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Original Paper

Rapid Recovery of a Functional MDR Phenotype Caused by MRP After a Transient Exposure to MDR Drugs in a Revertant Human Lung Cancer Cell Line

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Prior studies have shown that, in some human tumour cells, increased expression of the multidrug resistance gene *MDR1* can be induced in response to certain stress conditions such as a transient exposure to cytotoxic agents. Little is known about the possibility of increasing the expression of the recently cloned multidrug resistance-associated protein (MRP) in response to a transient exposure to cytotoxic drugs. In order to examine this possibility, we have used sensitive assays (RT-PCR, flow cytometry) and the sensitive large cell lung cancer cell line, COR-L23/P, and the revertant line (COR-L23/Rev), generated by growing the doxorubicin-selected, MRP-overexpressing resistant variant COR-L23/R without drug exposure for 24–28 weeks. COR-L23/Rev overexpresses MRP, but to a lesser extent than COR-L23/R. COR-L23/Rev rapidly recovered similar levels of MRP mRNA, protein expression, resistance and drug accumulation deficit as COR-L23/R after a 48–72 h exposure to cytotoxic concentrations of doxorubicin or vincristine but not cisplatin. The increase in MRP mRNA could only be detected 3 to 4 days after the transient exposure to drugs. However, when the parental line, COR-L23/P, was exposed to equitoxic doses of doxorubicin, vincristine or cisplatin, no increase in the levels of MRP mRNA could be observed at higher doses (5- to 10-fold the IC_{50}) of doxorubicin or vincristine (but not of cisplatin), we detected a transient increase in the levels of MDR1 mRNA immediately after short-term exposure. In conclusion, we have shown that a human revertant lung cancer cell line (COR-L23/Rev) has the ability to recover quickly, similar levels of MRP expression and resistance as COR-L23/R after a transient exposure to the MDR-drugs doxorubicin and vincristine.

Key words: MRP, MDR1, doxorubicin, vincristine, cisplatin, lung cancer, multidrug resistance

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INTRODUCTION

THE EXPRESSION of the *MDR1* gene is a well-known mechanism of multidrug resistance. The 170 kD product of this gene, P-glycoprotein (Pgp), is a drug efflux pump present in the membrane of cells of many cancers and normal human tissues [1]. More recently, a 190 kD transporter, the multidrug resistant-associated protein or MRP, has been isolated from cell lines that display a multidrug resistance phenotype, but do not overexpress Pgp [2]. The development of multidrug resistant cell lines (Pgp or non-Pgp mediated) usually involves one or more steps of cytotoxic selection with a prolonged exposure to the selecting drugs. These cells are cross-resistant to different natural product drugs such as anthracyclines, epipodophylotoxins or vinca alkaloids.

It has been shown that a rodent homologue of *MDR1* can be induced in several cell types in response to a short treatment with cytotoxic drugs [3]. Some studies have also shown that the expression of the human *MDR1* gene may be increased, under certain conditions of stress, by differen-

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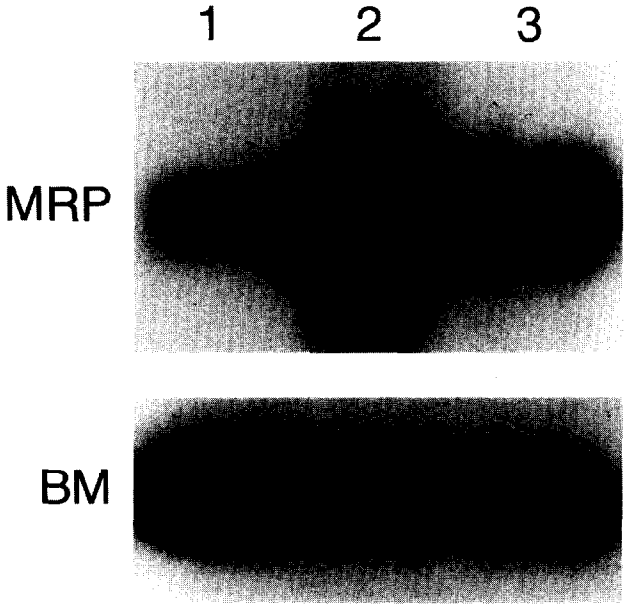

Figure 1. Relative MRP mRNA expression in COR-L23 cell lines as analysed by RT-PCR (as described in Materials and Methods). (1) COR-L23/P; (2) COR-L23/R; (3) COR-L23/Rev. BM, beta-microglobulin.

tiating agents [4], arsenite and heat shock [5]. However, only two previous reports have shown that a transient exposure to different drugs can induce MDR1 expression in human cancer cell lines *in vitro*. The first of these showed that a transient exposure to both MDR and non-MDR drugs can induce MDR1 expression in response to cellular damage in several drug-sensitive human tumour cell lines [6]. Another recent report demonstrated a rapid upregulation of MDR1 expression induced by short-term exposure to anthracyclines, but not to vinca alkaloids, in a leukaemia cell line with a low level of expression of Pgp following reversion of the multidrug-resistant phenotype in a cell line overexpressing Pgp [7]. However, the same phenomenon was not seen with the original parental sensitive leukaemia cell line.

It is not known whether such a transient exposure to drugs can result in increased MRP expression in human tumour cell lines from which resistant cells overexpressing MRP have been developed by stepwise selection *in vitro*. In order to examine the possibility of increasing the levels of expression of MRP after a transient exposure to cytotoxic drugs, we have used sensitive assays in the drug-sensitive large cell lung cancer cell line COR-L23/P and in the revertant line (COR-L23/Rev). COR-L23/Rev overexpresses MRP, but to a lesser extent than the resistant line. The relative expression of MRP mRNA of the three cell lines (COR-L23/P, COR-L23/R and COR-L23/Rev) is shown in Figure 1.

MATERIALS AND METHODS

Cell lines and drug treatment

The cell line used in this study, COR-L23/P, was derived from a human large cell lung carcinoma. The MDR variant (COR-L23/R) was developed by continuous exposure of the parent line to increasing concentrations of doxorubicin as described previously [8]. COR-L23/R has been previously shown to accumulate lower levels of doxorubicin than its parental line [8] and to overexpress MRP but not Pgp [9]. A revertant subline (henceforth COR-L23/Rev) was derived from COR-L23/R, by growing cells in the absence of doxorubicin for 24–28 weeks, and has been previously characterised [9]. The cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified incubator in 8% carbon dioxide. COR-L23 lines grow as attached monolayers on plastic. Cells ($1–3 \times 10^6$) were plated in 175 cm^2 plastic tissue-culture flasks in the absence or presence of doxorubicin, vincristine or cisplatin. Drug-treated cells were collected for RNA extraction, flow cytometric analysis or growth inhibition studies. For the growth inhibition studies, cells in triplicate were plated in 96-well plates (Falcon, Becton Dickinson, Cowley, U.K.) and allowed to grow in various concentrations of doxorubicin for a period of 6 days. Growth inhibition was determined in an MTT assay as described previously [10]. Signs of cytological damage, such as abnormal size or changes in granularity of the cells, were evaluated using phase-contrast microscopy at different time points during the exposure of the cells to the cytotoxic agents.

Chemicals

Doxorubicin and cisplatin were supplied by Pharmitalia (Milan, Italy) and Johnson-Matthey Technology Centre (Berkshire, U.K.), respectively. Vincristine hydrochloride was from Lederle (Gosport, Hampshire, U.K.). DL-Buthionine-S,R- sulphoximine (BSO) was from Sigma (Poole, Dorset, U.K.).

RNA preparation, complementary DNA synthesis and amplification by PCR

Total cytoplasmic RNA from drug-treated or untreated cells was prepared from PBS-washed cells lysed with guanidine hydrochloride, followed by ethanol precipitation and extraction with phenol/chloroform [11]. Complementary DNA (cDNA) synthesis and polymerase chain reaction (PCR) were carried out as described previously [9]. We determine the maximum number of PCR cycles providing for exponential amplification of the cDNA. The optimum number of PCR cycles was found to be 20 for MRP and beta-microglobulin, and 35 for MDR1 The MRP primers used are positioned at bases 4005–4024 (sense) and 4600–4619 (antisense). The MDR1 primers used are positioned at bases 2761–2780 (sense) and 3080–3099 (antisense). These sets of primers generate cDNA fragments of 614 bp for MRP and 340 bp for MDR1. In all experiments, amplification of a 297 bp fragment of the beta-microglobulin gene was included as an internal control of the amount of RNA and efficiency of transcription [12]. Only samples with adequate and similar levels of beta-microglobulin were compared. The PCR products were separated by subsequent electrophoresis in 1.4% agarose, transferred to Nylon filters by Southern blotting, hybridised with a [^{32}P]CTP oligolabelled DNA probe, and finally visualised by autoradiography. All the expression experiments with each of the three drugs, at the different concentrations and time-courses used, were repeated at least twice.

Cellular drug accumulation and flow cytometric assays

Cells in exponential growth were reduced to single cell suspension. In order to obtain single cell suspensions, the cells were subjected to two rinses with trypsin (0.4%) and versene (0.02%) in PBS and incubated for 15 min. Afterwards, the cells were harvested and resuspended ($2-5 \times 10^5$ cells/ml) in 20 mM Hepes-buffered, RPMI-1640 medium (pH 7.3) without sodium bicarbonate, but with 10% fetal bovine serum. In order to study the accumulation of doxorubicin, cells were incubated with the drug (2 μM) for 2 h at 37°C. Prior to the addition of doxorubicin and in order to examine the effect of BSO (MRP modifier) on the accumulation of doxorubicin, cells were cultured in the presence or absence of 25 μM BSO for 20 h.

The accumulation of doxorubicin was stopped by addition of ice-cold PBS. The cells were then pelleted in a microcentrifuge (1 min at 13 000 rpm) at room temperature. After removal of the supernatant, cells were resuspended in 0.5 ml of ice-cold PBS and the tubes were then kept on ice until cells were analysed by flow cytometry.

For the study of the expression of Pgp, COR-L23 cells (treated and non-treated) were labelled with FITC by indirect immunofluorescence with 2 μg of the primary antibody [antihuman Pgp specific mouse monoclonal antibody MRK-16 [13] (Kamiya Biomedical Company, Thousand Oaks, California, U.S.A.) or mouse IgG2a isotype control (Sigma)] and 5 μg of the secondary antibody [FITC-conjugated rabbit antimouse IgG (Dako Patts, High Wycombe, Bucks, U.K.)].

MRP protein expression

Membrane preparations were used to detect MRP by Western blotting with a rat monoclonal antibody MRPrl [14], kindly donated by Prof. R. Scheper. Membrane proteins were isolated from a post-nuclei homogenate by centrifugation at 60 000*g* for 1 h at 4°C. Membrane proteins (20 μg per well) were fractionated on a 7.5% (w/v) polyacrylamide gel and electroblotted on to nitrocellulose. The filters were first incubated for 24 h at 4°C in blocking buffer containing 5 mM EDTA, 0.25% gelatine, 0.01 M NaN_3, 0.15 M NaCl, 0.05 M Tris at pH 7.4, and 0.05 Nonidet P-40. They were then incubated in antibody MRPrl, diluted 1:3000 in blocking buffer for 5 h, and finally with [^{125}I]-labelled sheep antirat immunoglobulin (Amersham, Bucks, U.K.), diluted 1:1000 in blocking buffer before autoradiography.

RESULTS

Studies in the revertant cell line COR-L23/Rev

In order to examine the possibility of increasing MRP expression after a short-term exposure to different drugs, we initially tested doxorubicin (DOX) in the COR-L23/Rev cell line. Under the most frequently used treatment conditions (0.2 and 0.4 μM DOX for 48–72 h), we found an increase in the levels of MRP in the treated cells in comparison with the control non-treated cells. This increase could not be detected when the cells were collected immediately after the treatment, but was detected when the cells were kept in drug-free medium for 3–7 days after the experiment and then collected for analysis of protein expression (Figure 2). The increase in MRP was dose-related, being maximal with doses of 0.4 μM DOX. Higher doses of DOX did not result in further increase in the levels of MRP. Under conditions of maximal increase in the levels of MRP, the treated cells achieved nearly identical levels of MRP to those of COR-L23/R.

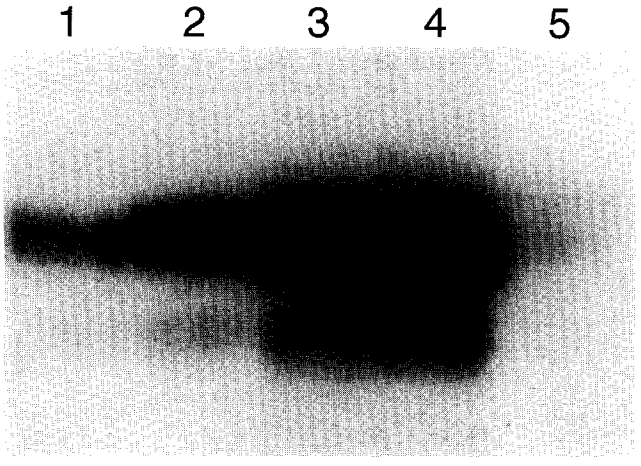

Figure 2. MRP protein expression in COR-L23/Rev treated (3-day exposure to doxorubicin) and resistant cells. Cells were kept in drug-free medium for 7 days after the experiment and then collected for membrane preparations and subsequent detection of MRP by Western blotting (as described in Materials and Methods). (1) COR-L23/Rev control; (2) COR-L23/Rev treated with 0.2 μM doxorubicin; (3) COR-L23/Rev treated with 0.4 μM doxorubicin; (4) COR-L23/R; (5) CEM/VBL (cell line overexpressing Pgp). The experiment was confirmed by repetition.

The concentrations of DOX used to increase MRP are higher than the IC_{50} value. Counting the number of cells before and after a 3-day exposure to DOX, we found a relative inhibition of growth (in comparison with the control cells) of: $36.2 \pm 6.2\%$ with 0.2 μM and $23.6 \pm 1.3\%$ with 0.4 μM (means and standard deviation (S.D.) of three independent experiments). We only observed the increase in MRP expression when signs of cytotoxic damage of the cells, such as increased granularity and cell swelling, were apparent. The levels of MRP mRNA did not increase when we used doses of 0.2 or 0.4 μM for 24 h or when we used doses of 0.1 μM for 3 days (two experiments), and accordingly after such drug exposures, signs of cytological damage were absent.

In each of the experiments carried out, Pgp could not be detected in the treated cells either by flow cytometry with the specific antibody MRK-16 or by Western blot using the antibody C219 (data not shown).

We also tested two other chemotherapeutic drugs at equitoxic dose levels: vincristine (VCR), which belongs to the MDR phenotype, and cisplatin which does not. We found an increase in the levels of MRP mRNA after treating the revertant cells with cytotoxic doses of VCR for 48–72 h (Figure 3).

To determine whether drug-induced MRP expression in the revertant cell lines was stable or transient, we treated the cells with cytotoxic concentrations of DOX or VCR for 3 days and the allowed them to grow in the absence of drugs. MRP mRNA expression in the surviving cells was analysed by RT-PCR at different time points. We found that the increased expression of MRP mRNA was maintained for at least 3 weeks after the drug exposure (Figure 3).

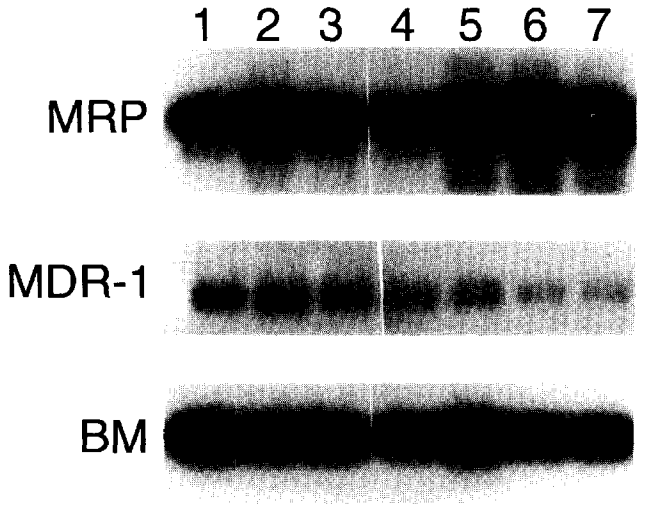

Figure 3. mRNA expression in COR-L23/Rev (all the treated cells were exposed to 3 days of drug treatment) as analysed by RT-PCR. (1) Control; (2) 0.2 μM doxorubicin at 2 weeks after the exposure; (3) 0.39 μM cisplatin at 1 week after the exposure; (4) 0.78 μM cisplatin at 1 week after the exposure; (5) 0.02 μg/ml vincristine at 1 week after the exposure; (6) 0.04 μg/ml vincristine at 18 days after the exposure; (7) 0.4 μM doxorubicin at 18 days after the exposure. BM, beta-microglobulin.

To explore further whether this increase in the MRP mRNA expression and protein level in the DOX treated cells were correlated with a modification in the functional status of these cells in comparison with the control cells, we performed growth inhibition studies and drug accumulation studies in parallel. The results of the MTT assays with increasing concentrations of DOX were in agreement with the increase in MRP levels. At 10 to 15 days after a 3-day exposure to DOX, the treated cells were more resistant than the control non-treated cells. The mean IC_{50} values of the treated cells increased 2–3.8-fold depending on the dose of DOX used (Figure 4). In the DOX treated cells, there was

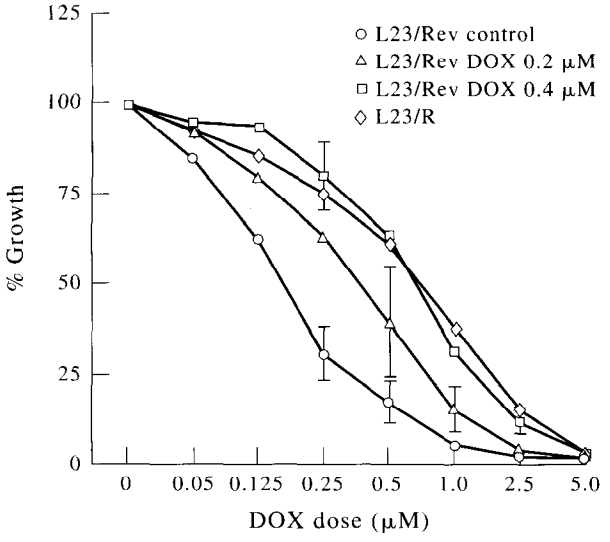

Figure 4. Cytotoxic effect of a 3-day exposure to doxorubicin (DOX) in COR-L23/Rev (the results are the mean and SD of three independent experiments).

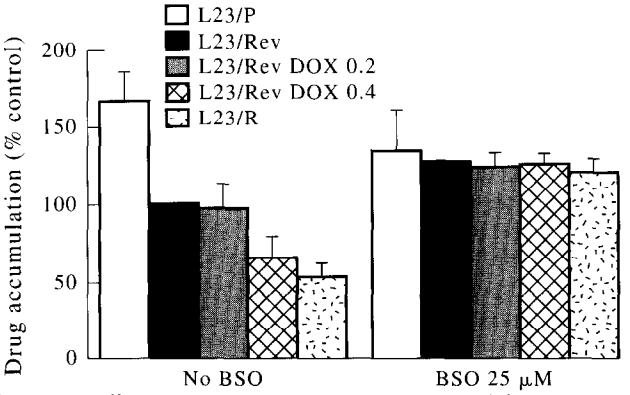

Figure 5. Effect of a 3-day exposure to doxorubicin upon the accumulation of doxorubicin in COR-L23/Rev. The cells were kept in drug-free medium for 10 days after the exposure and then collected for the experiment as described in Materials and Methods (the results are the mean of three independent experiments).

also a drug accumulation deficit in accordance with the increased levels of MRP (Figure 5). This accumulation deficit was reversed by a 20 h exposure to 25 μM BSO prior to the accumulation assay. It has been shown previously that BSO can revert the drug accumulation deficit induced by overexpression of MRP, but not that induced by Pgp [15].

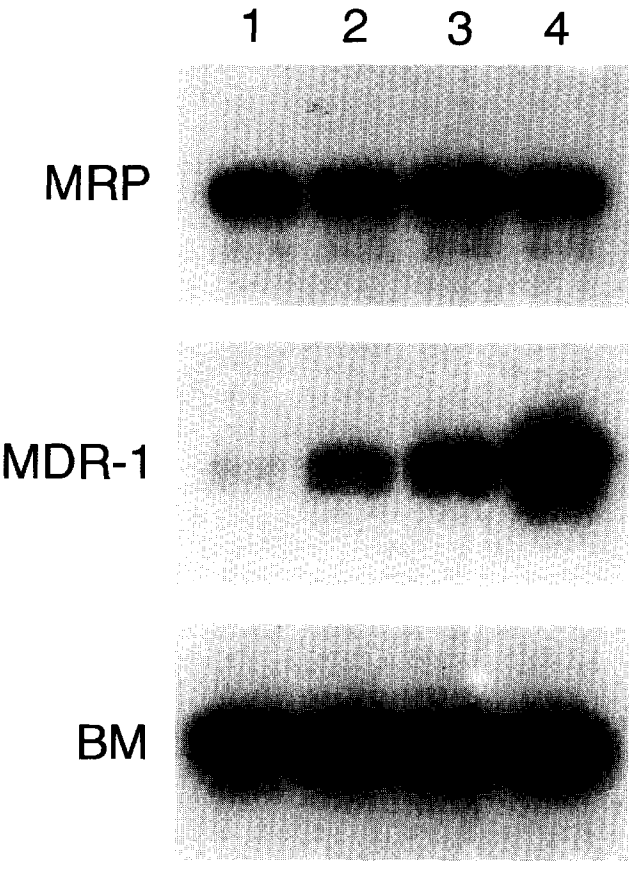

Figure 6. mRNA expression in COR-L23/P treated with a 3-day exposure of drugs (all the cells were collected immediately after the exposure). (1) Control; (2) 0.01 μg/ml vincristine; (3) 0.4 μM doxorubicin; (4) 1 μM doxorubicin. The experiment was confirmed by repetition. BM, beta-microglobulin.

A similar trend was observed when daunorubicin accumulation assays were performed (data not shown).

Studies with the parental line COR-L23/P

We did not find modifications in the levels of MRP mRNA after treating COR-L23/P cells with doses of DOX, VCR or cisplatin equitoxic to those used in COR-L23/Rev. However, we observed a dose-related increase in MDR1 mRNA levels after treatment with much higher concentrations of DOX and VCR (Figure 6) but not with cisplatin (data not shown). These results were confirmed with further experiments. MDR1 mRNA upregulation (but not MRP) was found in two to three experiments with a 3-day exposure to doses of DOX higher than 0.1 μM (range of doses used: 0.05–1 μM). These effects could not be observed when the same doses were used only for 1 day (range of exposures used: 1–6 days). Similarly, a 24-h exposure to the concentrations of VCR used (0.01 to 0.1 μg/ml) was not enough to increase MDR1 expression. The doses of DOX and VCR needed to increase MDR1 expression were 5–10 times higher than the IC_{50}. An increase in the level of protein expression as analysed by flow cytometry using the specific antihuman Pgp monoclonal antibody MRK-16 in the treated cells was not found, probably owing to lack of sensitivity to detect very low levels of Pgp (data not shown). In addition, the observed increase was not a stable effect. When we treated the cells with different cytotoxic drug concentrations for 3 days and then allowed them to grow in the absence of drugs for one week, we no longer detected the increase of MDR1 mRNA seen just after the drug exposure.

DISCUSSION

Although there are studies showing an increase in MDR1 expression in human cancer cell lines *in vitro* as a consequence of short-term exposure to cytotoxic drugs [6, 7], it is not known whether the same phenomenon can happen with the recently cloned *MRP* gene, overexpression of which also causes the appearance of the MDR phenotype in tumour cells.

We found that treatment with DOX and VCR of a revertant cell line from COR-L23/R, a human lung cancer cell line overexpressing MRP, resulted in an increased expression of MRP. This increase was maintained for at least 3 weeks and was accompanied by an increase in drug resistance and a drug-accumulation deficit. We observed that the incrase in MRP expression was related to the appearance of cytological damage. A prior report linked the appearance of cytological damage after drug exposure with the induction of MDR1 expression in different sensitive cell lines [6]. In this report, cytological damage, induced by short-term exposure to DOX or VCR, in a revertant cell line overexpressing MRP, did not induce any modification of the MDR1 expression, but did cause an increased expression of MRP. Interestingly, cytological damage induced by the same two drugs in the parental line COR-L23/P did not modify the basal MRP levels and induced a transient increase in the levels of MDR1 mRNA. In our study, we did not find any increase in the levels of MDR1 mRNA after treatment of the sensitive cell line COR-L23/P with different doses of cisplatin, a non-MDR drug. Chaudhary and Roninson [6] showed a stable increase in the levels of both MDR1 mRNA and Pgp after short-term exposure of several cell lines to different drugs, including drugs affected by the MDR phenotype and others not affected by this phenotype, such as cisplatin and ara-C. However, they failed to find such an increase in one of the sensitive cell lines studied.

After short-term exposure to drugs, we did not find a simultaneous increase in both MRP and MDR1. A more prolonged exposure is probably needed to obtain a stable coexpression of both genes, and apparently the appearance of MDR1 overexpression is a late event in some cells that initially overexpress MRP [16].

Under conditions causing maximal expression of MRP, we found that COR-L23/Rev quickly acquired increased levels of MRP and resistance nearly identical to those of the resistant cell line COR-L23/R, from which it is derived. Higher doses of DOX or VCR did not result in higher MRP levels. Although a continuous and prolonged exposure of the sensitive parental cell line COR-L23/P to the selecting drug was needed to achieve the high levels of MRP expression found in COR-L23/R, only 48–72 h of drug exposure of COR-L23/Rev was needed to recover the high levels of MRP present in COR-L23/R. This upregulation of MRP expression is apparently the consequence of selection rather than induction. There are several facts supporting this view. Firstly, although the upregulation of MRP occurs after short-term exposure to DOX or VCR, the increased levels of MRP mRNA could not be detected immediately afater the drug exposure, but only 3–4 days after drug removal. Secondly, treatment of the cells for less than 48 h with the most commonly used doses of drugs did not result in a consistent increase in MRP mRNA levels, and those lengths of exposure were not acccompanied by signs of cytological damage. Thirdly, only DOX and VCR (agents belong to the MDR phenotype) but not cisplatin, were found to increase the expression of MRP in the surviving cells. These agents can select cells with high expression of MRP. Whether induction contributed to some extent to the increased levels of MRP observed in the treated revertant cell line is not, however, clear and cannot be fully ruled out.

A prior report [7] showed how a revertant leukaemia cell line, derived from a MDR1 overexpressing cell line, can be induced to overexpress MDR1 shortly after a transient exposure to anthracyclines but not to vinca alkaloids. The levels of MDR1 expression of the induced revertant line were lower than those of the resistant line, but data comparing the levels of resistance (and the accumulation deficit) of the induced revertant leukaemia cell line with those of the resistant cell line from which it is derived, are not available. Therefore, it is not possible to know whether the treated cells recovered the full functional MDR phenotype present in the original resistant cell line. In our report, we show that a full functional recovery of the MDR phenotype caused by overexpression of MRP is rapidly achieved after a transient exposure to DOX or VCR of COR-L23/Rev. This ability of the revertant cell line to recover fully the MDR phenotype of the original resistant cell line is interesting because of its potential clinical implications. It could be that once tumour cells acquire high levels of MRP, the expression of MRP could be rapidly upregulated again after short-term exposure to certain chemotherapeutic drugs, even after a long off-therapy time, most probably as a consequence of selection.

Testing this hypothesis will be require further *in vitro* and clinical studies.

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