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In Vitro Uptake and Cytotoxicity of Adriamycin in Primary and Metastatic Lewis Lung Carcinoma*

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Abstract—*The* in vitro *capacity to take up adriamycin (AM) of murine Lewis lung carcinoma (3LL) cells, derived either from intramuscularly (i.m.) implanted tumor or from its lung metastases, was investigated together with the response of these 2 cell populations to AM treatment.*

Cell suspensions obtained by mechanical disgregation from primary and secondary tumors directly excised from tumor bearing mice, and primary cultures of these 2 cell populations were exposed to AM in 2 treatment conditions: a higher dose for a short time and a low dose for a longer time. No differences were observed in drug uptake, measured as binding to the cells between primary and metastatic tumor in both experimental conditions. However in the same treatment conditions on the same cell lines AM caused consistently greater cytotoxic effect on pulmonary 3LL metastases than on i.m. primary tumor cells.

These findings suggest that the different responses observed may be due to differences on the intrinsic sensitivity of primary and secondary tumor cell populations to AM.

INTRODUCTION

THAT metastases are more sensitive to chemotherapy than the primary tumor from which they are derived has been widely reported in experimental and clinical studies [1–6]. Differences in the biochemical, physiological and environmental conditions, besides the cell population characteristics of primary and secondary neoplasms, have been invoked to explain the different response.

Since chemotherapeutic agents are much more effective in killing rapidly proliferating cells [3, 7–9], the importance of the cell kinetics of the tumor cell population as a determinant of drug sensitivity has received particular attention [10, 11]; according to the Gompertzian function of tumor growth [12–14] metastases, which are small tumors, should have a shorter doubling time and should therefore be more sensitive to chemotherapy. In addition to the proliferative state of the tumor cells, another factor contributing to greater drug efficacy on metastases is better drug availability at the metastatic site [15]; this is taken to be the result of the greater blood supply usually present in smaller tumors [16–20]. The object of the present study is whether primary and secondary tumor cells take up or bind drugs to different extents, and how the response of these cell populations to therapy is related to cell drug concentrations. Cells of the Lewis lung (3LL) tumor system, an animal model devised to study the response of metastases to chemotherapy [21], were used in *in vitro* experiments. Cell populations of 3LL from the i.m. implantation site and from its lung metastases were exposed to adriamycin (AM), a drug to which 3LL responds [22], in 2 treatment conditions: a high dose for a short time and a low dose for a longer time. Drug uptake or binding to the cells together with the cytotoxicity of AM in these conditions were investigated.

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MATERIALS AND METHODS

Animals and tumor

C57Bl/6 male mice (22 ± 2 g body weight) obtained from Charles River, Italy, were used for this experiment. They were housed in

Makrolon cages (20 × 20 × 13 cm) at room temperature of 22°C and relative humidity about 60%, with free access to food and water. The mice received an intramuscular (i.m.) transplant of 2×10^5 viable cells of the syngeneic Lewis lung carcinoma (3LL), maintained by i.m. passages in the same strain every 2 weeks and known to give rise to macroscopic metastases to the lung within about 18 days of transplantation [21]. For the experiments cells from tumors and metastases of animals bearing i.m. implants at least 18 days old were employed.

Drug

Adriamycin (AM) was obtained from Farmitalia, Milan. It was assayed in tumor and metastasis cells as described by Schwartz [23], using $AgNO_3$ to release AM bound to DNA and RNA and to precipitate proteins, subsequently extracting the supernatant phase with *n*-butyl alcohol. Readings were taken in an Aminco Bowman spectrofluorometer at 470 nm excitation and 569 nm emission. In these conditions, recovery was 90% and sensitivity 0.01 μg/10^6 cells.

Cell suspension

Tumor and metastasis fragment were removed aseptically 25 days after implantation, minced with fine scissors and homogenized in phosphate buffer (pH 7.4 and osmolality 285 mOsm/kg) by mechanical disruption. Cell viability was assessed by the Trypan blue exclusion method and was found to be from 70 to 90% for both primary and metastatic tumor cells. The cell suspension concentration was then adjusted to 10^6 viable cells/ml in phosphate buffer. Samples of 10^6 primary and metastatic 3LL tumor cells in 1 ml phosphate buffer were incubated at 37°C in a Dubnoff shaker apparatus in the presence of scalar doses of AM for 15 min and in two other specific treatment conditions: 1 μg/ml for 5 min and 10 μg/ml for 1 min. Incubation was stopped by adding 9 ml of ice cold buffer and the tubes were centrifuged at 1500 rev/min for 10 min at 4°C. The cells were washed by adding buffer and centrifuging at 1500 rev/min for 10 min and the pellet was gently resuspended in 1 ml buffer. AM content was assessed as described before. The uptake of AM by the cells, measured as AM recovered from the cellular fraction, was expressed as a percentage of the total amount of drug in the 1 ml of buffer cell suspension.

The AM content in the incubation medium after removing cells, and in the buffer utilized for washing, was also measured. Drug loss during washing was about 3%, and the AM concentration found in the medium, compared to the amount of drug originally added, confirmed the uptake values obtained. The experiments were repeated 3 times and 5 samples per point were used in each experiment.

Cell culture

Tumors and lung metastases were removed aseptically, respectively 18 and 22 days after implantation. Cancer tissue was freed from surrounding material, finely minced with scissors and trypsinized in 0.3% BDH trypsin in phosphate buffer solution at 37°C for 30 min, under continuous stirring. The cell suspension was then filtered and washed twice in phosphate buffer solution (PBS).

Samples of 10^6 viable cells/ml were seeded in 25 cm^2 plastic bottles. Minimum essential medium (MEM) was used with an extra 3 × MEM vitamins, 3 × MEM non essential aminoacids, 15% fetal calf serum, and 50 μg/ml gentamycin. After 3–5 days, depending on each batch of cells ability to reach confluency, cells were freed into suspension by 0.125% trypsin in PBS for 10 min. Recovery studies on the cell population obtained from the original tumor showed that primary culture derived from 20% of the original population. These cells are resistant to further trypsinization procedures so the cell suspension for incubation with AM contained 90% of the viable cells existing in the primary culture. After washing the trypsin from the cells, a suspension containing 10^6 cells/ml was prepared for incubation with AM and the drug content was measured as described.

Cell culture for cytotoxicity experiments

Cytotoxicity experiments were made on monolayers obtained from explants of 3LL tumors and metastases in 16 mm Costar wells. Cells were grown for 4 days in the medium described for uptake experiments until there was sufficient outgrowth to permit application of a cytotoxicity score [24].

Statistical analysis

The Mann–Whitney test was applied to assess the significance of the differences between the amounts of drug taken up by primary and metastatic tumor cells.

RESULTS

The first experiment was designed to investigate the capacity of 3LL cells from the i.m. primary tumor or its lung metastases to take up AM, measured as binding to the cells, assumed to correspond to the amount of drug present in the cell extract. When cell suspensions were incubated at a constant concentration of 10^6/ml for 15 min at 37°C in the presence of scalar doses of AM, (Table 1) the quantity of cell-associated drug was proportional to its external concentration. In the range of concentrations from 0.1 to 10 μg/ml a cell volume of 10^6 cells/ml was not saturable by AM; average drug uptake was 80% (67.7–99.9) indicating that this compound is bound to cells to a large extent and very rapidly. No difference was seen between the amounts of drug in the cell extract of the 2 tumor cell populations. This means that primary tumor and metastases have much the same capacity to take up or bind AM. Since 10–30% of the cells in both populations are dead, the behaviour of viable and dead cells in drug uptake was investigated in order to ascertain that different amounts of dead cells did not interfere with correct measurement of drug uptake. Table 2 reports the concentration of AM bound to cell suspensions derived from the vegetating or the necrotic area of the Lewis carcinoma; no difference is detectable between cells assumed to be either viable or dead in accordance with the data of Skosgaard [25], indicating that AM undergoes a passive transport through the cell membrane.

Table 2. Uptake of adriamycin by cell suspensions of vegetating and necrotic intramuscular 3LL

	μg/10^6 cells	%
Vegetating cells	3.07 ± 0.38	30.7
Necrotic cells	2.86 ± 0.33	28.6 n.s.

10^6 cells/ml medium were incubated with AM (10 μg/ml × 1 min) at 37°C.
n.s. = not significant vs vegetating cells.

Table 1. Uptake of scalar doses of adriamycin by cell suspensions of intramuscular 3LL and its lung metastases

Adriamycin μg/ml	i.m. Tumor μg/10^6 cells	%	Lung metastases μg/10^6 cells	%
0.1	0.099 ± 0.001	99.7	0.099 ± 0.01	99.9 n.s.
0.2	0.13 ± 0.02	67.7	0.143 ± 0.006	71.6 n.s.
0.5	0.41 ± 0.05	81.8	0.38 ± 0.01	76.9 n.s.
1	0.81 ± 0.03	81.3	0.82 ± 0.02	72.6 n.s.
2	1.57 ± 0.01	78.4	1.69 ± 0.006	84.6 n.s.
10	7.05 ± 0.7	70.5	6.81 ± 0.4	68.1 n.s.

10^6 cells/ml medium were incubated at 37°C for 15 min.
n.s. = not significant (Mann–Whitney test) vs i.m. tumor.

The *in vitro* cell uptake of AM and its cytotoxic effect were investigated by exposing 10^6 cells of 3LL primary tumor and metastases to AM doses of the same order of magnitude as the concentrations found in serum of 3LL bearing mice after therapeutic injections of AM [22], i.e., 10 μg/ml × 1 min and 1 μg/ml × 5 min (Table 3). Again no difference was found between the two tumor cell populations, AM uptake at the lower dose being 66.1 and 70.7% and at the higher dose of 30.8 and 37.3% respectively for primary tumor and metastases. This means that at the lower dose 10^6 cells accumulate approximately as much compound in 5 min incubation as in 15 min, but the same cell volume is not able to bind more than 30–40% AM in 1 min, at least at the concentration of 10 μg/ml. In absolute amounts, however, 10^6 cells concentrate as much as 3–4 μg of AM in 1 min. The same AM concentrations at the same contact times were then tested for their cytotoxicity on primary explants of cells from i.m. primary and metastatic lung 3LL tumors. Figures 1 and 2 show that the morphology of the two populations is the same. Morphological evaluation of primary explants was made because the cells survive and reproduce for a relatively long time without requiring subculture in this condition. This enabled us to check the onset of toxicity during the first few days after treatment and to follow its course even at a submaximal degree; this is closer to the *in vivo* situation, where inhibition of tumor growth without complete destruction of the neoplastic mass is often the result of

Table 3. Uptake of adriamycin in two treatment conditions by cell suspensions of intramuscular 3LL and its lung metastases

AM treatment	i.m. Tumor $\mu g/10^6$ cells	%	Lung metastases $\mu g/10^6$ cells	%
10 $\mu g/ml \times 1$ min	3.08 ± 0.03	30.8	3.73 ± 0.06	37.3 n.s.
1 $\mu g/ml \times 5$ min	0.66 ± 0.02	66.1	0.71 ± 0.04	70.7 n.s.

10^6 cells/ml medium were incubated at 37°C in the presence of adriamycin.
n.s. = not significant (Mann–Whitney Test) vs i.m. tumor.

therapy. Cytotoxicity observed in these experiments is illustrated in Figs. 3 and 4; in both treatment schedules cultures of lung metastases appeared to be consistently more sensitive to AM than cultures of primary tumor cells. There was a striking difference between the responses of the tumor cells 24 hr after treatment, toxicity in the metastasis culture being more than 3 times higher than for the primary tumor with the 10 $\mu g/ml \times 1$ min dose and more than twice with the 1 $\mu g/ml \times 5$ min schedule. The difference between these two cell cultures was still present, although to a smaller extent, 48 and 72 hr after treatment. In view of this discrepancy between primary tumor and metastasis drug uptake and response to therapy, we decided to check whether the different environmental conditions for culture (see Methods) might be responsible. Cellular extracts from primary cultures of 3LL tumor and its metastases were exposed to the same AM treatments and their capacity to take up AM was investigated. Again no difference was seen between the primary and metastatic tumor cell populations (Table 4) although uptake was lower, especially at the 10 $\mu g/ml \times 1$ min treatment, than in the cell suspension experiment. This impaired binding of AM to cellular extracts of primary cultures might possibly be explained by the fact that in this experiment the cells were trypsinized before treatment and only trypsin resistent cells, which might have different permeability, were used for both cell populations.

DISCUSSION

The findings described show that cell suspensions of i.m. primary 3LL tumor accumulate AM to the same extent as cell suspensions of its lung metastases and the same is true for cellular extracts of these two populations in primary culture.

The fact that i.m. and lung 3LL tumor cells were exposed to the same tissue culture procedure long enough for their functional status to reach equilibrium might possibly explain the same drug uptake observed; however the finding that these cells, even when directly excised from the two tissues, do not show differences in drug uptake, lends consistency to this observation. Our data on AM uptake in tumor cells *in vitro* partially agree with the results of other authors [25–27] on different cell lines, i.e., that AM uptake is rapid, and depends on dose and time of contact. Our findings on drug binding to the cellular extract, however, show that AM uptake is very high (~80%), whereas the authors cited above observed only 20–40%. The observation that AM uptake is a passive process [25] and that dead cells bind AM to the same extent as viable cells suggests that the different drug uptake found was not in our hands due to injury to the cell membrane during cell preparation. This discrepancy might derive either from the different cell lines employed and their solution: cell volume ratio or from the possibility that gradients of drug

Table 4. Uptake of adriamycin in two treatment conditions by cells of intramuscular 3LL and its metastases in primary culture

AM treatment	i.m. Tumor $\mu g/10^6$ cells	%	Lung metastases $\mu g/10^6$ cells	%
10 $\mu g/ml \times 1$ min	0.98 ± 0.06	9.8	0.99 ± 0.01	9.9 n.s.
1 $\mu g/ml \times 5$ min	0.48 ± 0.001	48.3	0.44 ± 0.02	43.7 n.s.

10^6 cells/ml medium were incubated at 37°C in the presence of adriamycin.
n.s. = not significant (Mann–Whitney Test) vs i.m. tumor.

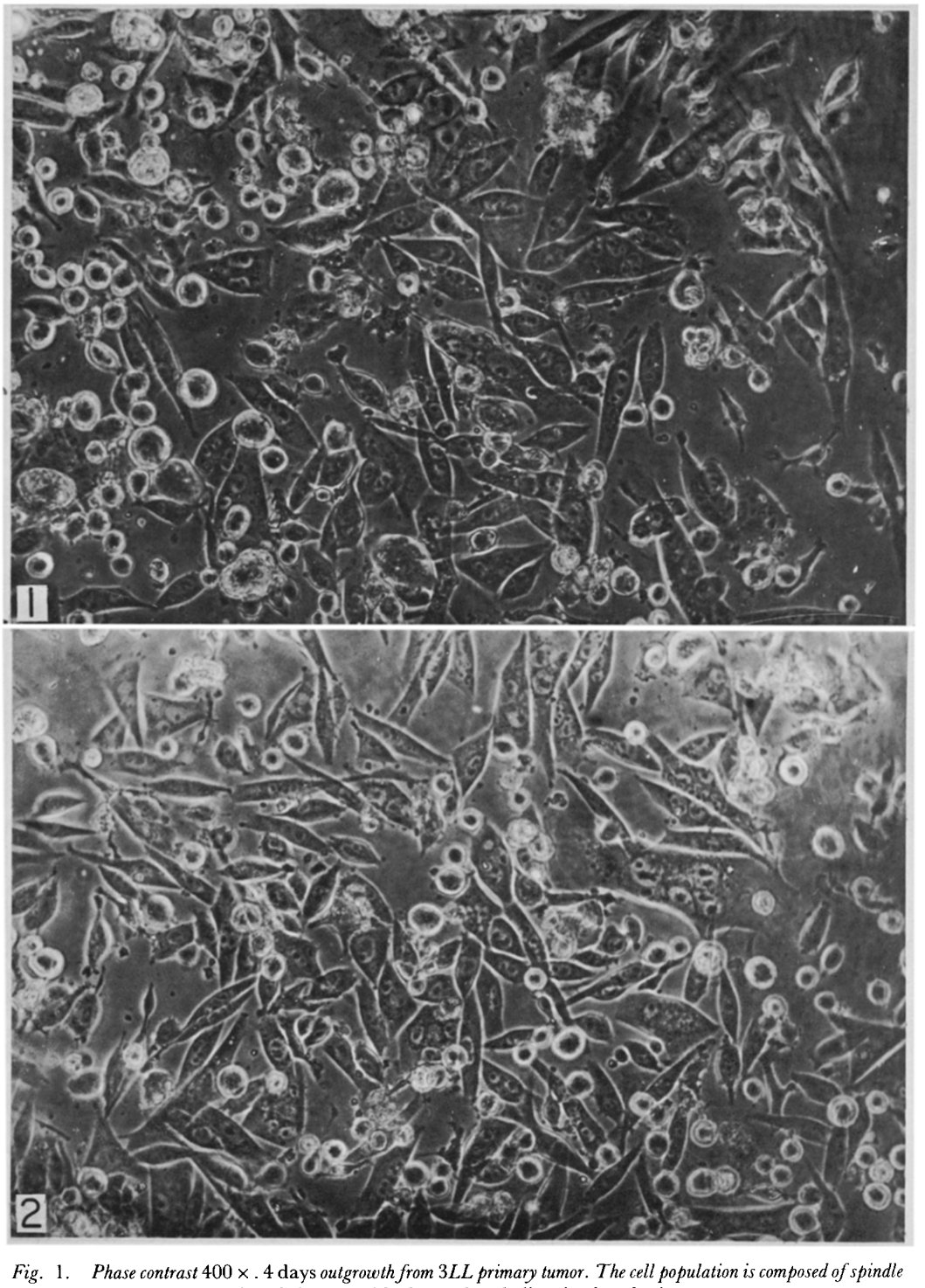

Fig. 1. *Phase contrast* 400 × . 4 days *outgrowth from 3LL primary tumor. The cell population is composed of spindle shaped elements with abnormal nucleoli and polynucleation.*

Fig. 2. *Phase contrast* 400 × 4 days *outgrowth from 3LL lung metastases. Morphological features are comparable to the population in Fig.* 1.

concentrations occurred in the incubation solution as a consequence of different shaking conditions. We chose the cell/medium ratio to ensure a good exchange between the two phases.

Since the therapeutic target of AM is intracellular [28, 29], cellular uptake is a decisive factor in its biological effect. Assuming that the drug concentration bound to the cellular extract represents the intracellular concentration, and that drug effect is a function of drug level at the receptor, the same amount of drug should result in equal cytotoxic activity.

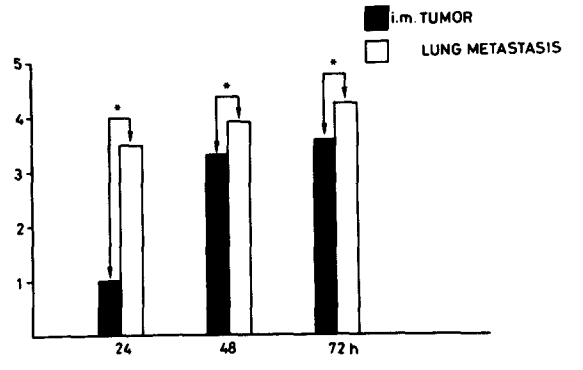

Fig. 3. Cytotoxicity of adriamycin (10 μg/ml × 1 min) *on 3LL intramuscular primary and pulmonary metastatic tumor explants. On the ordinate, toxicity score values. On the abscissa,* hr *after treatment.* (*) $P<0.01$ (Mann–Whitney test).

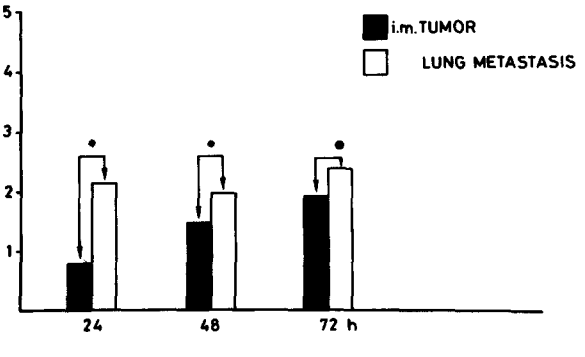

Fig. 4. Cytotoxicity of adriamycin (1 μg/ml × 5 min) *on 3LL intramuscular primary and pulmonary metastatic tumor explants. On the ordinate, toxicity score values. On the abscissa,* hr *after treatment.* (0) $P<0.02$ (*) $P<0.01$ (Mann–Whitney test).

In our conditions, however, *in vitro* cytotoxicity in the two cases was markedly different; pulmonary 3LL tumor cells were consistently more sensitive to AM than i.m. primary tumor cells over an observation time of 72 hr, and 24 hr after treatment they were 3 times more sensitive. According to our score test [24], which gives a continual estimate of toxicity on primary explants, the drug effect may differ depending on the dose used and the time of contact; this is in accordance with previous studies by our group [22]. Our findings that changes in the cytotoxic effect of AM cannot be correlated with changes in drug uptake by the tumor cells is in good agreement with studies by other authors [23, 30], who found that the cytotoxic effect of AM and DM on different cell lines *in vitro* is not always proportional to the intracellular amount of drug. In particular the results presented by Cherwinsky [26], showing that AM uptake is not appreciably different in L1210 cells sensitive or resistant to the drug, are consistent with our findings. As regards the greater response of metastatic 3LL tumors to AM treatment *in vitro*, good agreement is found with our group's *in vivo* results, indicating that AM, like other antitumoral agents, is more efficacious against lung metastases than against the i.m. primary tumor [15]. In particular, the same amount of drug results in higher cell kill at the metastatic site than at the primary tumor [31]. On the basis of *in vivo* drug distribution studies [15] it has been suggested that the higher concentration found in metastatic neoplasms could partly account for the drug's greater activity on metastases.

The hypothesis that there are differences between the intrinsic sensitivity to chemotherapy, of primary and metastatic tumor cells, not related to the medium or to the amount of drug taken up by the cells, seems to be supported by the present data.

For evaluating drug uptake or effect the two cell populations were exposed to the same treatment conditions and the same tissue culture technique, inducing migration at the same time, with comparable morphological features; this eliminates any possible influence of environmental stimuli and suggests that the different capacity to respond to therapy must be due to intrinsic differences in the two cell populations. In this regard *in vivo* studies by Fidler [32] indicating that two variant cell lines, weakly or highly metastatic, could be selected from the original B16 melanoma and the different cell properties are maintained regardless of the immune status of the host, confirm that intrinsic differences exist between primary and secondary tumor cells.

Further studies are warranted to investigate whether the different intrinsic sensitivity of primary and secondary tumor cells can be

confirmed in other experimental conditions and other tumor systems and to elucidate the cell metabolism and the mechanism of intrinsic factors distinguishing the tumor cell populations.

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