [5, 9, 16]. Although the influx of vinca alkaloids and anthracyclines is thought to occur via carrier-mediated, facilitated diffusion [1, 17], lower drug influx in resistant cells has been suggested in a daunomycin-resistant, Ehrlich ascites tumor line [15]. Alterations in membrane-associated enzymes in drug-resistant sublines of mouse lymphoblastoma L5178Y cells have been demonstrated [20], but the mechanism by which the reported alterations could affect drug actions are unknown. The calcium influx inhibitor, verapamil, has been shown to reverse resistance to adriamycin [22, 23] and to restore daunorubicin sensitivity to daunorubicin-resistant carcinoma cells [14, 18] and vincristine sensitivity to vincristine-resistant cells [21], probably by increasing drug accumulation in the resistant cell line.

The sodium pump is essential in all cells for maintaining ionic gradients that are important for the proper functioning of many cellular processes. Improper functioning of the sodium pump impairs the active, energy-dependent countertransport of sodium and potassium, resulting in increased intracellular levels of sodium and a decreased membrane potential. Membrane potential and/or sodium concentration is capable of controlling many membrane transport processes, and drug transport across the cell membrane may be influenced by these factors. A very small, relative change in cytoplasmic sodium can cause a large, relative change in free cytoplasmic calcium by the sodium-calcium antiport [19]. In addition, membrane potential also controls the degree of calcium channel opening [8], which would influence the cytoplasmic calcium concentrations. Thus, small changes in the rate of sodium pumping could have profound effects on the cell calcium, and hence affect drug movement as suggested by Tsuruo et al. [22, 23], largely in the efflux process. We decided to study the activity of the sodium pump in adriamycin-resistant and -sensitive cells; the results show that in adriamycin-resistant cells it appears to be less efficient than in nonresistant cells. Paradoxically, conditions which decrease membrane potential or inhibit sodium pumping increase adriamycin uptake in the adriamycin-sensitive cells but do not influence this uptake in the resistant leukemic cells. This finding would tend to rule out the sodium pump as the primary factor in the development of drug resistance, but both changes together may be caused by a common alteration in the cells.

Materials and methods

Cell culture. Murine leukemia cell lines P388/S (sensitive) and P388/ADR (adriamycin resistant) were obtained from the National Institutes of Health (Bethesda, Md, USA).

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The resistant cell line P388/ADR_1 was isolated by cloning the original P388/ADR line twice in soft agar containing 1×10^{-6} *M* adriamycin [3]. Under these conditions only 1 in 10^5 cells survived. P388/ADR_1 is 50-fold more resistant to adriamycin than the sensitive parent line P388/S. This resistant cell line is stable, as it retained the same degree of resistance even after 3 months of culturing in a drug-free medium. The sensitive and resistant cells were grown in RPMI-1640 medium supplemented with 10% horse serum, 10 μ*M* mercaptoethanol, 1 μg/ml bovine insulin, 100 IU/ml penicillin g, and 0.1 mg/ml streptomycin sulfate, in 5% CO_2 at 37° C. The doubling time of these cell lines was 12–14 h. There was no significant difference in the growth rates of the sensitive and resistant lines.

Cells were washed twice and suspended in a physiological salt solution (Krebs-Henseleit containing: 118 m*M* NaCl, 4.7 m*M* KCl, 1.4 m*M* KH_2PO4, 25 m*M* $NaHCO_3$, 1.2 m*M* $MgSO_4$, 2.5 m*M* $CaCl_2$, 11 m*M* glucose) supplemented with 1% w/v bovine serum albumin. This medium was used in all experiments. Cells were examined for viability in this medium for 2 h by the trypan blue exclusion method.

Rubidium uptake. Sodium pump activity was determined by the uptake of rubidium-86, which can substitute for potassium ions, and hence can function as a potassium ion tracer. Following a period of incubation in the physiological salt solution at 37° C, the cells were separated from the medium by centrifugation. The radioactivity in the pellet was suspended in a scintillation cocktail and counted. Suspensions of both resistant and sensitive cells contained 0.3–0.5 million cells per ml for all experiments.

Adriamycin uptake experiments. ^{14}C-Adriamycin with a specific activity of 30 mCi/mmol was kindly provided by Dr. R. Engle, National Cancer Institute (Bethesda, Md, USA). To determine the uptake of adriamycin 3 to 6×10^6 cells per ml were incubated at 37° C in a 1.0% bovine serum albumin-supplemented Kreb's solution containing the radiolabelled drug. After incubation for the appropriate period of time cells were transferred to Hopkins tubes, in which they were centrifuged through 0.25 *M* sucrose solution, present in the bottom of the tubes [7]. The supernatant was removed and the tubes were washed 3 times with water without disturbing the cell pellets which were then lysed in 0.5 *N* NaOH at room temperature for 30 min with occasional mixing. An aliquot of the lysate was removed and radioactivity counted in a xylene: PCS (Amersham): acetic acid (1:1:0.025) scintillation cocktail solution.

Student's unpaired *t*-test was used for comparing the resistant and sensitive cell lines in most of the experiments, and Anova block comparisons were used for some experiments where indicated.

Results

Viability tests indicated that both cell lines, P388/S and P388/ADR_1, were more than 95% viable for up to 2 h in the albumin-containing medium. The rate of rubidium uptake was determined at 4 different incubation times in the rubidium-containing medium. The results of such experiments, shown in Fig. 1, indicated that the rate of rubidium uptake was linear for at least 40 min in most experiments. In all subsequent experiments, the cells were incubated for 30 min. Background counts used in these studies were

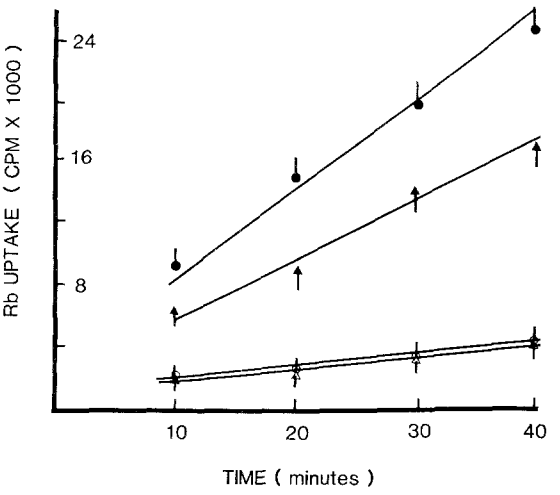

Fig. 1. Rate of rubidium uptake from five experiments, each being a mean of triplicates. Each sample had 1 million cells. The rate due to sodium pump activity which was sensitive to 2 m*M* ouabain is shown by *solid symbols* (*circles*, adriamycin-sensitive cells; *triangles*, adriamycin-resistant cell). Physiological salt solution, as described in Methods, contained 0.6 μCi rubidium-86/ml. The slopes of the two lines were different (statistical significance accepted at $P < 0.05$). *Open circles* (sensitive) and *open triangles* (resistant) denote ouabain-insensitive rubidium uptake in the presence of 2 m*M* ouabain

done with cells incubated at 4° C. Rubidium or potassium can be taken up into the cells by at least two processes: (a) by the sodium pump, and (b) by the sodium-potassium cotransport process. The data in fig. 1 show the rate of ouabain-sensitive rubidium uptake at 37° C (i.e. the difference between the total uptake and that in the presence of ouabain) as well as the uptake that was insensitive to the presence of ouabain. The ouabain-sensitive rubidium uptake was significantly lower in the resistant cells (by 30%, $P < 0.05$). Ouabain-insensitive rubidium uptake was similar in both cell types and contributed no more than 20% of the total rubidium uptake.

It was necessary to test whether adriamycin could directly affect rubidium uptake, because adriamycin resistance was developed by growing cells in the presence of adriamycin. The acute effects of adriamycin are shown in Fig. 2. The rubidium uptake of both cell types was unaffected by 0.01 m*M*. Hence, there must be other changes leading to the development of resistance that also cause the decrease in sodium pump activity, because the latter is not affected by the presence of adriamycin.

Decreased sodium pumping could have many consequences leading to drug resistance. One such effect could be the alteration of adriamycin uptake by the cell. This possibility was investigated in the present study. As can be seen in Fig. 3, the initial rate of adriamycin uptake and the drug content at steady state were lower in the resistant cell line; the results of 5 experiments each are summarized in Figs. 4 and 5. Incubating the cells at 4° C significantly inhibited both sodium pumping and the adriamycin uptake, suggesting uptake processes other than simple diffusion for adriamycin. Figure 5 shows that adriamycin uptake under control conditions was significantly lower in the resistant cells than in the sensitive cells when measured after 30 min incubation, but this difference was not significant

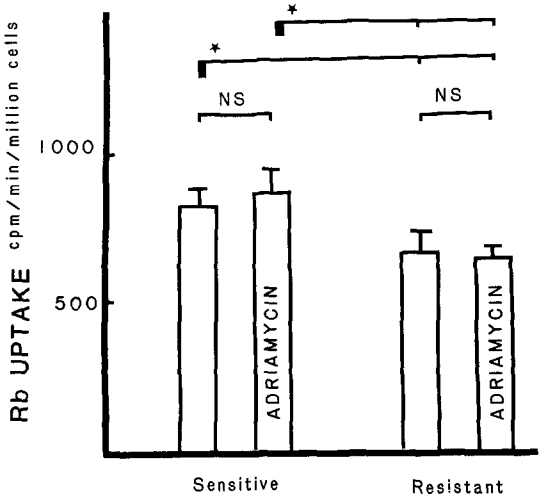

Fig. 2. Rate of rubidium uptake in the presence and absence of 0.01 m*M* adriamycin in the sensitive P388 and the resistant P388/ADR$_1$ cell lines. *NS*, no statistical difference as determined by Student's *t*-test. The two *asterisks* above indicate the statistical significance by Anova block comparisons. $P < 0.05$ for difference between the sensitive and resistant cell lines. $n = 6$ in each case

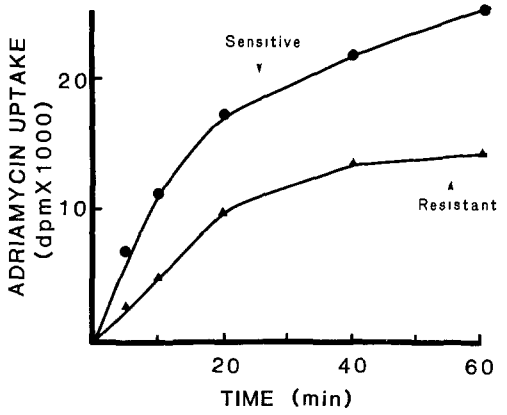

Fig. 3. Time course of uptake of ^{14}C-adriamycin by P388 (*solid circles*) and P388/ADR$_1$ (*solid triangles*) cells. Three million cells per ml were incubated at 37° C in a medium containing 0.06 μ*M* adriamycin. At different time points, cells were removed and counted as described in the text

($P < 0.06$) after 10 min incubation. Only in the sensitive cell line was an increase in adriamycin uptake observed in the presence of ouabain, which should have inhibited the sodium pump and lowered the membrane potential. Depolarizing the cells by increasing potassium concentration in the medium increased the adriamycin uptake in the adriamycin-sensitive cell line. In the adriamycin-resistant cells, this uptake was unaffected by either ouabain or a high potassium environment. After the cells had been incubated for 30 min, the efflux process contributed more to the total uptake measurements compared to cells that had been incubated for 10 min, at which point the influx process should be the major contributor to the cellular adriamycin content. The uptake at 4° C may represent a fraction that was bound to the cell membrane, in addition to the contribution of the diffusion process. However, this fraction of the adriamycin content was not significantly different in the sensitive and resistant cells.

The degree of stimulation of adriamycin uptake was not different in the presence of KCl from that in the presence of 0.1 m*M* ouabain. It appears from these studies that the sodium pump contributed to the major fraction of the rubidium uptake and was less efficient in the adriamycin-resistant cells. The ouabain-insensitive rate of rubidium uptake was similar in both cell lines and contributed to less than 20% of the total rubidium uptake. Most interestingly, the addition of ouabain or potassium did not influence the adriamycin uptake in the resistant cells, conditions which significantly stimulate this uptake in the adriamycin-sensitive cells.

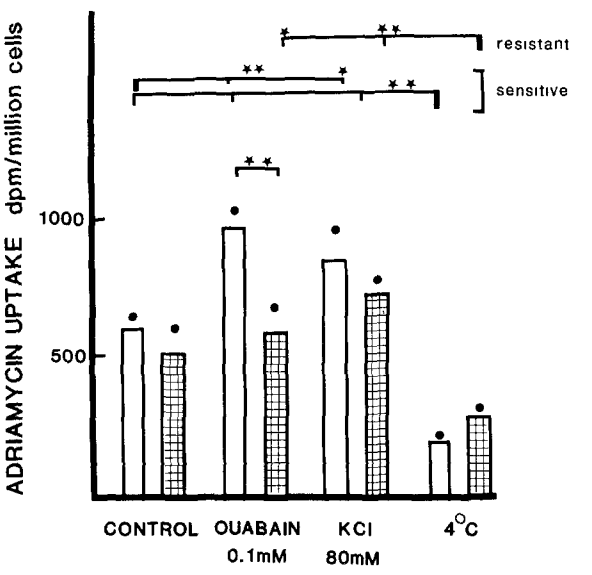

Fig. 4. Adriamycin uptake in six different samples of cells of the sensitive (*open bars*) and resistant (*filled bars*) P388 line. Adriamycin content was measured after 10 min incubation in the control medium or in the presence of 0.1 m*M* ouabain, 80 m*M* KCl, or after incubation in the control medium at 4° C. Anova block comparisons were used for statistical analysis. $*P < 0.01$; $**P < 0.001$

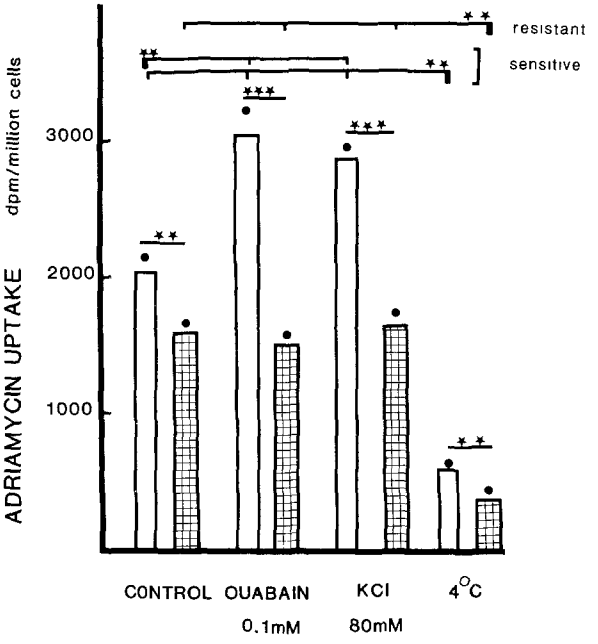

Fig. 5. Adriamycin uptake measurements similar to that described in Fig. 4, except that the incubation period was 30 min

Table 1. Data for ouabain-sensitive rubidium uptake shown as the means and SEM from five sets of experiments

	Sensitive cells	Resistant cells
Control	494 ± 193 ★	416 ± 162
Calcium 0.5 m*M*	569 ± 205	549 ± 189**
Verapamil 10 μ*M*	523 ± 219	501 ± 190*

★ Difference between sensitive and resistant cell lines significant at $P < 0.05$
Asterisks indicate significant difference from controls:
* $P < 0.05$; ** $P < 0.01$

The calcium channel blocker, verapamil, and extracellular medium containing 0.5 m*M* calcium were used to evaluate the effects of calcium on rubidium uptake. Table 1 summarizes data from such experiments. Both verpamil and the lowering of calcium significantly increased the rubidium uptake in the resistant cell line but had marginal effect on the sensitive cells. Calcium channel blocker was less effective than lowering the extracellular calcium to 0.5 m*M* in the adriamycin-resistant cells.

Discussion

Most living cells have negative potentials inside the cell membrane, along with low levels of calcium and sodium in their cytosol compared to the extracellular concentrations of these ions. The low concentrations of these ions are maintained with the help of energy-consuming pumps driven by the sodium-potassium ATPase and the calcium-dependent magnesium ATPase. The growth of some cell types in culture medium is dependent upon the sodium pump [11], but not necessarily related to the membrane potential [24]. In both 3T3 cells and Chinese hamster ovary cells, it has been shown that mitotic arrest occurred when there was a 5- to 6-fold increase in the membrane potential and a substantial decrease in intracellular sodium [4]. Conversely, increases in mitotic activity occurred with increases in intracellular sodium. Ouabain has been shown to produce inhibition of amino acid transport of skeletal myotubes in culture [24]. All these observations point to the importance of the sodium pump in cell growth and possibly in the transport of substances across the cell membrane.

We have shown that adriamycin-resistant cells appear to have a less efficient sodium pump than the drug-sensitive cell line. The decreased rate of sodium pump activity could result either from a reduced amount of sodium-potassium ATPase or from changes in factors that modulate its activity. Sugimoto et al. [20] have reported a change only in alkaline phosphodiesterase and sodium-potassium ATPase among several other enzymes in the cell membranes of lymphoblastoma L5178Y cells made resistant to adriamycin, aclacinomycin, and bleomycin. Since sodium pump activity was not measured in these cells, it is not known whether the changes in these activities could account for the lower pumping rate. Skovsgaard [15] was not able to show a difference in the uptake of daunomycin by daunomycin-resistant cells in the presence of 1 m*M* ouabain. Our results confirm his observations and, in addition, show that the ouabain-sensitive adriamycin uptake is present only in the adriamycin-sensitive control cells.

How, if at all, lowered sodium pump activity contributes to drug resistance is not known. Greater influx of sodium is expected to occur on depolarization with potassium in the sensitive cell line. If the influx of adriamycin were modulated by sodium, then more adriamycin would enter the cells of the sensitive type. Daunomycin retention varies equally with increasing calcium concentration in drug-sensitive and drug-resistant Ehrlich ascites cells in the presence of intact and interrupted active outward transport [14]. In the same study it was shown that energy-dependent efflux is more active in drug-resistant than in drug-sensitive cells, which is in agreement with the results of others [10, 15]. If a similar situation prevails in the present study then the net effect would be a significant decrease in the adriamycin concentration within the resistant cells.

Another contribution that decreased sodium pump activity would make is increase in cytosolic calcium levels. Verapamil, a calcium influx inhibitor, increased adriamycin cytotoxicity in human leukemic cells. It has also been shown to restore daunomycin sensitivity to daunomycin-resistant, Ehrlich ascites cells [22, 23]. Results from our laboratory suggest that an increase in rubidium uptake occurs in the presence of verapamil or in medium with 2 low calcium concentration. The role of calcium in the development of drug resistance is not understood, and in view of the report by Murray et al. [14], calcium-dependent efflux of daunorubicin did not appear to be different in the resistant cell line, even though the energy-dependent efflux was increased. In contrast, Chauffert et al. [2] were able to report a decrease in the enhanced efflux of adriamycin with verapamil in the adriamycin-resistant colonic cancer cell. Calcium may be involved in this energy-dependent efflux. If one infers from all these observations that the cytosolic level of calcium in the resistant cell line is greater than that in the sensitive cell lines then the depolarization with potassium or further inhibition of the sodium pump will not produce significant increases in cytosolic calcium in the resistant cells. The decreased pump activity could also be a consequence of increased cytosolic calcium, as has been shown for the red cell enzyme [6], Verapamil has been shown to increase drug accumulation in adriamycin-resistant Friend leukemia and P388 leukemia cells [13, 25].

In conclusion, it can be stated that the development of drug resistance in cell line P388 is associated with decreases in sodium pump activity, which could contribute to a decreased membrane potential and increased cytosolic calcium. Increased cytosolic calcium may then lead to the increased efflux of adriamycin and contribute to drug resistance. These observations may also explain the lack of effect of high potassium levels on the adriamycin uptake in the adriamycin-resistant cells. The paradoxical effect of potassium and ouabain on adriamycin uptake in the sensitive cells suggests that greater accumulation of sodium in the sensitive cell line during these treatments can increase drug influx and counteract the effects of elevated calcium on drug efflux under these conditions. Alternatively, other related changes could occur during the development of drug resistance.

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