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HIGH CELL DENSITY-DEPENDENT RESISTANCE AND P-GLYCOPROTEIN-MEDIATED MULTIDRUG RESISTANCE IN MITOXANTRONE-SELECTED CHINESE HAMSTER CELLS

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Abstract—Mitoxantrone (MIT) resistance has been studied in a colony selected from the CHO AA8 parental line in one step under a low degree of selective pressure (9 nM). The cells of the clonal isolate AA8/MIT C1(0) were sensitive to 9 nM MIT at low cell density but able to grow at high density. Parental AA8 cells were not able to grow under the latter condition. Decreased MIT accumulation (−20%) was observed at this step (step 0) in the absence of overexpression of *mdr* RNA coding for the drug efflux pump P-glycoprotein. Furthermore, AA8/MIT C1(0) did not exhibit cross resistance to vincristine, Adriamycin and etoposide at low cell density. During subsequent controlled growth for 2 months at high cell density in the presence of 9 nM drug, an additional selection occurred leading to a 4-fold MIT-resistant subline AA8/MIT C1(+). This subline was characterized at this step (step I) and after an additional 4 months of culture in the presence of 9 nM MIT (step II). Analysis of *mdr* gene expression and gene copy number showed an increase in *mdr* RNA and a pattern of *mdr* gene amplification which changed between step I and II. AA8/MIT C1(+)II exhibited a classical multidrug resistance phenotype with decreased accumulation of [^{14}C]MIT and cross-resistance to vincristine, Adriamycin and etoposide. The ability to form the cleavable complex in the presence of etoposide in DNA topoisomerase II-containing nuclear extracts was identical in AA8/MIT C1(+)II and AA8 cell lines. These results demonstrate a new sequence of events in MIT resistance: low level of drug resistance at high cell density followed by *mdr* gene amplification.

Development of cell resistance to anticancer drugs limits the activity of cancer chemotherapy against malignant tumors. Cell lines that have been selected *in vitro* for their resistance to one drug have alterations that may account for their resistance [1, 2]. Among these modifications, decreased drug accumulation via an overexpression of the membrane drug efflux pump P-glycoprotein (PGP†) coded by *mdr* gene (for a review, see Ref. 3) and DNA topoisomerase II alterations (for a review, see Ref. 4) are particularly important because they may confer cross-resistance to several unrelated chemotherapeutic agents.

Studies of the mechanisms of resistance to cytotoxic drugs carried out on cells exhibiting a high level of resistance have revealed genetic changes [5] which may have implications in cancer chemotherapy [6]. In the case of the multidrug resistance (MDR) phenotype mediated by a membrane PGP, *mdr* gene amplification is generally observed in resistant cell lines of different species [7–9]. However, it has been shown that an increased expression of *mdr*1 sequences can occur prior to gene amplification during the development of increasing levels of MDR in human cells [10]. Furthermore, it has been reported that selection of MDR human melanoma cells for a low level of resistance to *Vinca* alkaloids and colchicine resulted in a stable, increased *mdr* gene expression without DNA amplification [11] indicating that different genetic alterations may be responsible for low and high levels of resistance. Low level (up to about 10-fold) rather than high level (over 100-fold) resistance is likely to occur in refractory human tumors. Therefore, it is of interest to investigate mechanisms implicated in weak resistance to anticancer drugs.

Among new drugs designed from known chemical structures, mitoxantrone (MIT) is an active anthracene derivative used in the treatment of leukemias and breast cancer with less toxicity than Adriamycin (for a review, see Ref. 12). Like anthracyclines, MIT intercalates into DNA and induces both protein-associated and non-protein-associated DNA strand breaks [13]. In addition, this drug is able to stabilize DNA topoisomerase II–DNA cleavable complex [14]. Cross-resistance to MIT has been found in an epipodophyllotoxin-resistant cell line in which cleavage activity is poorly stimulated by etoposide, a DNA topoisomerase II inhibitor [15]. These results suggest that MIT might act via an interaction with DNA topoisomerase II. On the other hand, few experimental models of MIT resistance have been reported and the cellular

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† Abbreviations: PGP, P-glycoprotein; MDR, multidrug resistant or multidrug resistance; *mdr*, MDR-associated gene symbol in general; *pgp*, MDR-associated gene symbol in hamster species; MIT, mitoxantrone hydrochloride; PBS, phosphate-buffered saline; TE, Tris-EDTA (10 mM Tris–HCl, pH 7.5; 1 mM EDTA, pH 8).

mechanisms of resistance are not established. Human colon carcinoma [16], leukemia [17] and gastric carcinoma [18] resistant cell lines have been selected by continuous exposure to increasing concentrations of the drug. In these studies no PGP overexpression was observed but chromosome alterations, limited or pleiotropic cross-resistance, or membrane vesicle formation were reported.

In order to determine whether low MIT concentrations may select DNA topoisomerase II modifications, PGP overexpression or other phenotypic alterations, we isolated nine CHO colonies using a low degree of selection pressure. Some data suggested that the resistance of eight colonies depends on cell density. One of these colonies was further characterized and we found that the cells are initially resistant to MIT at high density only. During subsequent growth at high density in the presence of drug, a second selection occurred leading to a new MIT-resistant cell line. This report shows for the first time that MIT may select cells exhibiting a MDR phenotype mediated by PGP overexpression. Moreover, this new sequence of events may have implication for the clinical onset of resistance to MIT.

MATERIALS AND METHODS

Cell lines and cell culture. The parental cell line AA8 used for this study is a secondary clone isolated from SC1 Chinese hamster ovary cells [19]. Plating efficiency of AA8 cells is 50–80%. After selection with MIT, the selected colonies were designated AA8/MIT. Cells were grown as monolayer cultures in minimum essential medium α without ribo- and deoxyribo-nucleotides (Gibco, Paisley, U.K., catalog No. 072-2000) containing 5% fetal calf serum (Gibco), 2 mM glutamine, 125 U/mL penicillin and 125 μg/mL streptomycin at 37° and 5% CO_2.

Parental Chinese hamster ovary cell line AuxB1 and the colchicine resistant subline CH^RC5 [20] kindly provided by Dr R. M. Baker RPMI (Buffalo, NY, U.S.A.) were maintained in the same medium as above but containing ribo- and deoxyribonucleotides.

Drugs. MIT (Novantrone® 2 mg/mL or powder) was kindly provided by P. Magnet and D. Lecompte, Lederle Laboratories (Oullins, France) and stored at room temperature. [^{14}C]MIT hexahydrate, sp. act. 66 mCi/mmol, was a generous gift of W. E. McWilliams, American Cyanamide Co. (Pearl River, NY, U.S.A.). Vincristine sulfate (Oncovin® 1 mg/mL, stored at 4°) was purchased from Lilly France S.A. (Saint-Cloud, France). Adriamycin (Adriblastine®) was purchased from Laboratoire Roger Bellon (Neuilly-sur-Seine, France). Aliquots of 1 mg/mL aqueous solution were thawed just before use. Etoposide, kindly provided by M. Holava, Bristol Myers (Wallingford, CT, U.S.A.) was dissolved at a concentration of 10^{-2} M in dimethyl sulfoxide and aliquots stored at −20°.

Dose-dependent survival analysis. Drug cytotoxicity, was determined by a colony formation assay in the presence of drug. Exponentially growing cells were trypsinized and diluted to seed 1 mL containing from 2×10^2 to 5×10^4 cells in 60-mm petri dish containing 3 mL of complete medium. Then, 1 mL of 5 × drug dilutions was added to three replicate dishes for each determination. After 6–8 days incubation, drug-containing medium was discarded and petri dishes were washed with phosphate-buffered saline (PBS). Colonies were stained with 0.2% aqueous crystal violet solution, washed and counted under a stereomicroscope. Only colonies containing 50 cells or more were scored as survivors. Because of the shoulder observed in logarithmic-linear plots of survival in the presence of different drugs used, a D_{10} corresponding to 10% survival was used to determine a resistance index which is defined as the ratio of D_{10} subline/D_{10} parental line AA8.

Density dependence. Cell densities were defined as "low" from 50 to 2.5×10^3 cells/cm^2 and "high" from 5×10^3 to 2×10^4 cells/cm^2. In most experiments performed at high cell density, cells were seeded at $1.7–1.8 \times 10^4$ cells/cm^2. In order to obtain comparable results, the actual diameters of the culture dishes (Nuclon® delta, Nunc, Denmark) were measured and areas calculated from these diameters:

Commercial size (mm)	Actual size (mm)	Area (cm^2)	Volume of medium (mL)
"35"	33	8.6	2
"60"	51	20	5
"100"	84	55	15
"140"	135	143	40

Two types of experiment were carried out to check for density dependence. First, various cell densities were incubated in the presence of 9 nM MIT in 60-mm petri dishes using the same experimental procedure described for dose-dependent survival analysis. After staining, colonies were counted or a photograph of representative dishes was taken.

In the second type of experiment, 3 consecutive days growth at high density ($1.7–1.8 \times 10^4$ cells/cm^2) in 100- or 140-mm petri dishes was performed in the presence of 9 nM MIT. Every 3 days, cells were trypsinized, counted on a Coulter Counter ZM (Coulter Electronics, Luton, U.K.) and replated at the initial density with 9 nM MIT. Cells in suspension in the incubation medium were also counted before trypsinization. Extra petri dishes were saved each time for isolation of RNA and DNA.

RNA and DNA isolation and purification. Cells growing in 100- or 140-mm petri dishes were washed with 10 or 20 mL of sterile PBS at room temperature before freezing at −80°. RNA and DNA were extracted by the guanidine thiocyanate technique [21]. Briefly, about 3×10^7 cells were lysed in petri dishes by 5 mL of a guanidinium mixture. Cell lysate was layered on top of a 3-mL CsCl cushion (5.7 M CsCl in 25 mM sodium acetate, pH 5.5) and then centrifuged at 24,000 rpm for 20 hr at 15° in a SW41 rotor (Beckman Instruments, Gagny, France). Floating proteins were first discarded and banded DNA was separated from the RNA pellet.

The RNA pellet was washed by 0.7 mL of 70%

ethanol/H_2O and dried. After solubilization in 200 μL of 0.2% sodium dodecyl sulfate in Tris-EDTA (TE), RNA was further purified by phenol, phenol–chloroform and chloroform extraction. The RNA was then precipitated with 70% ethanol/0.3 M sodium acetate for 45 min at 4°. The RNA pellet obtained by centrifugation at 15,000 *g* for 15 min at 4° was dissolved in 50–200 μL H_2O.

The DNA fraction was diluted with the same volume of TE and extracted twice with chloroform. The precipitated DNA was then dried and solubilized in 0.25–0.5 mL TE.

Northern blots. A human *mdr* cDNA probe (1.2 Kb insert — encompassing nucleotide 3051 to poly A — in pUC 13 at EcoRI site, obtained from Dr T. Tsuruo, Tokyo, Japan) was used. This probe was labeled with [^{32}P]α-dCTP (Amersham International, Amersham, U.K.) at a specific activity of about 5×10^8 cpm/μg by random primed DNA synthesis. Northern blots were performed as described previously [22]. As a control of the amount of RNA loaded in each well, the amounts of actin mRNA and ribosomal RNA were checked in the same experiments. Actin gene expression was probed using transcripts of pBACT 5 which contains a 600 bp Taq I-Pst I fragment of mouse actin gene, as described previously by Dautry *et al.* [22]. In addition, before hybridization, the transfer membranes were photographed under UV light to assess the amount of ribosomal RNA by ethidium bromide fluorescence. A good correlation was observed between the two methods. Hence, ethidium bromide staining of ribosomal RNA was used to judge the amount of total RNA loading.

Southern blots. Each DNA sample in TE was first digested by EcoRI (Boehringer Mannheim GmbH, Germany) and purified by phenol-chloroform extraction. After determination of the concentration, 10 μg of each DNA sample were digested by EcoRI and electrophoresed on a 0.8% agarose gel containing 1 μg/mL ethidium bromide. Transfer onto an uncharged nylon membrane (Amersham Hybond N) was performed according to Maniatis *et al.* [23] and DNA was cross-linked by 254 nm UV light. The DNA amounts on the transfer membranes were assessed by ethidium bromide fluorescence. Prehybridization was performed at 42° for 6–7 hr in a buffer containing 50% deionized formamide, 750 mM NaCl, 75 mM Na citrate, 50 mM sodium phosphate buffer, pH 6.8, 0.1% sodium dodecyl sulfate, 2.5 × Denhardt's solution and 100 μg/mL denatured salmon sperm DNA. Hybridization was performed at 42° for 20 hr in the same buffer except that 0.5 × Denhardt's solution was used. The same *mdr* cDNA insert used in northern blots was used as probe.

Nuclear extracts. Preparation of 0.35 M NaCl nuclear extracts was performed according to Glisson *et al.* [24] with minor modifications described elsewhere [25]. Briefly, about 5×10^7 exponentially growing cells were trypsinized, washed with PBS, and nuclei were isolated at 4° by Dounce homogenization in a swelling buffer containing 0.3% Triton X-100. Nuclei were purified by centrifugation through a sucrose cushion, washed, and NaCl concentration was raised to 0.35 M. Protein concentration in the supernatant (0.35 M NaCl nuclear extract) was determined to equalize nuclear extracts from resistant colonies and parental AA8 cells. Aliquots in 50% glycerol were stored at −20° and used within one week.

DNA topoisomerase II-mediated DNA cleavage. Drug-stimulated DNA cleavage activity in nuclear extracts was assayed quantitatively by the generation of linear DNA from supercoiled pBR 322 DNA as described previously in detail [25]. Samples were analysed by agarose gel electrophoresis in the presence of ethidium bromide. Gels were photographed under UV illumination and negative films were scanned using Lecphor software on a Biocom 200 (Biocom, Les Ulis, France).

Uptake of MIT. Preliminary experiments were carried out to set the different parameters for uptake experiments. Time-course showed that MIT uptake reached a plateau after about 2 hr of incubation. Uptake of MIT was linear up to about 2×10^5 cells/35-mm diameter petri dish. Uptake at different external MIT concentrations (0.125–1 μM) showed a near linear relationship up to 0.4 μM between MIT accumulation and external MIT concentration. Above this concentration, accumulation continued to increase with a slower slope up to 1 μM. Therefore, the number of cells plated was adjusted to have about 1×10^5 cells/35 mm at the time of drug exposure; this corresponds to a high cell density (about 1.2×10^4 cells/cm^2). Then, MIT uptake was carried out by a technique described in detail elsewhere [26] after 4 hr contact with 1 μM [^{14}C]MIT (1 mL in a 35-mm petri dish) as external drug concentration in order to get the best precision. Experiments performed at low cell density (about 2.5×10^3 cells/cm^2) under the same conditions gave data which show considerable scatter. Increasing the number of cells by increasing the culture area in order to get better precision was not possible, as found previously [26]; this method led to more than 1 mL of cell suspension being placed on the 2-μm polycarbonate top filter and to much time was required to separate the cells from the surrounding solution). The same limitation was encountered with the cold buffer wash method [27] and the silicone oil technique [28] which are classically used with cells grown in suspension.

Cells were harvested on a 2-μm polycarbonate filter which was dried and transferred to a scintillation vial with 4.5 mL of Ultima Gold (Packard Instrument Co.). The radioactivity was measured in an MR 300 liquid scintillation counter (Kontron Instruments, France). Cell diameter was determined after trypsinization by microscopic measurements with a micrometer. Cell volume was calculated assuming a near spherical shape of trypsinized cells. Significance between mean values was analysed by Student's *t*-test for independent random samples.

RESULTS

Selection of colonies with MIT

We determined the cytotoxicity of MIT by cloning assay under continuous exposure to increasing concentrations of the drug. Exponential cell kill was observed above about 3 nM MIT (data not shown),

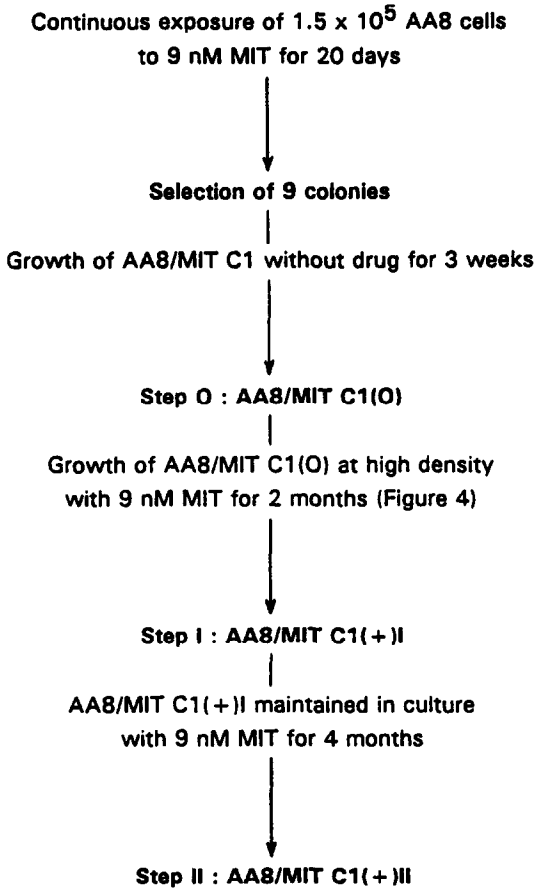

Fig. 1. Diagram showing the different steps at which characterization of the MIT-selected AA8/MIT C1 cells has been carried out. A new MIT-resistant cell line AA8/MIT C1(+)I has been selected at step I during growth of AA8/MIT C1(0) at high cell density.

suggesting that before reaching this concentration cells were able to accumulate sub-lethal damages and/or to repair toxic lesions [29]. The D_{10} value (reduction of relative plating efficiency to 10%) corresponded to 4 nM. In order to carry out a one-step selection of AA8 cells for low level of resistance to MIT, we chose a concentration of 9 nM MIT that corresponds to a frequency of 10^{-4}–10^{-3} surviving cells.

The selection was performed by seeding 15 100-mm petri dishes with 10^4 AA8 cells and growing cells in the presence of 9 nM MIT for 20 days (Fig. 1). A total of 64 colonies (three to eight per petri dish) arose from this culture, corresponding to a survival frequency of 9×10^{-4} (In this experiment, plating efficiency of AA8 control cells was 50%.) Nine colonies (one per petri dish) were isolated and designed AA8/MIT followed by a letter and a digit for identification. They were transferred into 24-well plates and then into 25-cm^2 flasks without MIT for about 3 weeks. Aliquots were then preserved (Fig. 1, step 0) as frozen stocks. Each of the nine clonal isolates obtained was then tested for MIT sensitivity.

Table 1. Resistance index of AA8/MIT C1 (0) cells to MIT and three MDR-related drugs

Drug tested	D_{10}* for AA8 (μM)	Resistance index of AA8/MIT C1(0)†
MIT	0.0040 ± 0.0003‡	0.8
Vincristine	0.023 ± 0.004	0.5
Adriamycin	0.059 ± 0.003	1.2
Etoposide	0.63 ± 0.07	1.2

* Drug concentration corresponding to 10% survival in a cloning assay under continuous exposure to drug as described in Materials and Methods.

† Ratio of D_{10} for the AA8/MIT C1(0) cells to that for the parental line AA8.

‡ Mean ± SD (4–7 experiments).

Eight of the nine colonies showed no resistance to MIT at this step. Attempts to culture separately these eight MIT-sensitive colonies in the presence of 9 nM MIT suggested that a high density was required for growth to occur. Therefore, we investigated further the cell density dependence of one colony AA8/MIT C1 (0) and the emergence of the mutant line AA8/MIT C1 (+) in two steps (Fig. 1, steps I and II).

Characterization of the MIT-selected AA8/MIT C1 (0) cells

The sensitivity of AA8/MIT C1(0) to MIT (Fig. 1, step 0) was first determined, using a cloning assay at low cell density (up to 5×10^2 cells/cm^2). As shown in Table 1, AA8/MIT C1 (0) are sensitive to MIT as compared to parental AA8 cells. No cross-resistance to Adriamycin and etoposide, and a 2-fold increased sensitivity to vincristine were also observed.

The ability of AA8/MIT C1(0) to accumulate [^{14}C]MIT as compared with the parental cell line was investigated. As shown in Table 2, AA8/MIT C1 (0) accumulated about 20% less drug than AA8 cells under the condition of high cell density (1.2×10^4 cells/cm^2) when the uptake is normalized to the same cell volume (because of the small standard deviations this difference is statistically significant). On the other hand, the poor precision obtained under the condition of low cell density (see Materials and Methods) precluded drawing any serious conclusion from these results.

The expression of *mdr* RNA and gene copy number were analysed by northern and Southern blot analysis, respectively. Comparable levels of *mdr* RNA (Fig. 5B, lane DO and Fig. 6A) and gene copy number (Fig. 6B) were found in AA8/MIT C1 (0) as compared with the parental AA8 cells.

Cell density dependence of AA8/MIT C1(0) cells

In order to determine whether cell cooperation might influence individual cell sensitivity, we investigated the effect of cell density on the cloning efficiency of AA8 and AA8/MIT C1(0) under continuous exposure to 9 nM MIT added at the time of plating. At low cell density (from 50 to

Table 2. Uptake of [^{14}C]MIT by parental AA8 cells, AA8/MIT C1(0) and the MIT-resistant subline AA8/MIT C1(+) at steps I and II

	Uptake* (not corrected) (pmol/10^6 cells)	Cell diameter† (μm)	Calculated cell volume ‡ (μm^3)	Uptake (corrected) (pmol/μL cell)
AA8	211 ± 16	12.5 ± 1.4	1023	206 ± 15
AA8/MIT C1(0)	454 ± 27	17.7 ± 3.1§	2903	156 ± 9§
AA8/MIT C1(+)I	211 ± 10	14.6 ± 3.3‖	1630	129 ± 6§
AA8/MIT C1(+)II	61 ± 11	15.4 ± 3.1¶	1912	32 ± 6§

Cells were incubated for 4 hr with 10^{-6} M [^{14}C]MIT, washed at 4° before harvesting and then processed for scintillation counting as described in Materials and Methods. To take account of the differences in cell size, uptake of MIT is corrected by the cell volume.

* Values are means ± SD obtained from two or three independent experiments in which determinations were made in triplicate.

† Determined on 60–90 cells in two or three independent series of measurements.

‡ Assuming a near spherical shape of trypsinized cells.

§ Significantly different compared with AA8, $P < 0.001$.

‖ Significantly different compared with AA8, $P < 0.05$.

¶ Not significant compared with AA8/MIT C1(+)I.

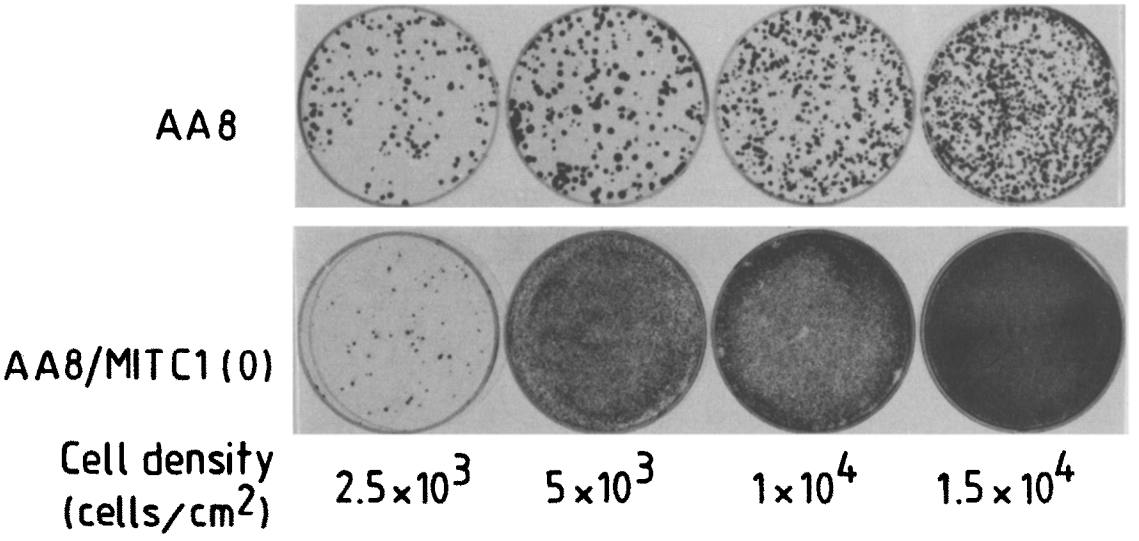

Fig. 2. Effect of cell density on the cloning efficiency or growth of AA8 and AA8/MIT C1(0) cells in the presence of 9 nM MIT. AA8 and AA8/MIT C1(0) cells were incubated with drug at the time of seeding for 7 and 5 days, respectively. Cells were stained before photographs were taken.

2.5×10^3 cells/cm^2) an expected linear relationship was observed between the number of colonies that appeared and the number of cells seeded for both cell lines (data not shown). Figure 2 shows that at high cell density (from 5×10^3 to 1.5×10^4 cells/cm^2) AA8 cells were still killed (the number of colonies is expected from the frequency of cell survival) in the presence of 9 nM MIT. On the contrary, AA8/MIT C1(0) cells were growing as a monolayer. These results indicate that MIT-selected cells were able to grow in the presence of drug at high density only.

In the previous experiment, microscopic examination showed that even at the highest cell density tested, cells were far from confluence at the time of plating. We determined the growth kinetics of AA8 and AA8/MIT C1(0) at two cell densities, 5.8×10^3 and 1.7×10^4 cells/cm^2, the latter being used in the following experiments. As shown in Fig. 3, both AA8 and AA8/MIT C1(0) cells were in exponential growth phase during the first 3 days with population doubling times of 17.6 ± 1.2 (mean ± SD) and 20.8 ± 0.7 hr, respectively. The saturation densities reached were 3.9 and 2.2×10^5 cells/cm^2 with AA8 and AA8/MIT C1(0), respectively. The larger cell volume of AA8/MIT C1(0) as compared with AA8 cells may account, at least in part, for the difference observed between the saturation densities.

In order to confirm the results of Fig. 2 quantitatively, we reseeded every 3 days AA8 and AA8/MIT C1(0) cells at 1.7×10^4 cells/cm^2 in the presence of 9 nM MIT. Figure 4 shows that during

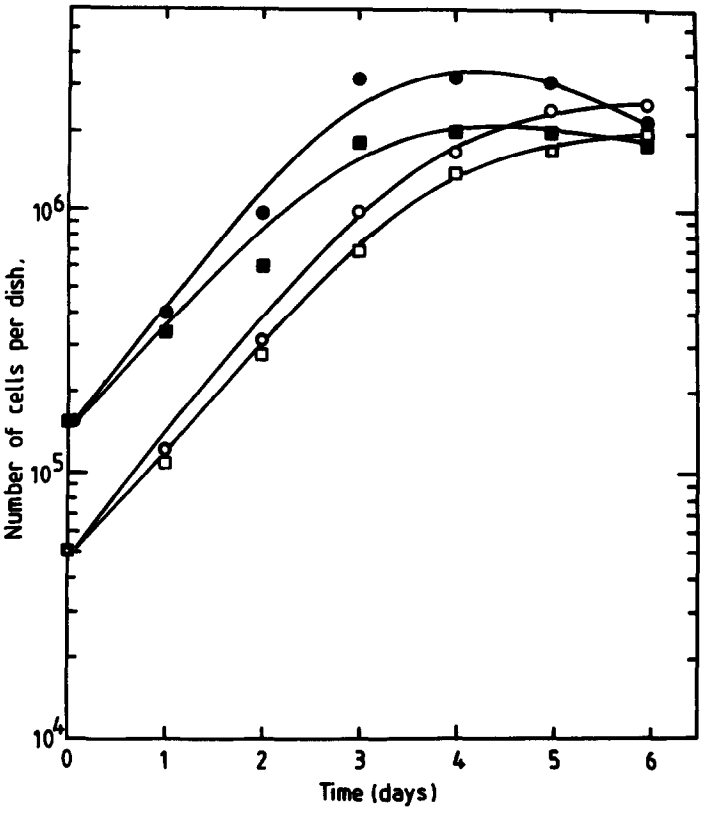

Fig. 3. Growth kinetics of AA8 and AA8/MIT C1(0) cells at two cell densities. The cells were seeded at 5.8×10^3 (open symbol) and 1.7×10^4 (closed symbols) cells/cm^2 in 35-mm petri dishes and medium was changed daily. AA8 (○, ●); AA8/MIT C1 (0) (□, ■). Each point corresponds to the mean of two experiments in which each determination was carried out in duplicate (SD < 10%).

each period of 3 days (each point corresponds to the number of viable cells after 3 days growth), AA8 and AA8/MIT C1(0) control cells grew to about 3 and 2×10^7 cells (2.1 and 1.4×10^5 cells/cm^2), respectively. These values correspond to the end of the exponential phase as observed in growth kinetics (Fig. 3). On the other hand, adding 9 nM MIT to these two types of cells resulted in two completely different effects. AA8 cells doubled in the first period of 3 days only and were then killed by the drug during the following periods, since the number of attached cells was, after each period of 3 days, less than the number of seeded cells. As expected from the frequency of surviving AA8 cells at 9 nM MIT (which is between 10^{-4} and 10^{-3}) the cells selected during the first 6–9 days resumed their growth at day 15 (Fig. 4). On the contrary, in the presence of 9 nM MIT, AA8/MIT C1(0) grew during each period of 3 days, at about half density as compared to control AA8/MIT C1(0). Taken together, these results indicate that AA8/MIT C1(0) cells have a capacity to grow at high density in the presence of drug whereas AA8 parental cells do not.

At high cell density, it is conceivable that most of the drug available in a given volume of culture medium could be taken up by the large number of cells. This could result in a new steady-state in which the external concentration of MIT would be much less than 9 nM. In our case, this might be true with AA8/MIT C1(0) but not with AA8. The data presented in Table 2 (1 μM MIT as external concentration) allow calculation of the percentage of MIT accumulated by these two cell lines. For example, 3×10^5 AA8 and AA8/MIT C1(0) cells (1.5×10^4 cells/cm^2) seeded in 5 mL MIT-containing medium (60-mm petri dish as in Fig. 2) do not take up more than 1.5 and 3% MIT, respectively (using the uptake not corrected by the cell volume, Table 2). As preliminary experiments showed that MIT accumulation is related to the external drug concentration for both AA8 and AA8/MIT C1 (0) (see Materials and Methods) these percentages would not exceed 6 and 12%, respectively, at 9 nM. This suggests that in AA8/MIT C1 (0) the 9 nM MIT concentration is still delivered at high cell density.

Selection of a MIT-resistant subline

Since AA8/MIT C1(0) cells were resistant to MIT via a density-dependent mechanism, we investigated further this ability over a longer period of time. AA8/MIT C1(0) cells were reseeded every 3 days in the presence of fresh MIT while dead cells suspended in the medium were counted. Using 100-

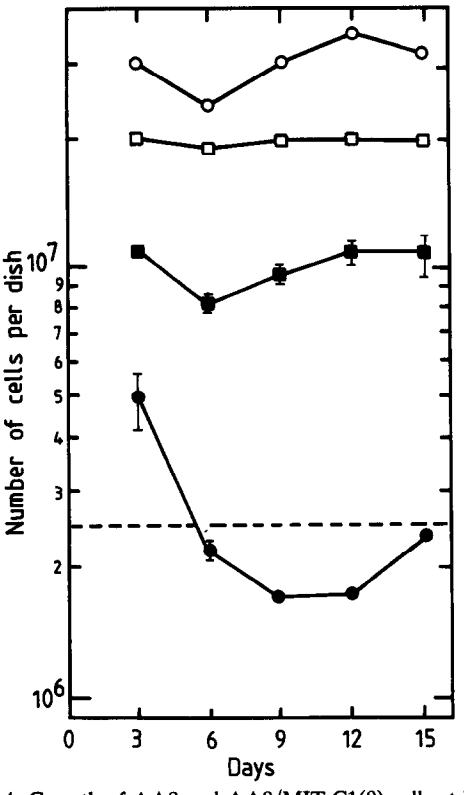

Fig. 4. Growth of AA8 and AA8/MIT C1(0) cells at high density in the presence of 9 nM MIT. Cells were reseeded every 3 days at 1.7×10^4 cells/cm^2 (dashed line) in 140-mm petri dishes with fresh medium with or without 9 nM MIT. AA8 control (○); AA8 with 9 nM MIT (●); AA8/MIT C1(0) control (□); AA8/MIT C1(0) with 9 nM MIT (■). Bars, ± 1 SD.

or 140-mm petri dishes, this type of experiment was repeated three times giving comparable results to those reported here. Figure 5A shows that over two months, the growth of AA8/MIT C1 is divided into three periods. The first period (day 0–24) corresponds to net growth of AA8/MIT C1(0) cells during which the number of dead cells was almost stable. As compared to the basal level found in AA8/MIT C1(0) (Fig. 5B, lane D0), no significant changes were observed in *mdr* RNA levels of cells growing in the presence of 9 nM MIT over this period (Fig. 5B, lane D6 and D21), nor in gene copy number (data not shown). Almost no net growth occurred during the second period which is rather short (day 24–30) while the number of dead cells increased. No aliquots were saved between day 30 and 36 because not enough cells were available. The third period (day 38–65) corresponds to the growth of selected cells which overexpress *mdr* RNA (Fig. 5B, lane D41 and D65). The number of dead cells decreased dramatically during this period. *mdr* gene amplification occurred each time that an overexpression of RNA was observed (one example given on Fig. 6B, lane C1(+)I) indicating that a new cell genotype arose in the initial population of AA8/MIT C1(0) cells; this new MIT-resistant cell line was named AA8/MIT C1(+).

Characterization of the MIT-resistant subline AA8/MIT C1(+)

Cells were first characterized after about 2 months of controlled growth in the presence of 9 nM MIT (Fig. 5, 60–70 days; step I in Fig 1). AA8/MIT C1(+)I cells were able to grow at low cell density in the presence of 9 nM MIT (plating efficiency was > 90% at this concentration). This cell line was maintained with drug for an additional 4 months and was then characterized further (step II in Fig. 1).

Analysis of *mdr* gene expression showed that *mdr* RNA levels were slightly increased in AA8/MIT C1(+) at step II as compared with step I (Fig. 6A). As shown on Fig. 6B an overall *mdr* gene amplification paralleled this increase in *mdr* RNA levels. However, the amplification of the 6.0-Kb DNA sequence at step I almost disappeared at step II.

About 2- and 6-fold decreased accumulation of [^{14}C]MIT was found in AA8/MIT C1(+) at step I and II, respectively, as compared with AA8 cells (Table 2). Verapamil, a calcium channel blocker which inhibits drug efflux by PGP by competition with drug binding, has been shown to increase drug accumulation in MDR cells [3]. Incubation of AA8/MIT C1(+)II with 4×10^{-5}M verapamil at the time of [^{14}C]MIT addition resulted in about a 6-fold increase in MIT accumulation. These results suggest that the PGP encoded by overexpressed *mdr* RNA in AA8/MIT C1(+) cells is an active drug-efflux pump.

Cross-resistance of AA8/MIT C1(+) to vincristine, Adriamycin and etoposide, which are known to be involved in the MDR phenotype, was observed at step II (Table 3). It is interesting to note that, at step II, AA8/MIT C1(+)II cells were 20–30-fold resistant to the three drugs but still only 4.4-fold resistant to MIT.

Etoposide and to a lesser extent, Adriamycin and MIT, are able to induce *in vitro* DNA topoisomerase II-mediated cleavage of DNA (cleavable complex) [14, 30]. Hence, a reduced ability to form the cleavable complex in the presence of drug could participate in the resistance of AA8/MIT C1(+)II to Adriamycin and etoposide in addition to recognition of these drugs by PGP. Drug-stimulated DNA cleavage of closed pBR 322 DNA by nuclear extracts from AA8/MIT C1(+)II cells was assayed in the presence of increasing concentrations of etoposide to which these cells are cross-resistant. AA8 and AA8/MIT C1(+)II nuclear extracts induced the same amount of linear pBR 322 DNA at the different etoposide concentrations used (1–128 μM, data not shown). This indicates that quantitative or qualitative DNA topoisomerase II modification is unlikely to participate in the resistance of AA8/MIT C1(+)II to etoposide, Adriamycin and MIT.

DISCUSSION

Low levels of resistance are likely to occur in refractory human tumors. During chemotherapy

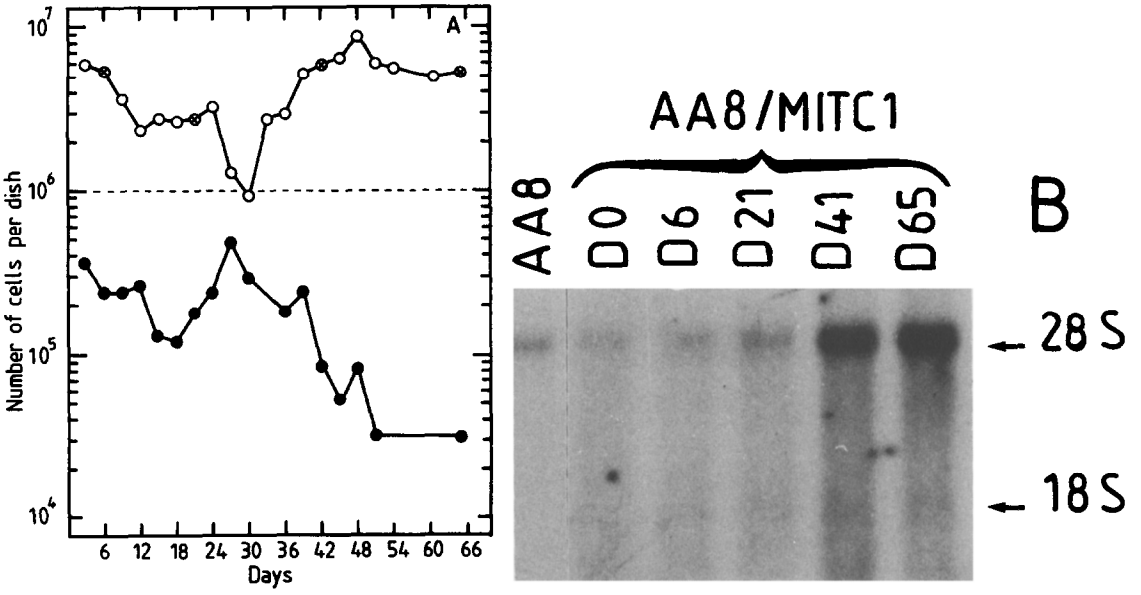

Fig. 5. Selection of *mdr* RNA overexpression during growth at high cell density. Panel A: growth of AA8/MIT C1(0) cells at high cell density in the presence of 9 nM MIT. Cells were trypsinized and reseeded every 3 days at 1.8×10^4 cells/cm^2 (dashed line) in 100-mm petri dishes with fresh medium containing 9 nM MIT. Cells in suspension in the supernatant before trypsinization (dead cells) (●); cells detached by trypsin (living cells) (○); time at which analysis of *mdr* RNA expression is shown (⊗). Panel B: northern blot analysis of *mdr* RNA expression at the indicated times (D = day).

regimens, repetitive drug treatments at the same dose are separated by intervals with no treatment. In order to investigate the possible mechanism(s) of resistance to MIT under these conditions, we have isolated nine colonies by a low selective pressure. After 3 weeks of culture without drug, the colony AA8/MIT C1(0) studied in this work exhibited cell density-dependent resistance. These MIT-selected cells were subsequently regrown at high cell density for 2 months in the presence of 9 nM MIT. A 4-fold MIT-resistant subline AA8/MIT C1(+), exhibiting an MDR phenotype, arose after about 4 weeks of culture under these conditions. Although less fully characterized, the same sequence of events has been found with another colony AA8/MIT D1 (0) suggesting that the results presented here are of some significance.

During the isolation of the colony AA8/MIT C1(0), it is likely that MIT was active for at least 6–7 doubling times [31]. Transient overexpression of the *mdr* RNA might have appeared in these cells during continuous exposure to the drug. It has been shown that *mdr*1 promoter can be activated directly on the addition of MDR-related drugs [32]. Moreover, *mdr* RNA levels increase in rodent cells following exposure to drugs that are known to be recognized by PGP [33]. If such was the case in the AA8/MIT C1(0) colony, overexpression of *mdr* RNA was lost during the 3 weeks of culture without drug after the selection.

The mechanism by which AA8/MIT C1(0) cells are resistant to MIT involves cell density dependence. Several reports have shown that stationary cells are usually less sensitive than exponentially growing cells [34–36]. In such cases, cell density as well as proliferation rates may be involved. In our case, both parental AA8 and AA8/MIT C1(0) cells are proliferating at high density in the absence of MIT (Fig. 3) whereas only AA8/MIT C1(0) cells are growing in the presence of 9 nM MIT, AA8 cells being killed under this condition (Fig. 4). This indicates that AA8/MIT C1(0) are resistant to MIT by a mechanism involving cell density only.

Resistance at high cell density might be the consequence of overall decreased external drug concentration. It seems that, under our conditions, resistance at high cell density and sensitivity at low cell density are not due to differences in drug:cell ratios. The difference between the low calculated percentages of MIT taken up by AA8 (6%) and AA8/MIT C1 (0) (12%) cells from the surrounding medium is unlikely to account for the density dependence observed with the MIT-selected cells as compared with parental AA8 at 9 nM MIT. Moreover, MIT-induced cell kill kinetics carried out on cells in suspension showed that MIT sensitivity begins to decrease substantially when cell density reaches 10^7 cells/mL [37]. Although, strictly, we cannot compare this situation to that of our attached cells, it appears that our conditions ($6–7 \times 10^5$ cells/mL) are beyond this value.

A slightly decreased accumulation of MIT (about 20%) without PGP overexpression was observed at high cell density in AA8/MIT C1 (0). A decrease in exposed surface area in proportion to an increase in cell density [38] may not be involved in our case because at the time of seeding cells are far from confluence (Fig. 3). Such decreased drug

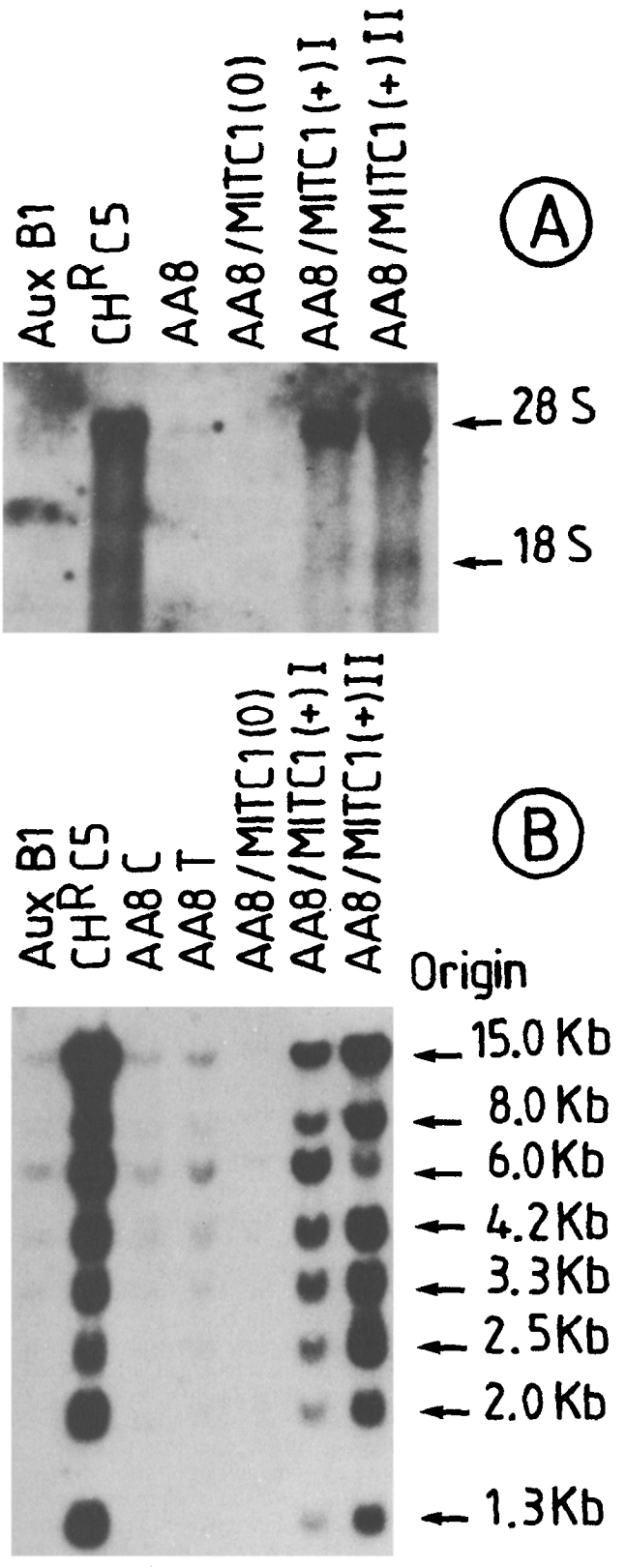

Fig. 6. Analysis of *mdr* RNA expression and gene copy number in AA8/MIT C1 cells at steps 0, I and II as compared with parental AA8 cells. Blot techniques were performed as described in Material and Methods using a 1.2-Kb human MDR1 cDNA probe labeled by random prime with [^{32}P]α-dCTP. The amounts of nucleic acids were identical judging by ethidium bromide fluorescence on the transfer membrane (excepted for AA8/MIT C1(0) DNA which was about 50% underloaded). CHO AuxB1 and CH^{R}C5 cells were used as negative and positive control, respectively. Panel A: autoradiography of northern blot analysis; the positions of 18S and 28S ribosomal RNA are indicated. Panel B: autoradiography of Southern blot analysis; lengths of EcoRI-digested DNA fragments are indicated in kilobases (Kb). Three fragments (15.0, 2.0 and 1.3 Kb) represent *pgp*1; three fragments (8.0, 4.2 and 3.3 Kb) represent *pgp*2; two fragments (6.0 and 2.5 Kb) represent *pgp*3 (according to Ref. 46).

Table 3. Resistance index of AA8/MIT C1(+) cells to MIT and cross-resistance at steps I and II

Drug tested	Resistance index* AA8/MIT C1(+)I	AA8/MIT C1(+)II
MIT	3.9	4.4
Vincristine	>4†	21
Adriamycin	>4‡	19
Etoposide	>2§	27

* See footnotes* and † in legend to Table 1.

† At the concentration corresponding with this resistance index, relative plating efficiency was still about 70% as compared with about 0.0001% for AA8 cells.

‡ At the concentration corresponding with this resistance index, relative plating efficiency was still 100% as compared with about 0.01% for AA8 cells.

§ At the concentration corresponding with this resistance index, relative plating efficiency was still 100% as compared with about 0.1% for AA8 cells.

accumulation has been described with Adriamycin [39] and more recently with anthracyclines in confluence-dependent resistance [40]. The decreased uptake that we observed might contribute to the resistance of AA8/MIT C1(0) at high cell density. However, imprecision in both drug accumulation and cell size measurements and the impossibility of determining precisely drug uptake per unit volume at low cell density preclude a definitive conclusion about this. Microspectrofluorimetry which allows the recording of individual cells [40] could be an alternative technique but cannot be used here because MIT is not fluorescent.

The mechanism of resistance of AA8/MIT C1(0) at high cell density remains unknown. It might be related to morphological alterations as these cells display an increased size and are flatter as compared with AA8 cells. A previous description of membrane vesicle formation in a MIT-resistant carcinoma cell line [18] led us to check for this alteration but no difference was observed between AA8/MIT C1(0) and AA8 cells whatever the cell density. Experiments to determine whether resistance of AA8/MIT C1(0) is related to other morphological alterations are in progress. On the other hand, the absence of cell-to-cell contact under our condition of high cell density at the time of plating suggests that soluble components are involved. Because a slightly longer population doubling time was observed with AA8/MIT C1(0) as compared with parental cells (Fig. 3), it is unlikely that the MIT-selected cells produce an autocrine factor which modulates their growth rate. Beside, it has recently been shown that alteration of the drug–DNA topoisomerase II interaction could be related to the resistance of stationary cells to DNA topoisomerase II inhibitors [41–43]. Although our conditions are different, target modification might occur in AA8/MIT C1(0) at high cell density. Because they are inhibited by MIT, DNA topoisomerase II [14], protein kinase C [44] and microtubule assembly [45] are potential candidates.

In addition to the cell density-dependent resistance, we report here the isolation of a new MIT-resistant cell line AA8/MIT C1 (+). Using a low degree of selective pressure and high cell density, we obtained a 4-fold resistance to MIT. These cells exhibit a MDR phenotype assessed by *mdr* gene amplification and RNA overexpression, decreased uptake (Table 2) reversed by verapamil, and cross-resistance to three MDR related drugs (Table 3). This is the first characterization of a MIT-resistant cell line overexpressing PGP. Recently, Ng *et al.* [46] identified three members in the hamster PGP gene family. Between steps I and II, a 6.0-Kb amplified DNA sequence, including a part of *pgp*3 gene, disappeared (Fig. 6B). Experimental evidence and homology between hamster, mouse and human *mdr* gene family members suggest that *pgp*3 gene overexpression cannot confer MDR phenotype while *pgp*1 and *pgp*2 can [3, 47, 48]. The different overexpressed *mdr* RNA species rather than the quantity of total *mdr* RNA (Fig. 6A) may account for the significantly decreased uptake observed at step II as compared with step I. At step II, a good agreement was observed between the uptake and the level of resistance to MIT. In addition, the ability to form the cleavable complex in the presence of etoposide was not reduced in AA8/MIT C1(+) II cells indicating that DNA topoisomerase II is not modified in this subline. On the other hand, MIT has not been described as a DNA topoisomerase I inhibitor [4] and has little propensity for activation to free radicals [12]. Therefore, it is unlikely that a DNA topoisomerase I modification or alteration in the glutathione pathway occurs in AA8/MIT C1(+) II. Hence, the PGP-mediated MDR phenotype is probably the only mechanism of resistance to MIT in this subline. AA8/MIT C1(+) II cells display a high cross-resistance (5–7-fold the resistance to MIT, Table 3) to drugs which are usually recognized by PGP. While the highest degree of resistance is generally observed with the drug used for the selection [49], some reports describe resistant cell lines which are more resistant to other agents [50–53]. In these cases, higher cross-resistance is generally limited to one class of drugs which is most often *Vinca* alkaloids. Our data, showing cross-resistance to three MDR related drugs belonging to different series, suggest that MIT is not well recognized by PGP in our model.

In conclusion, this study shows that under a low degree of selective pressure, MIT may select a subset of cells which, after 3 weeks of growth without drug, are able to grow in the presence of drug at high cell density. Within a few weeks of growth under this last condition, a new cell genotype with *mdr* gene amplification arises. Sequential emergence of distinct resistance phenotypes has been described previously during selection of Adriamycin-resistant cells with stepwise increasing drug concentrations [54, 55]. Alternatively, we report that using sequential exposures to an identical low concentration of MIT, two different mechanisms of resistance are selected consecutively. Our experimental conditions are reminiscent of what may happen during successive treatments in cancer chemotherapy. Thus, the first mechanism might be relevant in some clinical situations in which early steps of selection of resistant cells must be studied to better circumvent drug

resistance. Further investigations of the different steps at the molecular level should give new information and permit a better understanding of acquired drug resistance in tumors.

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REFERENCES

1. Curt GA, Clendeninn NJ and Chabner BA, Drug resistance in cancer. *Cancer Treat Rep* **68**: 87–99, 1984.
2. De Vita VT Jr, Principles of Chemotherapy. In: *Cancer Principles and Practice of Oncology* (Eds. De Vita VT Jr, Hellman S and Rosenberg SA), pp. 278–281. JB Lippincott Co., Philadelphia, 1989.
3. Georges E, Sharom FJ and Ling V, Multidrug resistance and chemosensitization: therapeutic implications for cancer chemotherapy. *Adv Pharmacol* **21**: 185–220, 1990.
4. Schneider E, Hsiang YH and Liu LF, DNA topoisomerases as anticancer drug targets. *Adv Pharmacol* **21**: 149–183, 1990.
5. Siminovitch L, On the nature of hereditable variations in cultured somatic cells. *Cell* **7**: 1–11, 1976.
6. Goldie JH and Coldman AJ, The genetic origin of drug resistance in neoplasms: implications for systemic therapy. *Cancer Res* **44**: 3643–3653, 1984.
7. Riordan JR, Deuchars K, Kartner N, Alon N, Trent J and Ling V, Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines. *Nature* **316**: 817–819, 1985.
8. Scotto K, Biedler JL and Melera PW, Amplification and expression of genes associated with multidrug resistance in mammalian cells. *Science* **232**: 751–755, 1986.
9. Van der Bliek AM, Baas F, Van der Velde-Koerts T, Biedler JL, Meyers MB, Ozols RF, Hamilton TC, Joenje H and Borst P, Genes amplified and overexpressed in human multidrug-resistant cell lines. *Cancer Res* **48**: 5927–5932, 1988.
10. Shen DW, Fojo A, Chin JE, Roninson IB, Richert N, Pastan I and Gottesman MM, Human multidrug-resistant cell lines: increased *mdr*1 expression can precede gene amplification. *Science* **232**: 643–645, 1986.
11. Lemontt JF, Azzaria M and Gros P, Increased *mdr* gene expression and decreased drug accumulation in multidrug-resistant human melanoma cells. *Cancer Res* **48**: 6348–6353, 1988.
12. Shenkenberg TD and Von Hoff DD, Mitoxantrone: a new anticancer drug with significant clinical activity. *Ann Intern Med* **105**: 67–81, 1986.
13. Durr FE, Wallace RE and Citarella RV, Molecular and biochemical pharmacology of mitoxantrone. *Cancer Treat Rev* **10** (Suppl B): 3–11, 1983.
14. Tewey KM, Rowe TC, Yang L, Halligan BD and Liu LF, Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* **226**: 466–468, 1984.
15. Glisson B, Gupta R, Hodges P and Ross W, Cross-resistance to intercalating agents in an epipodophyllotoxin-resistant Chinese hamster ovary cell line: evidence for a common intracellular target. *Cancer Res* **46**: 1939–1942, 1986.
16. Dalton WS, Cress AE, Alberts DS and Trent JM, Cytogenetic and phenotypic analysis of a human colon carcinoma cell line resistant to mitoxantrone. *Cancer Res* **48**: 1882–1888, 1988.
17. Harker WG, Slade DL, Dalton WS, Meltzer PS and Trent JM, Multidrug resistance in mitoxantrone-selected HL-60 leukemia cells in the absence of P-glycoprotein overexpression. *Cancer Res* **49**: 4542–4549, 1989.
18. Dietel M, Arps H, Lage H and Niendorf A, Membrane vesicle formation due to acquired mitoxantrone resistance in human gastric carcinoma cell line EPG85-257. *Cancer Res* **50**: 6100–6106, 1990.
19. Thompson LH, Fong S and Brookman K, Validation of conditions for efficient detection of HPRT and APRT mutations in suspension cultured Chinese hamster ovary cells. *Mutat Res* **74**: 21–36, 1980.
20. Kartner N, Evernden-Porelle D, Bradley G and Ling V, Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. *Nature* **316**: 820–822, 1985.
21. Chirgwin JM, Przybyla AE, MacDonald RJ and Rutter WJ, Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294–5299, 1979.
22. Dautry F, Weil D, Yu J and Dautry-Varsat A, Regulation of pim and myb mRNA accumulation by interleukin 2 and interleukin 3 in murine hematopoietic cell lines. *J Biol Chem* **263**: 17615–17620, 1988.
23. Maniatis T, Fritsch EF and Sambrook J, *Molecular cloning*, pp. 383-386. Cold Spring Harbor Laboratory, New York, 1982.
24. Glisson B, Gupta R, Smallwood-Kentro S and Ross WE, Characterization of acquired epipodophyllotoxin resistance in a Chinese hamster ovary cell line: loss of drug stimulated DNA cleavage activity. *Cancer Res* **46**: 1934–1939, 1986.
25. Charcosset JY, Saucier JM and Jacquemin-Sablon A, Reduced DNA topoisomerase II activity and drug-stimulated DNA cleavage in 9-hydroxyellipticine resistant cells. *Biochem Pharmacol* **37**: 2145–2149, 1988.
26. Charcosset JY, Salles B and Jacquemin-Sablon A, Uptake and cytofluorescence localization of ellipticine derivatives in sensitive and resistant Chinese hamster lung cells. *Biochem Pharmacol* **32**: 1037–1044, 1983.
27. Yalowich JC and Goldman ID, Analysis of the inhibitory effects of VP-16-213 (etoposide) and podophyllotoxin on thymidine transport and metabolism in Ehrlich ascites tumour cells *in vitro*. *Cancer Res* **44**: 984–989, 1984.
28. Vistica DT, Toal JN and Rabinowitz M, Amino acid-conferred protection against melphalan: characterization of melphalan transport and correlation of uptake with cytotoxicity in cultured L1210 leukemia cells. *Biochem Pharmacol* **27**: 2865–2870, 1975.
29. Drewinko B, Roper PR and Barlogie B, Patterns of cell survival following treatment with antitumor agents *in vitro*. *Eur J Cancer* **15**: 93–99, 1979.
30. Chen GL, Yang L, Rowe TC, Halligan BD, Tewey KM and Liu LF, Non-intercalative antitumor drugs interfere with the breakage-reunion reaction of mammalian DNA-topoisomerase II. *J Biol Chem* **259**: 13560–13566, 1984.
31. Lecompte D, Bousselet M and Magnam J, Stability of mitoxantrone (Novantrone®) after dilution in PVC bags for infusions. *J Pharm Clin* **9**: 159–165, 1990.
32. Kohno K, Sato S, Takano H, Matsuo K and Kuwano M, The direct activation of human multidrug resistance gene (*mdr*1) by anticancer agents. *Biochem Biophys Res Commun* **165**: 1415–1421, 1989.

33. Chin KV, Chauhan SS, Pastan I and Gottesman MM, Regulation of *mdr* RNA levels in response to cytotoxic drugs in rodent cells. *Cell Growth Diff* **1**: 361–365, 1990.
34. Bhuyan BK, Fraser TJ and Day KJ, Cell proliferation kinetics and drug sensitivity of exponential and stationary populations of cultured L1210 cells. *Cancer Res* **37**: 1057–1063, 1977.
35. Epifanova OI, Smolenskaya IN and Polunovsky VA, Responses of proliferating and non-proliferating Chinese hamster cells to cytotoxic agents. *Br J Cancer* **37**: 377–385, 1977.
36. Drewinko B, Patchen M, Yank LY and Barlogie B, Differential killing efficacy of twenty antitumor drugs on proliferating and nonproliferating human tumor cells. *Cancer Res* **41**: 2328–2333, 1981.
37. Ohuma T, Arkin H and Holland JF, Effects of cell density on drug-induced cell kill kinetics *in vitro*. *Br J Cancer* **54**: 415–421, 1986.
38. Robinson JH and Smith JA, Density dependent regulation of proliferation rate in cultured, androgen-responsive, tumour cells. *J Cell Physiol* **89**: 111–122, 1976.
39. Born R and Eichholtz-Wirth H, Effect of different physiological conditions on the action of adriamycin on Chinese hamster cells *in vitro*. *Br J Cancer* **44**: 241–246, 1981.
40. Pelletier H, Millot JM, Chauffert B, Manfait M, Genne P and Martin F, Mechanisms of resistance of confluent human and rat colon cancer cells to anthracyclines: alteration of drug passive diffusion. *Cancer Res* **50**: 6626–6631, 1990.
41. Sullivan DM, Glisson BS, Hodges PK, Smallwood-Kentro S and Ross WE, Proliferation dependence of topoisomerase II mediated drug action. *Biochemistry* **25**: 2248–2256, 1986.
42. Robbie MA, Baguley BC, Denny WA, Gavin JB and Wilson WR, Mechanism of resistance of non cycling mammalian cells to 4′-(9-Acridinylamino) methanesulfon-*m*-anisidide: comparison of uptake, metabolism, and DNA breakage in log- and plateau-phase Chinese hamster fibroblast cell cultures. *Cancer Res* **48**: 310–319, 1988.
43. Schneider E, Darkin SJ, Robbie MA, Wilson WR and Ralph RK, Mechanism of resistance of non-cycling mammalian cells to 4′-(9-acridinylamino) methanesulfon-*m*-anisidide: role of DNA topoisomerase II in log- and plateau-phase CHO cells. *Biochim Biophys Acta* **949**: 264–272, 1988.
44. Ballas LM, Holmes WD, Sachs CW and Fine RL, Mitoxantrone inhibits serine/threonine kinases associated with signal transduction and mitogenesis. *Proc Am Assoc Cancer Res* **32**: 394, 1991.
45. Ho CK, Law SL, Chiang H, Hsu ML, Wang CC and Wang SY, Inhibition of microtubule assembly is a possible mechanism of action of mitoxantrone. *Biochem Biophys Res Commun* **180**: 118–123, 1991.
46. Ng WF, Sarangi F, Zastawny RL, Veinot-Drebot L and Ling V, Identification of members of the P-glycoprotein multigene family. *Mol Cell Biol* **9**: 1224–1232, 1989.
47. Rothenberg M and Ling V, Multidrug resistance: molecular biology and clinical relevance. *J Natl Cancer Inst* **81**: 907–910, 1989.
48. Endicott JA, Sarangi F and Ling V, cDNAs encoding the Chinese hamster P-glycoprotein gene family. *Proc Am Assoc Cancer Res* **31**: 372, 1990.
49. Bradley G, Juranka PF and Ling V, Mechanism of multidrug resistance. *Biochim Biophys Acta* **948**: 87–128, 1988.
50. Fojo A, Akiyama S, Gottesman MM and Pastan I, Reduced drug accumulation in multiply drug-resistant human KB carcinoma cell lines. *Cancer Res* **45**: 3002–3007, 1985.
51. Dalton WS, Durie BGM, Alberts DS, Gerlach JH and Cress AE, Characterization of a new drug-resistant human myeloma cell line that expresses P-glycoprotein. *Cancer Res* **46**: 5125–5130, 1986.
52. Schurr E, Raymond M, Bell JC and Gros P, Characterization of the multidrug resistance protein expressed in cell clones stably transfected with the mouse *mdr*1 cDNA. *Cancer Res* **49**: 2729–2734, 1989.
53. Yang LY and Trujillo JM, Biological characterization of multidrug-resistant human colon carcinoma sublines induced/selected by two methods. *Cancer Res* **50**: 3218–3225, 1990.
54. Baas F, Jongsman APM, Broxterman HJ, Arceci RJ, Housman D, Scheffer GL, Riethorst A, van Groenigen M, Nieuwint AWM and Joenje H, Non-P-glycoprotein mediated mechanism for multidrug resistance precedes P-glycoprotein expression during *in vitro* selection for doxorubicin resistance in human lung cancer cell line. *Cancer Res* **50**: 5392–5398, 1990.
55. Slapak CA, Daniel JC and Levy SB, Sequential emergence of distinct resistance phenotypes in murine erythroleukemia cells under adriamycin selection: decreased anthracycline uptake precedes increased P-glycoprotein expression. *Cancer Res* **50**: 7895–7901, 1990.