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Initial and early effects of adriamycin in murine sarcoma 180 cannot be restored in a resistant subline by increasing the uptake and external concentration of the drug

Jaroslav Šonka, Ute Schossig, Marlies Vogt-Schaden, and Manfred Volm

Institute of Experimental Pathology, German Cancer Research Center, D-6900 Heidelberg, Federal Republic of Germany

Summary. We demonstrate the early effects (1 day) of Adriamycin (ADM) on proliferation-stimulated and quiescent sensitive, and ADM-resistant cells of the murine tumor sarcoma 180 (S 180). By investigating cell-cycle distribution and thymidine labeling, it can be shown that sensitive cells are strongly affected by the drug, even in a proliferationarrested state. A remarkable but slow DNA synthesis is the prominent effect of this drug treatment on sensitive cells, even under nonstimulating conditions. In resistant cells, neither an increase in concentration nor a variation in drug uptake can induce effects that could be compared with those observed in the sensitive line. From these results we conclude that the early effects of ADM are not modulated by drug uptake.

Introduction

A well-established membrane pump system depending on a supply of metabolic energy ([6, 33]; for review see [16, 17]) is assumed to be the main mechanism of acquired anthracycline resistance. This phenomenon was later included in the field of so-called multiple drug resistance (Mdr) (for review see [16, 29]). According to the abovementioned papers, the basic phenomenon of resistance is the lowered content of drug in resistant cells caused by an active extrusion process with an unknown mechanism. Frequently found membrane markers (see [29] for review) have been introduced in this context as a possible part of the presumed mechanism. By intensified research on amplified genes of resistant cells, some specific sequences have been isolated and sequenced [4, 11]. These sequences fit well with the presumed membrane structures; however, their systematic comparison with already known sequences revealed relationships with not only bacterial transport proteins, but also proteins of the DNA-repair complex.

Offprint requests to: J. Šonka

Abbreviations: ADM, Adriamycin; AMSA, 4'-(9-acridinylamino)-methanesulfon-m-anisidide; FCM, flow cytometry; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; Mdr, multiple drug resistance; PBS, phosphate-buffered saline; PI, propidium iodide; R 123, rhodamine 123

Some evidence has recently been accumulated that the so-called drug pump might not be the only mechanism responsible for the described type of resistance. Colofiore et al. [5] could increase the cellular survival of drug-resistant cells by inhibiting the production of metabolic energy, whereas Danø [6] and Skøvsgaard [33] used the same inhibitors to impair the drug-pumping system. Hence, similar treatment caused opposite effects depending only on the time schedule of application. Siegfried et al. [32] could not correlate the variations in drug uptake and extrusion with the degree of resistance of the respective lines. Lothstein and Horwitz [21] found that the originally resistance-associated pumping mechanism was retained in revertant lines. Mirski et al. [22] selected an adriamycin (ADM)-resistant line that confirms all the phenomenology known with the exception that it does not express the resistance-specific P-glycoprotein. In a previous report [35], we demonstrated differences in the pumping status in drug-resistant cells in situ (proliferation-stimulated) and in vitro (depleted of stimuli). This fact may explain the contradictory results of the studies mentioned above. Besides the known means of impeding the pumping process – Tween and verapamil [3, 28, 30] – we can now alter drug uptake in ADM-resistant murine sarcoma 180 (S 180) lines through relatively natural means using proliferation-stimulating or -inhibiting conditions [35].

Most experiments designed to explore such drug resistance *select* resistant lines and *test* them in long-term experiments evaluating the fraction of surviving cells and correlating that data with measurements of drug uptake. Seldom have additional data been collected on the status of the cell that demonstrate the respective drug uptake as function of, e.g., the cell-cycle phase [39] or the course of cellular death in different lines. To overcome this lack of data on short-term effects of anticancer drugs, Hill and Schimke [12] demonstrated changes in prelabelled chromosomes in an elegant system. Although the drug used (methotrexate) is unrelated to the Mdr group, the approach toward observing the fate of cells which, with the exception of a very small fraction, will not survive the drug treatment is exemplary. Little evidence can be compiled about such effects of ADM on resistant cells [37], whereas some data have been presented about the effects of this drug on cell-cycle passage in cells with no previously acquired resistance [1].

The crucial question as to the validity of the transport concept in Mdr involves comparisons of short-term effects

in sensitive cells and those resistant cells in which drug uptake has been manipulated to the level of sensitive cells. If these effects are not comparable, additional mechanisms of resistance must be taken into account, irrespective of the later fate of the different cells. We present such an approach.

Materials and methods

Murine sarcoma 180 (S 180) cells were cultivated in RPMI 1640 supplemented with 10% fetal calf serum (FCS). An ADM-resistant line, S 180/ADM210, was previously developed in mice by applying increasing suboptimal doses of the drug to weekly passages. The final therapy dose was 4 mg/kg body weight. After transfer into cell culture, the cells could grow in a maximum of 10 mg/l ADM with no growth delay and were held stable by one drug application per passage for over 2 years. For the experiments, cells that had not previously been exposed to the drug within the respective passage were used. The drug was kindly supplied by Farmitalia Carlo Erba (Freiburg, FRG).

Experiments demonstrating differential drug uptake were carried out with ascites tumors induced by the i.p. inoculation of NMRI mice with 10^6 cells, which yielded a logarithmically growing cell suspension at day 7 post-implantation. This approach helps to reduce time-consuming and cell-damaging procedures (trypsinizing, scraping, washing, and centrifugation) needed for the preparation of cells propagated in cell culture. From the ascites fluid, which demonstrates a very high cell density, suspensions of 2×10^6 cells/ml can easily be obtained by simply diluting a standardized droplet of the original fluid into 20 ml Hanks' balanced salt solution (HBSS). Cell counts were checked after the experiments and revealed only minor deviations from the desired values. This sequence of experimental steps reduces the preparative procedure and allows uptake measurements to be made within the intended time period. The uptake of daunomycin was determined according to Plagemann and Wohlhueter [25]. The cell suspensions were layered over an oil layer (Silicon oil DC 550 – Serva/Parafinoil 7174 – Merck; 91/9 parts) and mixed with [^{3}H]-daunomycin (New England Nuclear), for a resulting activity of 250 µCi/ml at a concentration of 0.5 m*M*. After an incubation of 5 min, the tubes were mounted into a centrifuge and the cells were spun through the oil layer, destroyed by hypotonic shock, and analyzed by liquid scintillation counting in Unisolve 1 scintillation mixture (Koch Light Labs). For measurements carried out after 150 min, the cells of the originally diluted suspensions were stored on ice and incubated shortly before the experiment at 37° C. The experiments were repeated four times at five samples per respective value.

For other experiments, the cells were inoculated into cell-culture flasks at a density of approximately 20,000 viable cells/cm^2 and cultivated for 3 days in RPMI containing 10% FCS. These growing cultures were proliferation-arrested by incubation in RPMI 1640 without serum for a further 2 days. The 24-h treatment was subsequently carried out in four groups: one-half of the flasks were supplied with fresh serum-free medium and the rest, with serum-supplemented (10%) medium; both groups were further subdivided and cultivated with and without ADM, respectively. Sensitive cells were incubated with 10 mg/l ADM and resistant cells, with 100 mg/l. In a test system developed by Volm and Lindner [40], these concentrations cause a >50% inhibition of the incorporation of [^{3}H]-uridine. It should be pointed out that the application of the drug to media containing different amounts of FCS results in different effects, since the drug is trapped to a considerable extent by the serum proteins [8]. As these differences would in any case cause effects impeding the conclusion drawn (cf. Table 1), they need not be taken into account. Following the first incubation, a first set of samples was investigated by measuring its cell-cycle distribution by DNA fluorimetry. A second set of samples was uniformly supplied with RPMI 1640 containing 10% FCS and similarly investigated after an additional 24-h cultivation. Finally, a third set of samples was checked for viability by propidium iodide (PI) exclusion assay [14].

DNA fluorimetry was carried out as previously described [34]. Briefly, cells treated with RNAase and pepsin were subsequently stained with a mixture of PI and diami-

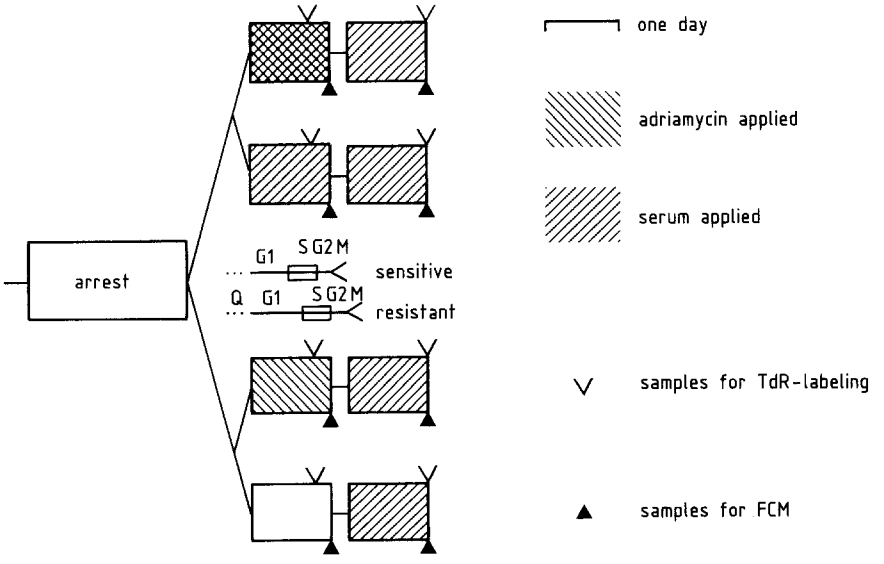

Fig. 1. Flow-through diagram showing the schedule for treating sensitive and resistant S 180 cells with various media

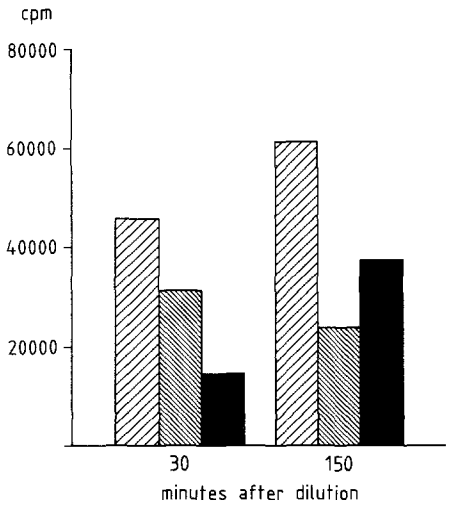

Fig. 2. Uptake of [^{3}H]-daunomycin over a 5-min period 30 and 150 min after the preparation of the cell suspension. Cells for both determinations were prepared at the beginning of the experiment. The suspension for the later measurements was stored on ice and used after a short incubation at 37° C. ▨ sensitive; ▧ resistant; ■ difference

dinophenylindol and measured in an ICP 22 flow cytometer. The experiments were repeated at least twice; the effect of Tween on the uptake of rhodamine 123 was checked repeatedly in several independently resistant lines. As a further control, our previous data [35] reproducibly show the behavior of cells arrested by serum depletion and repopulated by refeeding with serum-containing medium.

For the purpose of thymidine labeling, the cells were plated onto cover slides, grown under stimulating conditions for 1 day, and subsequently arrested by serum-depleted medium. Following 48 h cultivation in serum-free RPMI 1640, they were treated as described above. However, the time points of fixation of the samples were altered, since this had to take place during the presumed S-phase periods [35]. The respective time points are indicated below. At 2 h before fixation, 10 μCi [^{3}H]-thymidine (20 μCi/mmole; Amersham Buchler) was added to 2 ml medium. The samples were fixed with ethanol/acetic acid (3:1), washed in ethanol, and air-dried. They were subsequently washed with a 1,000-fold excess of cold thymidine and processed for radioautography by dipping into emulsion NTB2 (Kodak). After a 26-day exposure, the slides were developed, weakly stained with toluidine blue, and photographed with Ilford PAN F film. These experiments were repeated twice at two samples per respective value, all giving identical results. Figure 1 illustrates the treatment schedule

Results

In Fig. 2 we demonstrate cumulative 5-min uptake values of daunomycin at two different points after the in situ/in vitro transfer of the cells. Ascites cells were punctured immediately after each animal's death and diluted without any further preparative delay to approximately 10^6 cells/ml with HBSS. The cells were then preincubated in this solution for the indicated intervals and used for drug-uptake measurements. The *solid bars* demonstrate the difference between the respective values of sensitive and resistant

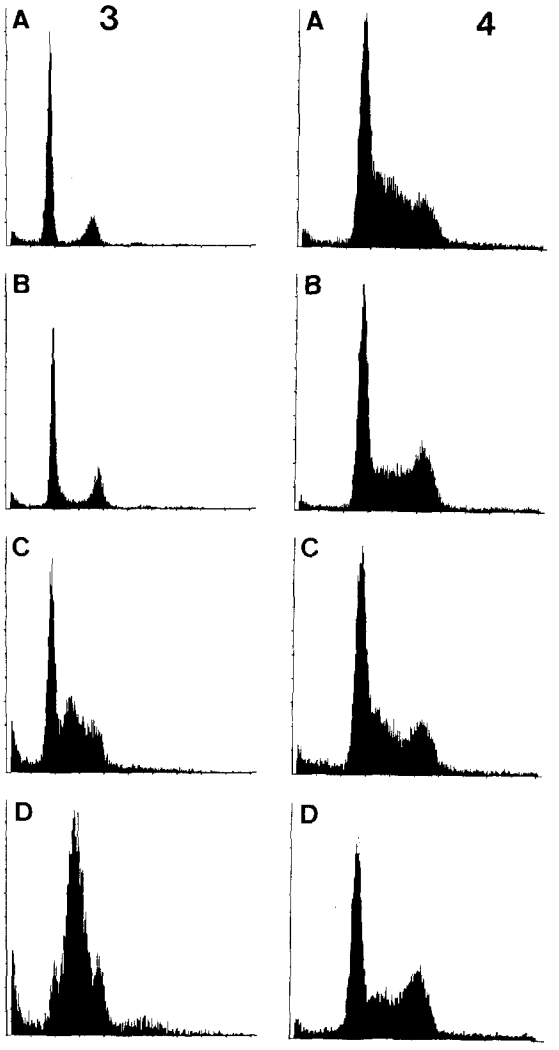

Fig. 3. Cell-cycle diagrams of sensitive S 180 cells after 24 h treatment. The cells were incubated 48 h before treatment in serum-free RPMI 1640. *A*, controls treated with fresh serum-free medium; *B*, cells treated with fresh medium plus 10% FCS – the first cycle is already finished; *C*, cells treated with 10 mg/l ADM under serum-free conditions; *D*, cells treated with 10 mg/l ADM and 10% FCS

Fig. 4. Cell-cycle diagrams of resistant S 180 cells 24 h after treatment: pretreatment and experimental variants are the same as in Fig. 3. Note that the duration of the cell cycle in resistant cells is longer (cf. [35])

cells. In a previous study [35] we observed very quick reactions shortly after the in situ/in vitro transfer; therefore, in the present study we did not attempt to approach the zero values.

After serum depletion, the incorporation of thymidine by sensitive and resistant S 180 cells was reduced to minimal values within hours; after 36–48 h, >80% of the cells appeared in the G1 region of the cell cycle ([34]; Fig. 3). Following this treatment, resistant cells demonstrated a drug-extruding status, which was not the case under serum-stimulated conditions or at any stage in sensitive cells. We treated the cells with ADM over a 24-h period after 48 h arrest. Figure 3 demonstrates cell-cycle distributions following the continuous 24-h treatment of sensitive cells with 10 mg/l ADM under serum-depleted and

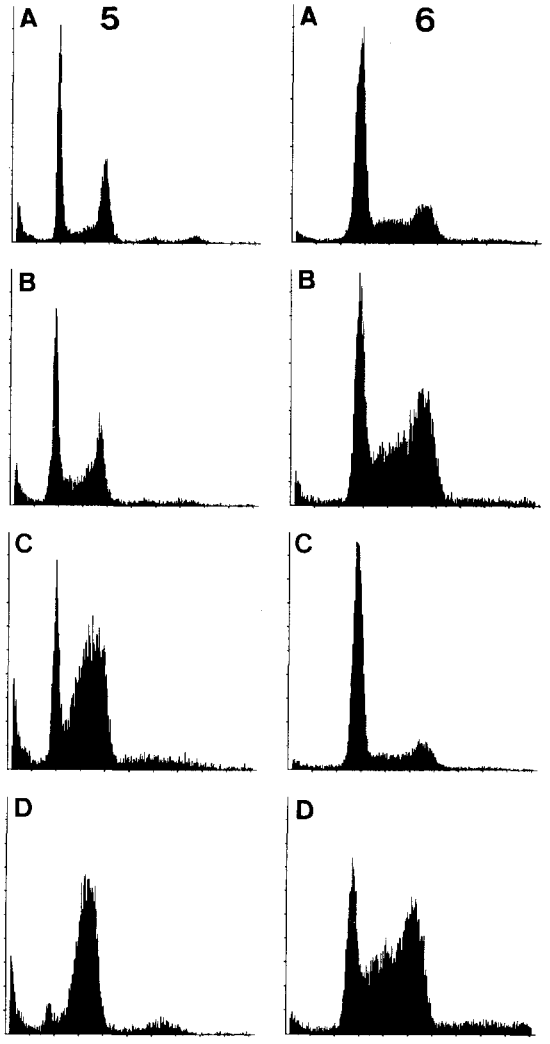

Fig. 5. Cell-cycle diagrams of sensitive S 180 cells after 24 h treatment (same as in Fig. 3) and a uniform subcultivation for an additional 24 h in drug-free RPMI 1640 with 10% FCS

Fig. 6. Cell-cycle diagrams of resistant S 180 cells after 24 h treatment (same as in Fig. 3) and a uniform subcultivation for an additional 24 h in drug-free RPMI 1640 with 10% FCS

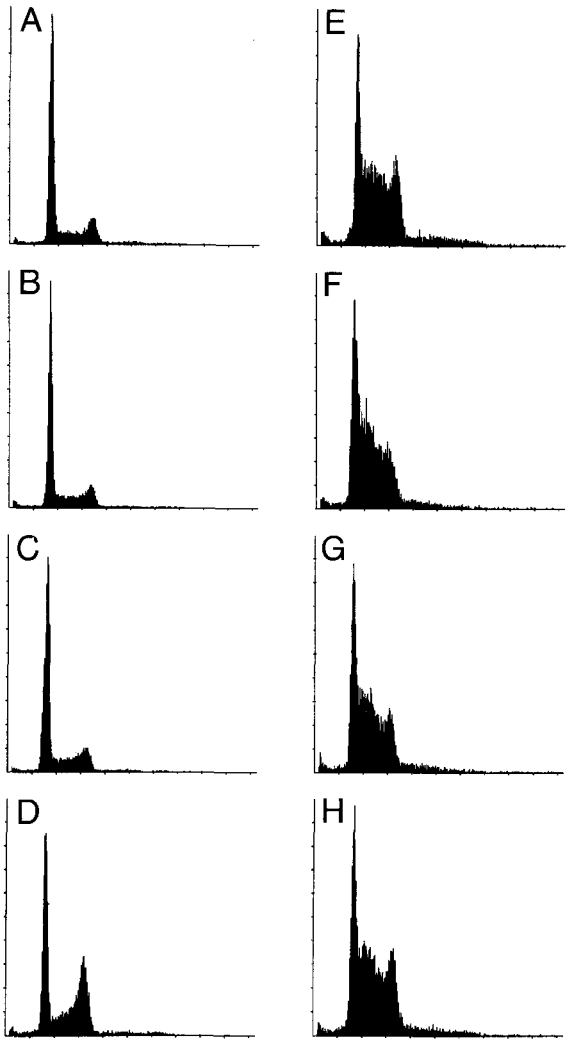

Fig. 7. Cell-cycle diagrams of resistant S 180 cells in serum-free RPMI 1640, treated with 100 mg/l ADM and 20 mg/l Tween-20 after 24 h treatment (*A–D*) and after a uniform subcultivation for an additional 24 h in drug-free RPMI 1640 with 10% FCS (*E–H*). *A*, control; *B*, +ADM; *C*, + Tween-20; *D*, + ADM and Tween-20

Table 1. Cell counts (relative units, coulter counter) and dead/viable ratios (PI fluorescence)

First incubation	Sensitive		Resistant	
	cell counts	Dead/viable	cell counts	Dead/viable
−FCS −ADM	9,480	10.0%	9,100	7.3%
+FCS −ADM	11,803	12.0%	11,020	3.9%
−FCS +ADM	6,907	24.0%	9,120	14.3%
+FCS +ADM	5,488	17.0%	6,750	7.6%

serum-stimulated conditions (10% FCS after 48 h in serum-free conditions). Under the latter conditions in the absence on the drug, sensitive cells had already passed through their first cycle ([35]; Fig. 3B). ADM-treated sensitive cells appeared to be stimulated into the S-phase irrespective of the presence of serum. However, the time course of the apparent stimulation was rather unexpected (Fig. 3C, D), since the regular duration of the S-phase and of the entire cell cycle usually would not at that time allow an accumulation of cells at that point of DNA synthesis.

Analogous cell-cycle distributions in resistant cells are demonstrated in Fig. 4. No effects of the drug are evident, although a tenfold concentration was used (100 mg/l). The application of serum stimulated the cell cycle (Fig. 4B, D) but did not cause any of the associated effects of ADM that were seen in cell-cycle distribution in sensitive cells.

For further analysis of the effects of the above-mentioned treatments, we refed parallel samples of all demonstrated variants with RPMI containing 10% FCS and collected them for DNA fluorimetry after an additional period of 24 h with no further treatment. Figure 5 shows cell-cycle distributions in sensitive cells. ADM-treated sensitive cells remained blocked in the apparent S-phase irrespective of serum stimulation. Cell-cycle distributions in resistant cells are shown in Fig. 6; again, no effect of the pretreatment with the drug was observed in these cells.

The demonstrated cell-cycle distributions do not give data on cell counts or cell loss. Table 1 shows the cell counts and dead/viable cell ratios for the above experiments at the end of the drug-free postincubation. Any major contribution by the amount of cell death (as determined by PI fluorescence) can be excluded. After the drug treatment only a slight increase in dead-cell ratio could be seen; however, this does not necessarily hold true for the following time periods. A comparison of the total cell counts demonstrates reduced values in ADM-treated samples irrespective of the proliferation status of sensitive cells and an inhibition that depends on serum stimulation in resistant cells. The comparison of cell counts and the cell cycle diagrams suggest that the effect of ADM on sensitive cells involves an initial stimulation of replicative processes followed by an arrest at intermediate DNA content. In resistant cells a slowdown of the cycle passage could be observed only in the serum-stimulated version. We omitted experiments at other concentrations of drug because of the high nonspecific toxicity of higher concentrations toward

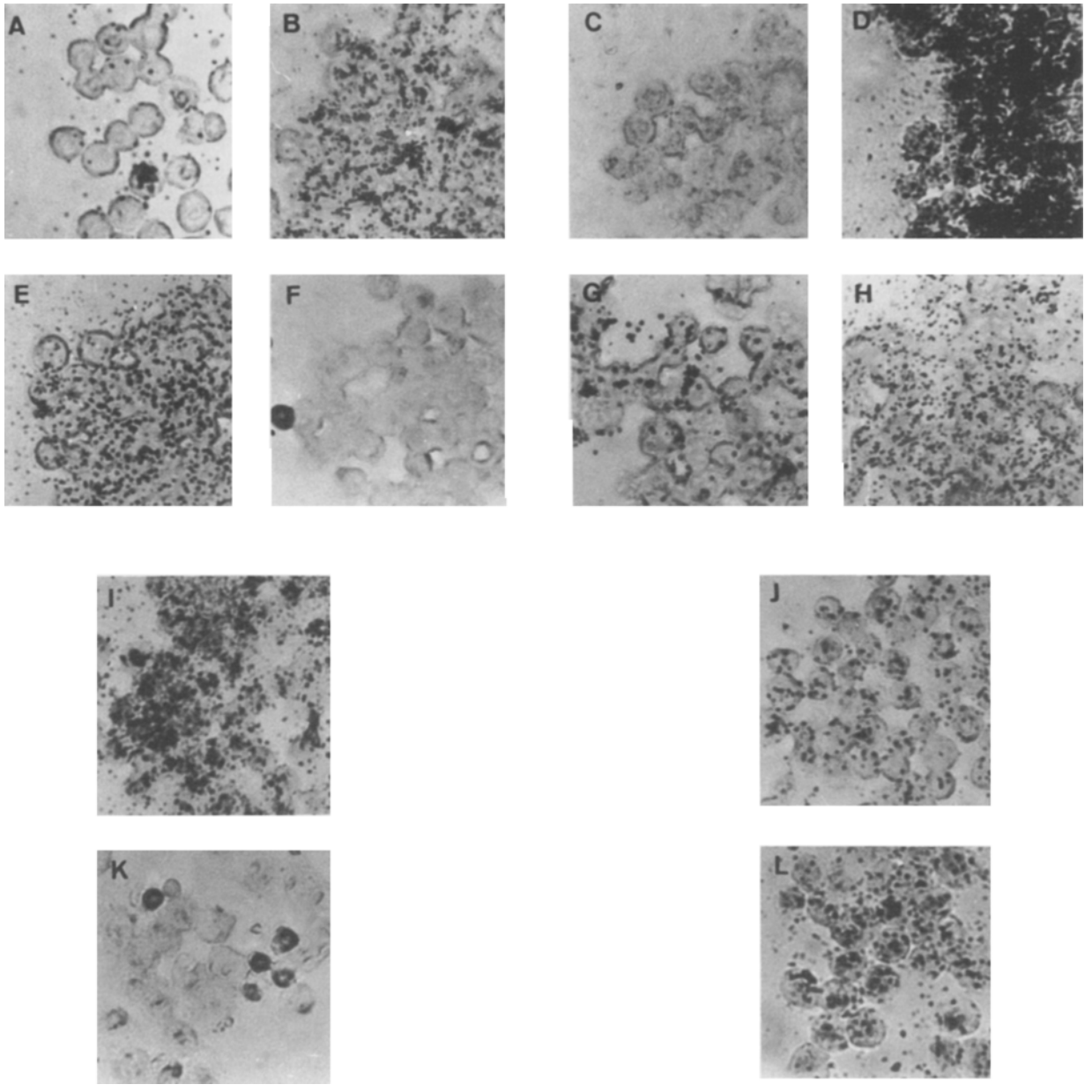

Fig. 8. Thymidine labelling (2 h) of sensitive and resistant S 180 cells under different treatments (all arrested by 48-h incubation in serum-free medium). *A*, sensitive cells after 18 h in serum-free medium; *B*, sensitive cells stimulated for 18 h with 10% FCS; *C*, resistant cells after 18 h in serum-free medium; *D*, resistant cells stimulated for 18 h with 10% FCS; *E*, sensitive cells after 18 h in serum-free medium with 10 mg/l ADM; *F*, sensitive cells stimulated for 18 h with 10% FCS + 10 mg/l ADM; *G*, resistant cells after 18 h in serum-free medium with 100 mg/l ADM; *H*, sensitive cells stimulated for 18 h with 10% FCS + 100 mg/l ADM; *I*, sensitive cells treated as in *B* and cultivated for 1 additional day in RPMI 1640 with 10% FCS; *J*, resistant cells treated as in *D* and cultivated for 1 additional day in RPMI 1640 with 10% FCS; *K*, sensitive cells treated as in *F* and cultivated for 1 additional day in RPMI 1640 with 10% FCS; *L*, resistant cells treated as in *H* and cultivated for 1 additional day in RPMI 1640 with 10% FCS

sensitive cells, on the one hand, and the lack of appreciable effects of lower concentrations on resistant cells, on the other.

Besides modulating drug uptake by serum stimulation, we also used Tween-20, a detergent that increases the net uptake of drug in resistant cells without stimulating them into the cell cycle. In Fig. 7 we demonstrate the effects of Tween-20 in resistant cells (we omitted data on sensitive cells since they did not reveal any effects [35]). Although treated with this repeatedly used substance [3, 28, 30], the resistant line did not acquire the behavior of the sensitive line.

To specify further the state of the cells after the respective treatments, we labeled the cells with [^{3}H]-thymidine and analyzed them by radioautography. In Fig. 8 we show the degree of labeling in sensitive and resistant cells after 18 h under serum-free and serum-stimulated conditions, respectively, both with and without ADM. The best picture of cells arrested by the serum-free medium was revealed by the resistant cells (Fig. 8C), which were also best stimulated by serum (Fig. 8D). The addition of drug caused increased labeling in cells held in serum-free medium, a fact which may be attributable to unscheduled (repair) DNA synthesis (Fig. 8G). This effect clearly demonstrates the primary attack of ADM even in quiescent, drug-extruding cells (see also Fig. 2 and [34]). The heavy label of the serum-stimulated cells (Fig. 8D) could not be demonstrated in drug-treated cells (Fig. 8H), in correlation with the inhibition of proliferation demonstrated in Table 1. Sensitive cells demonstrated a residual label even after 48 h arrest (Fig. 8A); they were also easily stimulated by serum (Fig. 8B), although the degree of stimulation seemed to be less pronounced. The addition of drug caused some unscheduled DNA synthesis (Fig. 8E) that was also observed in the DNA-fluorimetric data (Fig. 3) presented above. However, this synthesis was apparently not a regular excission repair because of the accumulation of newly synthesized DNA (cf. Fig. 3) as well as the accumulation of label during a period of longer than 1 day. The latter also argues against use of the term S-phase in this case. Additionally, in Fig. 8 control samples are shown that were subcultivated after the various treatments for a further 24 h in fresh drug-free medium containing 10% FCS. All resistant cells, irrespective of the previous treatment, could be stimulated to proliferation by FCS (we present only those that were treated in serum-containing medium – Fig. 8J, L). Sensitive cells of both drug-treated variants (again, we show only those that were treated in serum-containing medium – Fig. 8K) were no longer stimulatable by FCS and demonstrated heavily fragmented nuclei (not shown) irrespective of the apparent viability assayed by PI extrusion (Table 1). The controls were normally stimulatable (Fig. 8I).

Discussion

According to our previous results [35], one should expect that the uptake of anthracyclines by cells at different points of time after their in situ/in vitro transfer would exhibit considerable differences, which would be superimposed on the true uptake kinetics. Therefore, we compared values of the uptake of labeled daunomycin (the only labeled anthracycline available at the time of the study) within a stable 5-min interval measured at different points after the in situ/in vitro transfer. The uptake values demonstrated in Fig. 2 for cells preincubated 150 min in HBSS resemble those previously found by others [32] in different resistant lines of the same cell line (S 180) that we used. Recently published data on the shape and optical density of the presented cell lines [19] underline these facts: resistant cells acquired greater nuclei; hence, the difference found becomes more significant.

However, our values reveal considerable differences between the respective points, thus underlining the existence of a factor apart from the often shown transport kinetics [6]. An extrapolation of these results toward zero time and the previously published results concerning the uptake of the fluorescent dye R123, which belongs to the cross-resistant agents [20, 35], allow one to argue that under conditions of growth stimulation, ADM-resistant S 180 cells do not differ from sensitive ones in their drug uptake. This can be confirmed by the fluorescence microscopy of proliferation-stimulated (FCS) and serum-depleted (quiescent) monolayer cells treated with ADM or R123 (data not shown). However, this method does not allow quantitative evaluation. In conventionally prepared experiments, rhodamine 123 and thus the anthracyclines are pumped out of resistant cells that have lost their stimulating conditions, e.g., by suspension in HBSS or phosphate-buffered saline (PBS) (cf. [35]). This leads back to the results of previous studies by Darzynkiewicz et al. [7], Goldstein and Korczak [10], and Summerhayes et al. [36], in which benign cells took up rhodamine 123 only after having been stimulated to proliferation.

Facts that support this explanation were recently published [29]; the observed changes in drug uptake and extrusion developed by resistant lines were accompanied by changes in the transport of amino acids and other substances. According to Glassy and Furlong [9], the transport systems for these substances are dependent on the serum dose and cell-cycle stage.

For the investigation of cell kinetics, we chose two different methods, DNA fluorimetry and thymidine labeling, because of the complementarity of their -esolving power. Recently published results [26] underline the suspicion that these methods measure quite different processes, and the common assumption that "S-phase ratios" determined by one method can be substituted by those determined by the other becomes rather problematic. Frequently, singular data are presented and interpreted that require a complementary approach to obtain a cross-control. Thus, a decline in thymidine labeling is interpreted as an arrest of cells outside the S-phase (e.g., G1/S-border [27]). The lack of other data makes it impossible to detect an arrest within the S-phase, much less an arrest occurring after the rather unusual replicative steps shown in the present paper. On the other hand, Hill et al. [13] have interpreted flow cytometric (FCM) data showing intermediate or 2C-DNA content to be an arrest in the respective part of the cell cycle, without taking other replication or amplification possibilities into account. Such non-S-phase DNA synthesis has clearly been demonstrated [23] using a dual approach (total DNA/antibody to bromodeoxyuridine-substituted DNA).

From the results presented and the above-summarized background, the question arises as to whether the effects of anthracyclines on sensitive and resistant cells would not simply fit into the old theory of proliferation-dependent drug action [2]. The observed pump would then be a consequence of cellular quiescence and yet be symptomatic for a reversion to a status capable of quiescence.

The results of the present study demonstrate that the initial (minutes to hours) and early (up to days) effects of ADM observed in sensitive cell lines cannot be restored in resistant cells by annihilation of the pumping process, even in experiments where tenfold higher concentrations of the drug are used. Sensitive cells – although condemned to death after several days – are stimulated by the drug into an apparent S-phase but cannot continue the cycle. This arrest can clearly be demonstrated by prolonging the cultivation by 1 day under stimulating (10% FCS) conditions and may cause the inevitable death of the cells. It was rather unexpected that stimulation by the drug could be shown even in cells cultivated in serum-free RPMI 1640, a condition that normally inhibits the cell-cycle progress. The resulting cell counts (based a.o. on the proliferation on the last day of the experiment, when all samples are stimulated by serum) are reduced in both ADM-treated variants, irrespective of serum stimulation during application of the drug. Hence, it can be stated that proliferation enhances the effects of ADM in sensitive cells.

The observation of ADM's effects on proliferation-arrested cells seems to corroborate previous results [15] demonstrating the protection of cells against ADM by an inhibitor of protein synthesis, anguidine. Two (partly coupled) factors can be held responsible for the effects of this substance: the inhibition of protein synthesis and the resulting cytostasis. According to our data as extended by achieving natural arrest by the depletion of stimuli, we assume that the first factor rather than the second causes the protection of cells from the effects of the drug. Further, it is hardly possible to term the demonstrated DNA synthesis an S-phase; its early onset and long duration seem to exclude this description. Such slow processes, instead of repeated cell-cycle events, could be the basis for the slow and continuous increase in dihydrofolate reductase amplification previously demonstrated by Tlsty et al. [38] and Kleinberger et al. [18]; however, these authors did not use proliferation-arrested cells.

To obtain assessable data (important especially when concerning unscheduled DNA synthesis by radioautography), we treated resistant cells with a tenfold higher concentration of the drug. The results demonstrate reduced cell counts after ADM treatment only in stimulated samples and thus, at least partly, proliferation-dependent effects. However, no stimulation of net DNA synthesis by the drug could be seen; moreover, no subsequent arrest in the S-phase could be observed, even with increased drug uptake. On the other hand, even those cells that usually extrude ADM due to nonstimulating conditions clearly exhibited unscheduled incorporation of [^{3}H]-thymidine when treated with the drug; again, this points out that the extrusion of the drug is not sufficient to impede its effects on the cells. Similar conclusions can be drawn by comparing the reduction in drug uptake of ADM-resistant S 180 cell lines with the degree of their resistance and the intracellular drug binding [32]. In addition, the use of the nonionic detergent Tween 20, which can break resistance and increase the uptake of the drug [3, 28, 30, 35], is not suitable for the restoration of drug-sensitive behavior in the first hours of treatment.

These results clearly show that the original effects observed in sensitive cells do not reappear in resistant cells if the pumping system is put out of function. We conclude that the pumping system in anthracycline-resistant cells is only partly responsible, if at all, for the phenomenon of resistance. Recently, Lothstein and Horwitz [21] could separate resistance and drug-uptake phenomena in a similar way; the reduction in drug uptake persisted even after the reversion of the cells to a sensitive status. Hence, additional explanations for the resistance phenomenon must be sought. The following discussion concerns two factors of particular interest.

Differences in regulation of the cell cycle

In a previous paper we demonstrated that the time needed for the induction of proliferation in serum-starved cells by serum application differs between sensitive and resistant cells, which suggests a better regulated passage of this part of the cell cycle in resistant cells. The proliferative fraction within the resistant tumor seemed to be reduced (from originally 100% [31]); accordingly, the reduction in drug sensitivity might to a considerable extent be caused by these changes in cell-cycle settings.

Differences in repair capacity

The results presented above suggest an insufficient repair of DNA in sensitive cells. Sensitive cells cannot carry out a complete replicative cycle. By the methods used we cannot decide if the observed DNA synthesis caused by the drug is an insufficient S-phase or an insufficient repair (lacking a previous excission), or even nonspecific proliferation-unrelated DNA amplification. Yet, as resistant cells can pass the S-phase even with increased uptake and a higher concentration of the drug, they must be able to obviate the damage. This suggests improved DNA repair, which should be further investigated. The data of Nelson et al. [24] demonstrating interactions of AMSA, an agent belonging to the cross-resistant group of the anthracyclines, with DNA topoisomerase support this suggestion.

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