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Verapamil reverses the ultrastructural alterations in the plasma membrane induced by drug resistance

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Two P388 cell sublines with different levels of resistance to daunomycin (DNM), P388/20 and P388/100 cells (~ 20- and 100-fold resistance, respectively), undergo a significant (~ 2-fold) increase in the number of intramembrane particles (IMPs) present at their plasma membrane, as compared to that exhibited by the parental, drug-sensitive P388 (P388/S) cell line. Regardless of the level of resistance, incubation of drug-resistant cells with verapamil, a well known reverting agent of anthracycline resistance, restores the morphology of the plasma membrane in these cells, yielding a pattern in which the number and size distribution of IMPs at both leaflets of the bilayer, become undistinguishable from those displayed by drug-sensitive cells. Furthermore, verapamil did not affect the ultrastructural organization of the plasma membrane of drug-sensitive cells. It is possible that the alterations in the structural organization of the plasma membrane of the antineoplastic-resistant tumor cells, might represent a reliable 'marker' for early diagnosis of drug resistance.

Cellular drug resistance; Verapamil; Intramembrane particle; Freeze-fracture

1. INTRODUCTION

The acquisition of the multidrug resistance phenotype (MDR) by tumor cells involves a pleitropic response which include alterations in the molecular components [1,2] as well as in the dynamic properties [3] of the cell membrane. In molecular terms, the best known and widely spread change associated with MDR is represented by the overexpression of P-glycoprotein, a plasma membrane protein [4], which acts as an ATP-dependent efflux pump able to eliminate drugs and lipophilic substances from the cells [5,6]. The reduced drug accumulation observed in drug-resistant cells, can be abolished by several agents including calcium channel blockers, calmodulin inhibitors, etc.. These compounds increase intracellular drug levels presumably by interfering with anti-neoplastic binding and transport by P-glycoprotein [7,8].

In spite of the fact that the plasma membrane of tumor cells appears as a cellular structure critically involved in the development of MDR [9,10], few studies have been devoted to analyze the incidence of acquiring the resistant phenotype on the ultrastructural organization of these membranes [11–14]. Nevertheless, Arsenault et al. [12] using drug-resistant Chinese hamster ovary (CHO) cells and human leukemia cells, Garcia-Segura et al. [13] using daunomycin (DNM)-resistant P388 cells and Sehested et al.[14] working on Ehrlich ascites tumor cells, coincide in that acquisition of drug resistance is associated to a pronounced increase in the number of intramembrane particles (IMPs) at the plasma membranes of these cells. In order to further explore the relevance of these alterations to the MDR phenotype, we have analyzed the influence of the resistance-reverting agent verapamil (VRP), on the structural organization of the plasma membranes from two P388 cell sublines increasingly resistant to DNM.

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Abbreviations: DNM, daunomycin; VRP, verapamil; IMPs, intramembrane particles; P388/S, DNM-sensitive P388 cells; P388/20, ~ 20- fold DNM-resistant P388 cells; P388/100, ~ 100-fold DNM-resistant P388 cells; MDR, cellular multidrug resistance; CHO, Chinese hamster ovary cells; P and E leaflets, protoplasmic and exoplasmic leaflets of the plasma membrane, respectively.

2. MATERIALS AND METHODS

2.1. *Cells*

Parental DNM-sensitive P388 murine leukemia cells (P388/S) and two cell sublines selected for resistance to DNM (~ 20- and ~ 100-fold resistance, respectively), were maintained in culture as described previously [1]. The level of resistance was determined by the ratio of the IC_{50} values for DNM exhibited by drug-resistant and drug-sensitive cells. IC_{50} is defined as the concentration of DNM that produces 50% inhibition of cell growth, upon 48 h incubation of the cells (in log phase), in the continuous presence of the drug.

2.2. *Intracellular drug accumulation measurements*

Cell suspensions (10^6 cells/ml) in 10 mM HEPES buffer, pH 7.2,

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containing 130 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$ and 1 mM $MgCl_2$ (HBS solution), were incubated with 3 μM DNM, in the absence or in the presence of 5 μM verapamil (VRP) added to the cells 2 h prior to the uptake assay. The intracellular level of the anthracycline was estimated under steady-state conditions by flow cytometry using an Epics Profile I instrument, as previously described [15].

2.3. *Freeze-fracture studies*

Freeze-fracture replicas of the plasma membrane of P388/S, P388/20 and P388/100 cells growing in log phase and incubated for 48 h in the absence (control) or in the presence of 5 μM of VRP, were obtained and analyzed as described previously [13].

3. RESULTS AND DISCUSSION

Previous reports concerning the characterization of the MDR phenotype [11–14], have described that acquisition of drug resistance by tumor cells is accompanied by marked alterations in the structural organization of the plasma membrane. Arsenault et al. [12] as well as Sehested at al. [14], found an increase in the density of IMPs in the protoplasmic leaflet of the plasma membrane of drug-resistant sublines compared with wild-type cells, which correlated well with the level of overexpression of P-glycoproteins. Garcia-Segura et al. [13], however, reported an almost identical morphological alteration in a P388 cell line with a low level of resistance to DNM, similar to that displayed by the P388/20 cells, which did not overexpress P-glycoproteins. The observations reported here (Fig. 1), indicate that the changes in the architecture of the plasma membrane in the two P388 cell lines increasingly resistant to DNM, seem independent of the level of resistance. Thus, the increased number and size distribution of IMPs in these two cell lines are very similar (Table I). The two DNM-resistant P388 cell lines exhibit an approximately 2-fold increase in the total number of IMPs with respect to that found in the P388/S cells at both, the P and E faces of the plasma membrane. Although the most significant increase occurs in the number of IMPs having a diameter of 10–15 nm, the density of IMPs with lower (< 10 nm) or larger (> 15 nm) size, is also increased in the DNM-resistant cells. This last point, further emphasizes the difficulties in assigning the observed differences in size distribution of IMPs between DNM-sensitive and DNM-resistant cells, to only one molecular entity having a defined molecular size such as the P-glycoprotein and rather suggests the occurrence of global changes in the architecture of the membranes.

Table I

Number[a] and size[b] distribution of intramembrane particles in plasma membranes from P388 cells

Intramembrane particles per μm^2	P388/S	P388/20	P388/100
Control			
Protoplasmic face:			
diameter < 10 nm	133 ± 16	196 ± 15	192 ± 14
diameter 10–14.9 nm	785 ± 91	1342 ± 105*	1421 ± 106*
diameter > 15 nm	208 ± 24	306 ± 24	312 ± 23
Total	1126 ± 131	1844 ± 144*	1925 ± 143*
Exoplasmic face:			
diameter < 10 nm	29 ± 8	25 ± 3	21 ± 3
diameter 10–14.9 nm	112 ± 29	396 ± 44*	401 ± 57*
diameter > 15 nm	48 ± 12	54 ± 6	48 ± 7
Total	189 ± 49	475 ± 53	471 ± 67*
5 μM Verapamil			
Protoplasmic face:			
diameter < 10 nm	206 ± 22	218 ± 19	221 ± 32
diameter 10–14.9 nm	899 ± 94	824 ± 71	798 ± 117
diameter > 15 nm	264 ± 28	298 ± 26	242 ± 36
Total	1369 ± 143	1340 ± 116	1261 ± 185
Exoplasmic face:			
diameter < 10 nm	32 ± 8	30 ± 7	29 ± 6
diameter 10–14.9 nm	155 ± 38	108 ± 26	98 ± 20
diameter > 15 nm	61 ± 15	39 ± 9	91 ± 19
Total	248 ± 60	177 ± 42	218 ± 45

[a] Each value is the mean ± S.E. from 20 cells. For each cell and membrane face, at least 1 μm^2 of membrane area were evaluated.

[b] The size of the IMPs was measured as described in Ref. 13. Statistical evaluation of the data was carried out by analysis of variance for multiple comparisons. Individual means were compared with the multiple range test of Duncan. A level of confidence of $P < 0.001$ was selected.

*Significant differences ($P < 0.001$) versus the values of P388/S cells.

Table II

Effect of verapamil on the cytotoxicity induced by DNM in P388 leukemia cells

Verapamil (μM)	IC_{50} (μM DNM)		
	P388/S	P388/20	P388/100
0	0.029 ± 0.003	0.556 ± 0.073	2.909 ± 0.459
5	0.040 ± 0.003	0.097 ± 0.005	0.287 ± 0.029
10	0.035 ± 0.006	0.080 ± 0.009	0.219 ± 0.059
20	0.035 ± 0.004	0.061 ± 0.004	0.152 ± 0.049

The most intringuing findings reported in this paper concern the effects of a drug resistance-reverting agent, the calcium antagonist VRP . Tsuruo et al. [16] first reported that VRP increases the intracellular drug accumulation in drug-resistant cells, thus enhancing the susceptibility of these cells to the cytotoxic activity of the anti-neoplastics. The drug-enhancing activity of VRP seems to be independent of its effects on calcium channels [17] and it has been suggested to occur mainly by competition with antitumor drugs for binding to P-glycoproteins [7,8]. In our P388 cells, VRP at concentrations ranging from 5 to 20 μM that do not affect cell survival, increases the sensitivity (reduced IC_{50} values) to DNM of the drug-resistant cells without affecting that of the parental, drug-sensitive P388/S cells (Table II). Furthermore, as expected from a drug-resistance reverting agent, the level of intracellular accumulation of DNM under steady-state conditions increases substantially in the presence of VRP in drug-resistant cells,

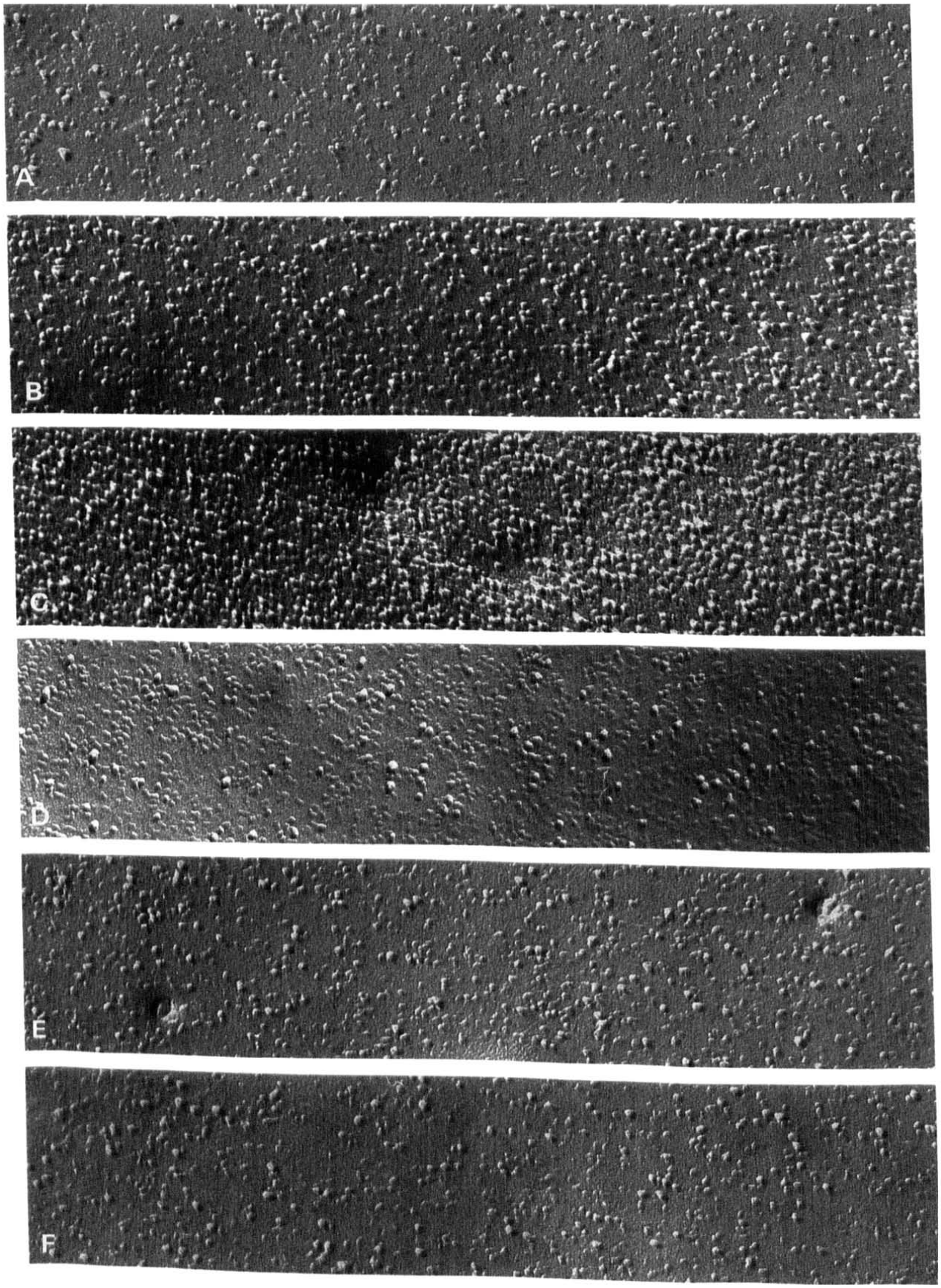
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Fig. 1. Freeze-fracture replicas of the protoplasmic face of the plasma membrane of P388/S (A,D), P388/20 (B, E) and P388/100 (C,F) cells. A, B and C correspond to plasma membrane from cells incubated in the absence of VRP. D, E and F correspond to plasma membranes from cells incubated for 48 h in the presence of 5 μM VRP. To avoid possible bias, photographic prints were coded and evaluated without knowledge of the experimental group from which pictures were taken. Magnification: 125,000 ×.

remaining nearly unaffected in the case of drug-sensitive cells (Fig. 2). It should be noticed, that the effect of VRP in increasing both, the sensitivity to DNM and the intracellular accumulation of the drug, occurs in the two P388/100 and P388/20 drug-resistant cell lines. This strongly suggests that regardless of the possible occurrence of P-glycoproteins in the P388/100 cells, the expression of which in P388 cells seems to be associated with high levels of resistance [18], the mechanism(s) through which VRP causes reversion of drug resistance, are not neccesarily restricted to the presence of P-glycoproteins. In fact, other alterations such as the intracellular redistribution of anti-neoplastics induced by VRP, have also been proposed to explain the reversal of MDR by the calcium antagonist [19].

Reversion of DNM-resistance by VRP in P388 cells has profounds effects on the ultrastructural organization of the cellular plasma membrane. Fig. 1D,E and F show that incubation of either P388/20 or P388/100 cells with 5 μM VRP, a concentration at which the cells retain full proliferative capacity, results in freeze-fracture images of the plasma membranes from drug-resistant cells undistinguishable from those of drug-sensitive cells (see Table I for quantitative analysis of IMPs in the freeze-fracture replicas). Sehested et al. [14] working on cultured Ehrlich ascites tumor cells reported that incubation with VRP, under different conditions (higher concentrations of VRP and shorter time exposures) to those employed in the present study, promoted clustering of IMPs in both drug-sensitive and drug-resistant cells. This seems not to be the case for P388 cells since as observed in Fig. 1, all the membrane samples including those exposed to VRP, display a homogeneous distribution of IMPs without formation of clusters or patches. In a previous study [13], we suggested that the different morphology between DNM-resistant and DNM-sensitive cells could be somehow related to the increased plasma membrane traffic in anthracycline-resistant P388 cells reported by Sehested and coworkers [20]. These authors hypothesized that endosomal drug trapping followed by vesicular extrusion to the extracellular medium (the volume and membrane area of endosomes is higher in anthracycline-resistant than in sensitive P388 cells), represents a mechanism of cellular drug resistance that may account for the reduced intracellular level of anti-neoplastics in drug-resistant cells. This possibility seems to be further supported by the effects of VRP in restoring the architecture of the plasma membranes of DNM-resistant cells, since VRP is able to inhibit the increased membrane traffic in these cells [20].

In summary, our observations indicate that in addition to specific interactions with P-glycoproteins as proposed by others [7,8], reversion of cellular drug resistance by verapamil involves global changes affecting the assembly and the overall structural organization of the membranes.

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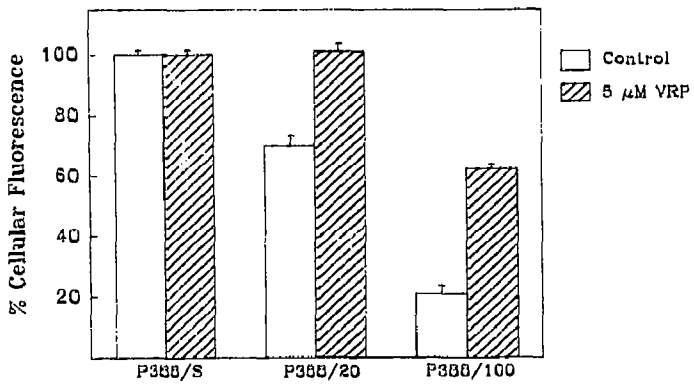

Fig. 2. Total accumulation of DNM by P388 cells as estimated by laser flow cytometry. Cells were incubated with 3 μM DNM during 90 min (steady-state conditions), in the absence or in the presence of 5 μM VRP in HBS solution at pH 7.2. The Y axis indicates the percentage of drug fluorescence associated to each cell population. 100% refers to the level of fluorescence exhibited by drug-sensitive cells. bars ± S.D.

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