

Structural Differences between Sensitive and Resistant L1210 Cells

B. UHRÍK, A. H. EL-SAGGAN, M. ŠEREŠ, L. GIBALOVÁ, A. BREIER
AND Z. SULOVIÁ

*Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences,
Bratislava, Slovakia*

Abstract. The main structural differences between sensitive L1210 mouse leukaemic cells and their multidrug resistant counterpart, obtained by adaptation of the parental cell line to vincristine (VCR), concern the size and shape of the cells, their surface properties and changes in organelles involved in proteosynthesis and transport of substances. The resistant cells are larger with higher density of microvilli. In light and electron micrographs containing a group of cells, cells were found to be closer to each other in L1210/VCR cells than in L1210 cells. This difference in cell aggregation suggests different surface properties which could be visualised by decreased staining of L1210/VCR cell surface coat (glycocalyx) with a polycationic dye ruthenium red. A decrease in surface to volume ratio as a consequence of increased cell size in resistant cells is compensated by proliferation of villi and cytoplasmic protrusions of the cell surface. L1210/VCR cells were further distinguished by higher amount of euchromatin, increase in density of rough endoplasmic reticulum, more developed Golgi apparatus and aggregation of free ribosomes into tetrameric and pentameric polyribosomes. These structural changes may be interpreted as a sign of increase in proteosynthesis and transport of substances.

Key words: Multidrug resistance — P-glycoprotein — L1210 cell line — Ruthenium red — Ultrastructure — Cell clustering

Introduction

Mouse leukemic cell line L1210, first described by Law et al. (1949), can be turned into multidrug resistant (MDR) cell line expressing P-glycoprotein (P-gp) by stepwise adaptation to vincristine (VCR) as checked at mRNA and protein levels (Poleková et al. 1992; Fiala et al. 2003). P-gp, a member of ABC transporters family (classified as ABCB1), represents an efflux ATPase that is responsible for membrane transport of lipophilic substances, including anticancer drugs and some

Correspondence to: Branislav Uhrík, Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Vlárská 5, 833 34 Bratislava 37, Slovakia
E-mail: branislav.uhrík@savba.sk

fluorescent substrates, against their concentration gradients (Orlický et al. 2004; for a review see Breier et al. 2005). P-gp mediated MDR in L1210/VCR cells may be reversed by pentoxifylline or its analogues (Breier et al. 1994a; Kupsáková et al. 2004; Dočolomanský et al. 2005).

Basic ultrastructural features of L1210 cells were described by Brandes et al. (1966). Both the solid and ascites forms of L1210 cells were investigated, the solid form being studied in lymph nodes and in spleen.

The data on structural differences between parental, drug-sensitive L1210 cells and the resistant L1210 cells overexpressing P-gp are relatively scarce.

Breier et al. (1994b) have found an increase in the mean cell diameter in the VCR-resistant L1210 cells. In a comparative study, Uhrík et al. (1994) described parental L1210 cells exhibiting a relatively smooth elliptic shape whereas the appearance of resistant cells was altered by numerous villous projections and cytoplasmic protrusions of the cell surface. These cytoplasmic protrusions contained cytoplasm with a number of ribosomes. In a subsequent study (Breier et al. 1994a), both damaged sensitive L1210 cells and damaged resistant L1210 cells, pre-treated with a reversal agent pentoxifylline, after their exposure to VCR were shown.

Since a high metabolic burden on cells overexpressing P-gp may be assumed, significant differences in ultrastructural appearance between sensitive cells and cells resistant against drugs and transporting these drugs against concentration gradient may be expected. The study of these differences and their underlying mechanisms may help elucidate the characteristics of the cellular machinery in both sensitive and resistant cell lines.

In the present comparative study, the main attention was paid to surface properties of cells and to structures and organelles engaged in protein synthesis and transport (nuclei, ribosomes, endoplasmic reticulum, Golgi apparatus). The main results of this study have been reported and published in abstract form (Uhrík et al. 2005).

Materials and Methods

Cells and cultivation conditions

Two sublines of murine leukaemic cell line L1210 were used: the sensitive (parental) cell line L1210, and the MDR cell subline L1210/VCR. The resistant cell subline was obtained by long-term adaptation of L1210 cells to VCR (Poleková et al. 1992). Cell cultivation was carried out in standard RPMI 1640 medium (Life Technologies Ltd., Paisley, Scotland), supplemented with heat inactivated foetal bovine serum (4%, Life Technologies Ltd. Paisley, Scotland), glutamine (1 mg/ml) and gentamycin (1 mg/l) in an atmosphere of 5% CO₂ at 37°C. The resistant cell line was cultured in the absence or presence of 0.2 mg/l of VCR.

Light microscopy

Cells in Petri dishes with culture medium were observed under an Axiovert 40C (Carl Zeiss) microscope and photographs were taken with a Canon PowerShot G6 digital camera.

Preparation of cells for electron microscopy

After cultivation, cells were centrifuged at $1000 \times g$ for 3 min, washed in phosphate buffer saline (PBS) and fixed for 60 min with 2% glutaraldehyde in 0.1 mol/l Na-cacodylate buffer (CB) at pH 7.2. During fixation, the cells were centrifuged at $1000 \times g$ for 3 min to form a sediment. The specimen was then washed 3 times for 7 min with CB and postfixed with 1% osmium tetroxide in CB for 60 min. The samples were rinsed in H₂O, stained overnight with 2% uranyl acetate, dehydrated in increasing concentrations of ethanol, cleared in propylene oxide and embedded in Durcupan. Ultrathin sections were cut with a microtome Porter-Blum MT2, stained with lead citrate and examined in a JEOL JEM-1200 EX electron microscope at 80 kV.

Staining of the cell surface with ruthenium red

Polycationic dye ruthenium red (RR) stains negative binding sites in external coats of cells and has been used as a marker of glycoconjugates in cells of different origin (Luft 1964, 1971a,b; Vorbrodt and Koprowski 1969; Rosenfeld et al. 1973). The cell coat is rich in carbohydrates incorporated into glycoproteins, glycolipids, and proteoglycans carrying a negative charge at physiological pH. The cell pellet after centrifugation was stained with 0.5 mg/ml RR in PBS for 60 min at 37°C, fixed with 2% glutaraldehyde in CB with 0.5 mg/ml RR for 75 min, and washed (3×10 min) in the same buffer with RR. Next the pellet was postfixed with 1% OsO₄ in the same buffer with RR for 75 min, then washed 3×10 min in a water solution of RR (0.5 mg/ml) and 2 min in 0.5 mg/ml RR in 50% ethanol. After dehydration in absolute ethanol, the samples were cleared in propylene oxide and embedded in Durcupan. Ultrathin sections were cut from the periphery of the pellets where the RR staining was maximal (El-Saggan and Uhrík 2002) and examined either unstained or stained with lead citrate.

Results

The shape and size of the cells

Sensitive L1210 cells have a relatively smooth elliptic shape and the surface is covered with villous projections (Fig. 1); resistant L1210 cells are generally larger with higher density of microvilli (Fig. 2).

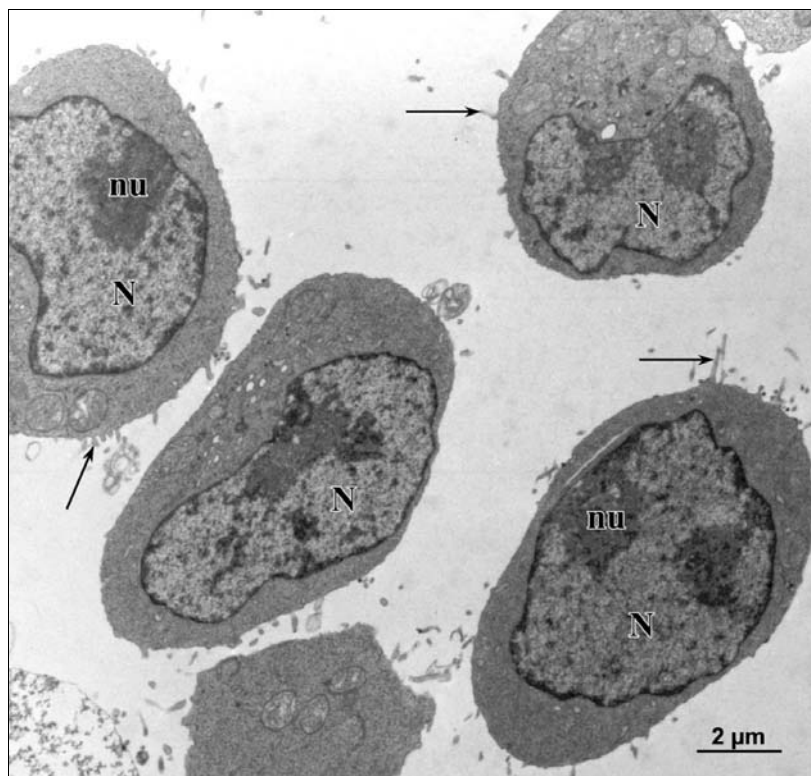


Figure 1. Sensitive L1210 cells. They have mostly a round or elliptic shape. The surface is covered with microvilli. The nuclei (N) are compact with a major part of the DNA contained within condensed heterochromatin masses. nu, nucleoli; arrows – microvilli.

Cell clustering

In electron micrographs containing a group of cells, cells were found to be closer to each other in L1210/VCR cells (Fig. 2) than in sensitive cells (Fig. 1). This tendency to aggregation in L1210/VCR cells resulted frequently in compact clusters with barely visible cell borders (Fig. 3). The same pattern of cell clustering could be observed also in light micrographs (Fig. 4).

These differences in cell aggregation persisted even after staining of external coat (glycocalyx) of cells with a polycationic dye RR (Figs. 5, 6). In accordance with previous findings (Fiala et al. 2003), the layer stained with RR was compact and thick in sensitive cells in contrast to resistant cells, where the layer was thin and discontinuous at places.

Nuclei

The DNA in nuclei of sensitive L1210 cells is arranged predominantly as hete-

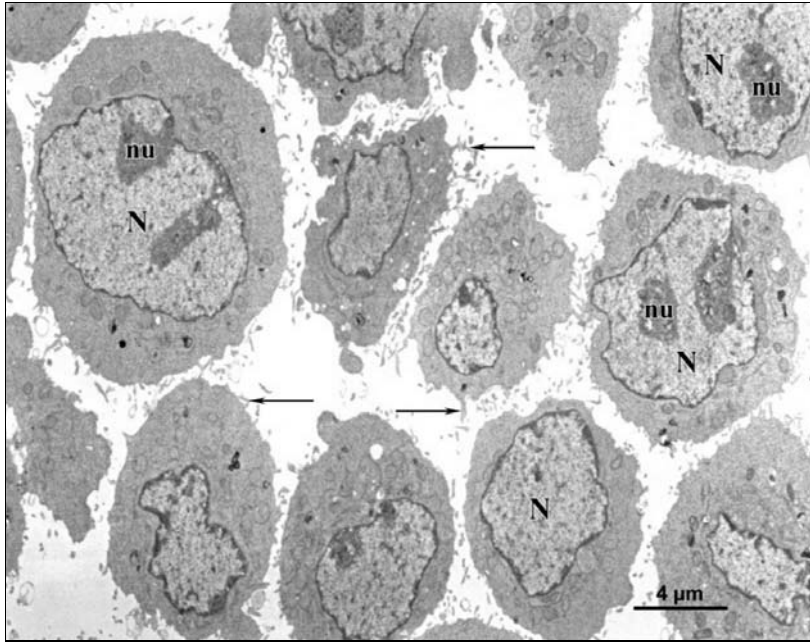


Figure 2. Resistant L1210/VCR cells cultured in the presence of VCR with finger-like protrusions and microvilli. The surface is more corrugated than in sensitive cells. In nuclei (N), a higher amount of euchromatin is present. nu, nucleoli; arrows – microvilli.

rochromatin. In resistant L1210/VCR cells, a higher amount of euchromatin was marked. Nucleoli are well developed, especially in resistant cells (compare Figs. 1 and 2).

Cytoplasmic organelles

In both L1210 and L1210/VCR cells, mitochondria have a round shape with irregular cristae (Figs. 7, 8, 9). Golgi apparatus consists of flattened saccules, vesicles and vacuoles, is located in the vicinity of nucleus and is especially well developed in resistant cells (Fig. 7). The cytoplasm is densely studded with ribosomes. Both smooth and rough endoplasmic reticulum is purely developed in sensitive cells (Fig. 8). In resistant cells, an increase in density of rough endoplasmic reticulum is marked, especially in cells grown in the presence of drugs (Fig. 9). Free ribosomes in sensitive cells showed little tendency to aggregate into polyribosomes (Fig. 8) whereas in resistant cells this aggregation into tetrameric and pentameric polyribosomes was pronounced (Figs. 7b and 9). Lipid droplets (Fig. 7a) were present in both L1210 and L1210/VCR cells, multivesicular bodies (Fig. 8) could be seen occasionally.

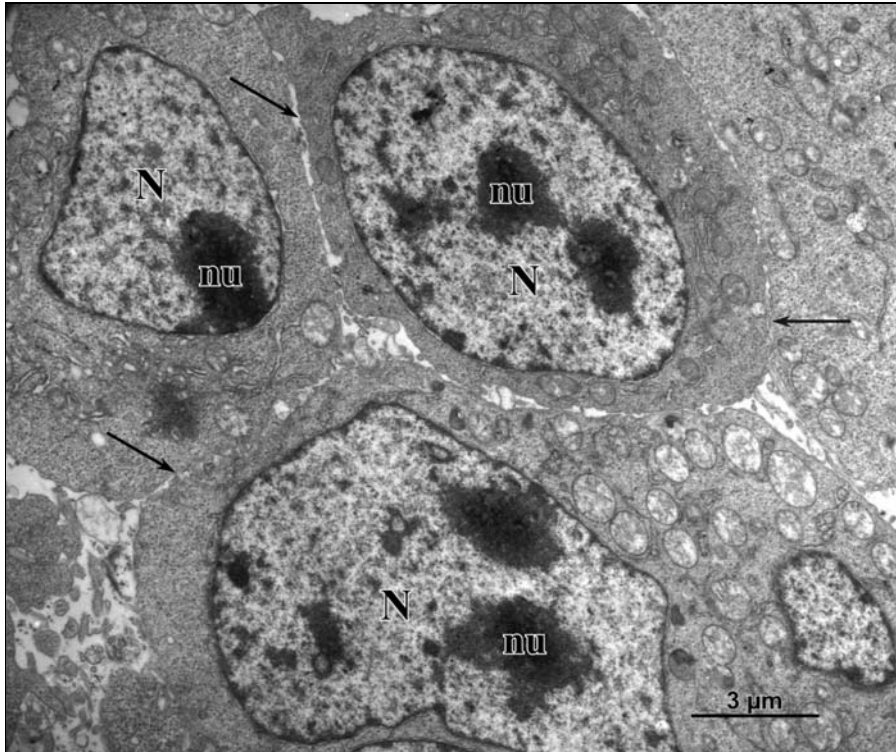


Figure 3. A cluster of tightly packed L1210/VCR cells cultured in the presence of VCR. N, nuclei; nu, nucleoli; arrows – cell borders.

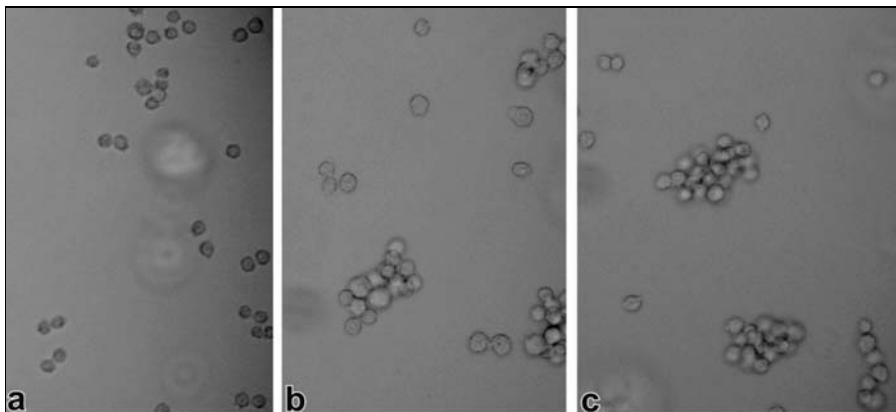


Figure 4. L1210 cells as seen in the light microscope. a) Sensitive cells in the culture medium. b) Resistant cells in the culture medium without VCR. c) Resistant cells in the culture medium with VCR. Note clustering of cells in b and c.

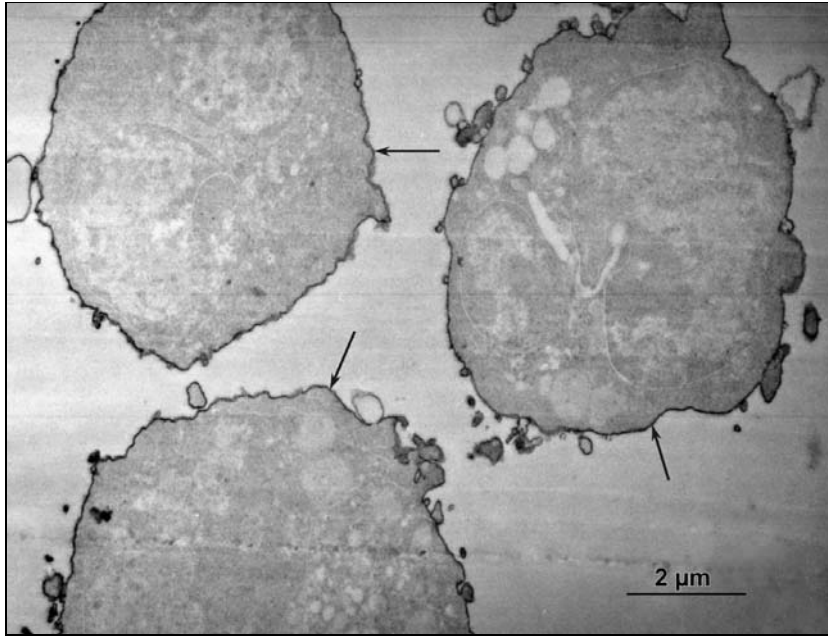


Figure 5. A heavily stained compact layer of RR (*arrows*) at the periphery of the sensitive L1210 cells.

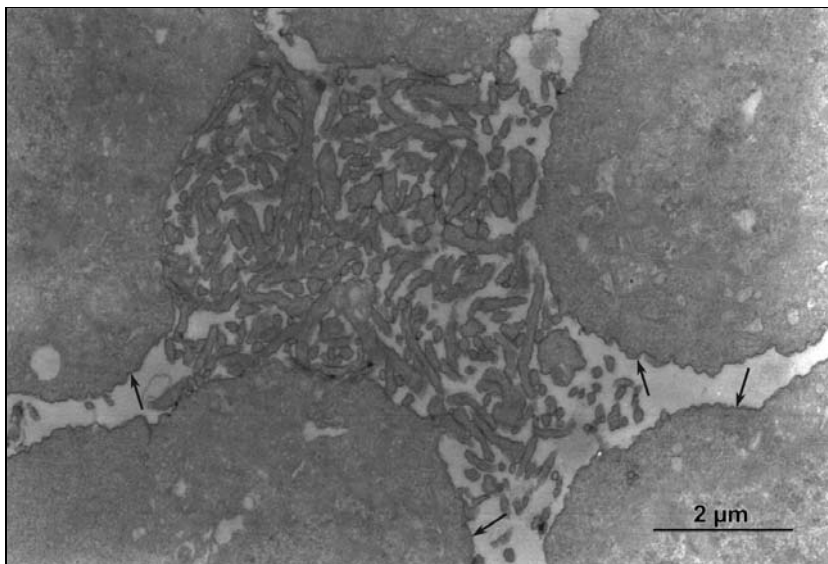


Figure 6. A cluster of 5 resistant L1210/VCR cells stained with RR with intermingled microvilli. Note the faint staining (*arrows*) of the glycocalyx.

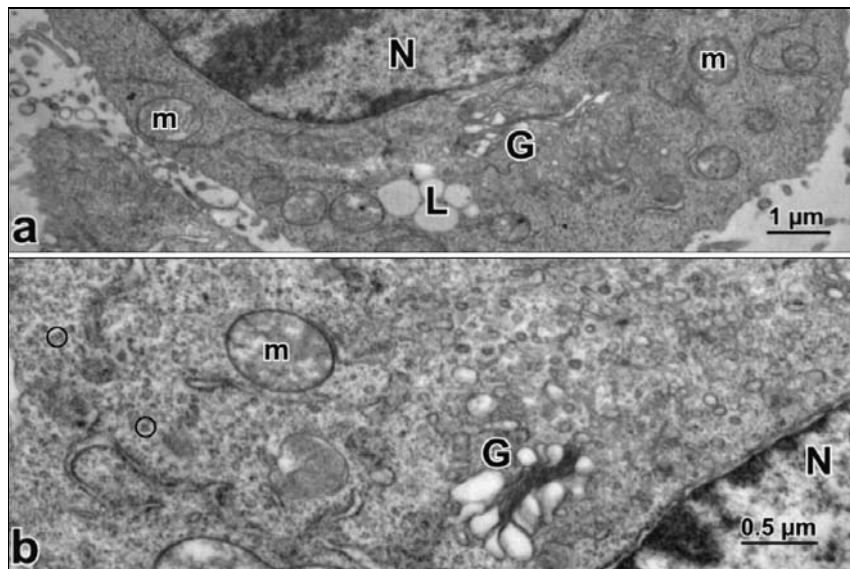


Figure 7. a) Resistant L1210 cell with Golgi apparatus (G) and lipid droplets (L). b) Resistant L1210 cell with prominent Golgi apparatus and free ribosomes aggregated into polyribosomes (*circles*). The cells in a and b were grown in the presence of VCR. N, nuclei; m, mitochondria.

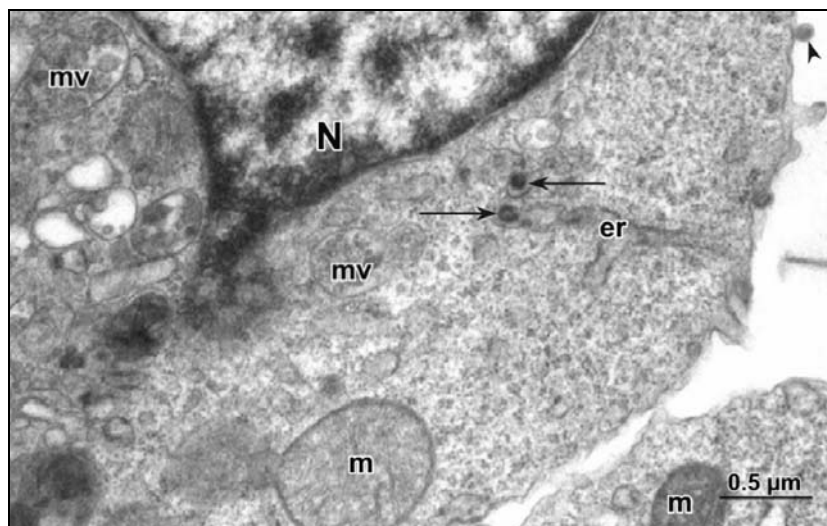


Figure 8. Sensitive L1210 cell with poorly developed endoplasmic reticulum (er) with virus particles inside (*arrows*). Free ribosomes are mostly dispersed with only limited tendency to aggregate into polyribosomes. N, nucleus; m, mitochondria; mv, multivesicular bodies; *arrowhead* – virus particle budding from the cell membrane.

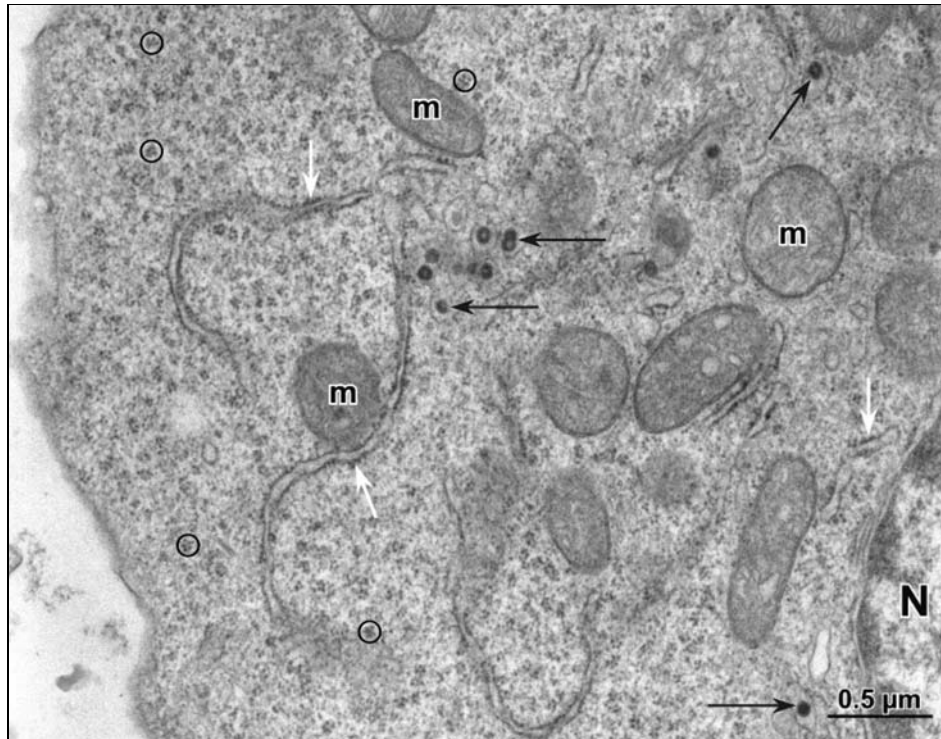


Figure 9. Resistant L1210 cell grown in culture medium with VCR. Free ribosomes are aggregated into tetrameric or pentameric polyribosomes (*circles*). N, nucleus; m, mitochondria; *white arrows* – rough surfaced endoplasmic reticulum; *black arrows* – virus particles.

Virus particles

Virus particles are a characteristic feature of L1210 cells and can be encountered in the cytoplasm, predominantly inside of vesicles of endoplasmic reticulum (Figs. 8, 9). They leave the cell (Fig. 8) and may be found also in the extracellular space. Frequency and localization of virus particles was similar in both L1210 and L1210/VCR cells.

Discussion

Structural differences between sensitive and resistant L1210 cells are pronounced and concern especially cell membranes with external coat (lamina externa, glycocalyx). Proliferation of villous projections and cytoplasmic protrusions of the cell surface is a characteristic feature of resistant cells. The highly corrugated cell membrane increases efficiently the area necessary for drug extrusion *via* P-gp transporters.

Decrease in the number of negative binding sites in the lamina externa of resistant cells, as visualised by staining with the polycationic dye RR, is not only a sign of alteration in oligo- and polysaccharides metabolism (Fiala et al. 2003) but may be related also to changes in cell aggregation, with a tendency of resistant cells to form clusters. If this tendency is determined mainly by changes in the number and distribution of negative charges in glycocalyx or by expression of adhesion molecules (for a review, see Vitte et al. 2005), should be investigated in future studies. No correlation between P-gp expression and expression of an adhesion molecule CD56 in acute myeloid leukaemia has been reported recently (Raspadori et al. 2002; Suvannasankha et al. 2004). The aggregation of resistant L1210/VCR cells observed in our study was rather weak, the cells were disaggregated readily by gently shaking the culture dishes.

Differences in structures involved in proteosynthetic machinery and transport of substances are evidently an expression of different metabolic conditions in sensitive and resistant L1210 cells. Increased density of free ribosomes and their aggregation into tetrameres and pentameres in resistant cells together with a more developed rough surfaced endoplasmic reticulum and vesicles of Golgi apparatus are a sign of enhanced proteosynthetic activity. A higher proportion of euchromatin to heterochromatin in nuclei of L1210/VCR cells may be interpreted along the same line. Similar changes in chromatin texture have been described by Dufer et al. (1995) in MDR human leukaemic cell lines.

Immunocytochemical studies on different drug resistant neoplastic cell lines performed at light and electron microscopic levels have demonstrated the presence of P-gp not only at the plasma membrane but also on the membranes of intracellular organelles, predominantly vesicles of the Golgi system and the lysosomal system (Boiocchi et al. 1992; Molinari et al. 1994; Bobichon et al. 1996).

MDR-associated drug sequestration in intracellular vesicles is an active process and also depends upon intracellular pH gradients. Cleary et al. (1997) have found that in addition to P-gp, which mediates a reduction in cellular drug accumulation in the human lung cell line DLKP-A10, an alternative transport mechanism – an ATP-dependent vesicular sequestration is present. The VCR trapping in these vesicles and its transport out of cell may be pH-dependent: since VCR molecule is a weak base, it is accumulated in acidic membrane vesicles, and any shift in cellular pH affects the rates of vesicular transport and exocytosis (Dalmark 1981; Van Adelsberg and Al-Awqati 1986).

Virus particles in both sensitive L1210 and resistant L1210/VCR cells did not differ in their localization, predominantly in vesicles of endoplasmic reticulum. Development of multidrug resistance has evidently no influence on the frequency and site of expression of virus particles. They can be considered a sign of an intact proteosynthetic machinery and of a functioning intracellular vesicular transport.

In conclusion, it can be stated that overexpression of P-gp by VCR in L1210/VCR cells is accompanied by changes in cell metabolism which are reflected by modification of cell architecture, decreased number of negative binding sites at the external coat and also by altered Ca^{2+} sensitivity and cytochemically determined

Ca²⁺ distribution as described previously (Sulová et al. 2005). Interestingly, P-gp overexpression induced by doxorubicin was associated with similar changes both in ultrastructure and in mechanisms of multidrug resistance (Boháčová et al. 2006) as in the present study.

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