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Is Better Drug Availability in Secondary Neoplasms Responsible for Better Response to Chemotherapy?*

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Abstract—*The response of intramuscular Lewis Lung carcinoma (3LL) and its pulmonary metastases to graded doses of adriamycin (AM) was investigated in* C57Bl/6 *mice given the drug i.v.* 11 days *after tumor implantation and the effect was quantitated by recording tumor or metastases weight at various intervals after treatment. In the same experimental tumor model the distribution of AM in primary and secondary neoplasms was studied by a fluorimetric procedure. The results indicate that, compared to the primary 3LL implant, AM has a much more pronounced effect on a percentage basis on the lung nodules, where the drug reaches 3–5 times the levels in the intramuscular tumor. In order to clarify the role of this better drug availability in determining the higher response at the metastic site, the experimental correlation law between AM amount (peak level or area under the concentration versus time curve, AUC) and the drug effect (the smallest ratio of mean tumor or metastases weights in treated to untreated animals) was investigated for both primary and secondary tumors, and the concentration–response curves thus constructed were compared with the dose–response curves. If the effect is related to drug concentration, there is definitely less difference between the response of intramuscular 3LL and its metastases to AM and it even disappears at certain concentrations. The* ED_{50} *for the primary tumor is* 10 *times higher than for the lung nodules if derived from the dose–response curve, and only* 2–3 *times higher if derived from the concentration–response curve. Moreover, the lack of linear relationship and the biexponential correlation between the variables of effect and dose or peak concentration or AUC, either for the intramuscular or pulmonary 3LL, indicates that the effect does not increase proportionally to the drug amounts, suggesting that other factors beside AM concentration may contribute to the better drug response at the metastatic site.*

INTRODUCTION

IT HAS been frequently reported in experimental and clinical studies of malignant diseases [1–6] that drugs have greater therapeutic efficacy on metastases than on the primary tumor. Even when drug treatment of advanced primary tumors is ineffective, complete cure of metastatic foci depending on the dose and metastatic burden at the time of therapy may be observed after surgical removal of the primary tumor mass [7]. The differences between the sensitivity of primary and secondary neoplasms have been attributed to various factors, among which the different cell kinetics of primary and secondary neoplasms [8, 9] as a determinant of drug sensitivity have received particular attention. In addition, better drug availability at the metastatic site which is considered to be the consequence of the greater blood supply usually present in smaller tumors [10–14] has been recently reported by this laboratory [15]. It has been shown that some antitumoral drugs accumulate preferentially in the metastases of different experimental tumors in the mouse and rat.

In order to examine quantitatively the significance of the drug concentration in tumoral tissue, the correlation between the concentration of adriamycin (AM) reached in the intramuscular Lewis Lung carcinoma (3LL) or in its pulmonary metastases and the *in vivo* response was investigated in the present study.

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MATERIALS AND METHODS

(1) *Animals and tumor*

C57Bl/6 male mice (22 ± 2 g), obtained from Charles River (Calco, Italy) received an i.m. transplant of 2×10^5 viable cells of the syngeneic 3LL, maintained by i.m. passages in the same strain every 2 weeks and known to give rise to macroscopic metastases to the lungs within about 18 days of transplantation.

For the evaluation of antitumoral activity, adriamycin was injected i.v. at the dose of 1.875, 3.75, 5.625, 11.25 and 15 mg/kg to 10 mice per group on day 11 after tumor transplantation when the tumor weighed approximately 1.7 ± 0.2 g and gross metastases were not yet evident. Thirty animals were employed for the control group.

The inhibition of growth of the primary tumor of treated and untreated mice was followed by recording maximum and minimum diameters with a Vernier caliper at intervals of 2–5 days and calculating tumor volume by the formula $V = \frac{e W^2}{2}$, where e is the average length in mm and W the width of the tumor; the volume is converted to weight in mg assuming unit density and a spheroidal shape, where the short axes are of the same length (width and depth).

It is assumed that leg diameters are directly related to tumor weight; that the assumption is reasonable for this tumor has been shown by Steel [4].

The inhibition of growth of pulmonary metastases was evaluated by killing groups of 10 animals every day after the onset of metastases and recording the weight of the lung nodules.

(2) *Quantification of antitumoral effect*

The antitumoral effect exerted by AM on the primary tumor was quantitated by the function:

$$\frac{S \text{ treated } (t)}{S \text{ controls } (t)}; \qquad (1)$$

S treated (t) being the tumor size (calculated from the tumor diameters) on day t for each of the 10 mice per group given AM and S control (t) the mean tumor size of all control animals available (30 mice) on the same day t.

The mean of the smallest values for this ratio calculated for each animal measures drug effect in terms of tumor shrinkage (reduction in volume) after treatment and was assumed to give an indication of the percentage weight of cells surviving, the remainder representing the cells killed by AM as compared to the number of cells present in the tumor of controls on the same day t.

For quantitating drug effect on lung metastases, curves of tumor size ratios (1) for treated over untreated animals were calculated differently. As the animals were killed every time the metastases were weighed, the tumor inhibitory effect of AM could not be followed in the same animal.

An attempt to plot curves of tumor size ratios (1) utilizing the weight of metastases from one mouse chosen every day at random did not prove reliable, because of the broad variability of these weights. A single curve was therefore plotted utilizing the ratios (1) of mean metastases size of treated over untreated mice from day 11 after implantation ($t > 11$). For each AM dose the lowest value on this curve was assumed to represent the effect of the drug on metastases expressed as % of cell survival after treatment.

Statistical analysis to calculate the standard error and confidence intervals on these data was done on the basis of the following function [16]:

$$\mathrm{Var}\left(\frac{S \text{ treated}}{S \text{ controls}}\right) = \frac{\mathrm{Var}\ (S \text{ treated})}{(\text{Mean } S \text{ controls})^2}.$$

If this second procedure for evaluating the drug effect was followed for primary tumors too, the effect values obtained were fully comparable with the results of the first procedure averaging the curves for the single mice.

The high significance $r^2 = 0.97$ of the regression line ($y = 1.06 \pm 0.91x$) between the effect calculated by the two methods and the fact that it does not differ from the bisecting line of the two axes proved that the two procedures are interchangeable. Therefore the second procedure was also utilized for primary tumors in order to obtain comparable results of drug effect at the two sites.

(3) *Drug assay*

For drug assay in primary and secondary tumors adriamycin was injected i.v. at the same doses as for evaluation of antitumoral activity on days 11 and 25 after tumor transplant, when the i.m. tumor weighed approximately 1.7 ± 0.2 g and 7.2 ± 1.1 g, respectively, and its pulmonary metastases were

either absent or numbered about 27 ± 2.7 per mouse with an average weight of 169 ± 35 mg per mouse. The drug concentrations in primary and metastatic 3LL were assayed at different times after administration by the fluorimetric procedure described by Schwartz [17], with minor modifications. In these conditions recovery was $75 \pm 3\%$ and sensitivity was about 0.5 μg/g tissue. This procedure does not distinguish between fluorescence due to AM or metabolites but it represents well the amount of unchanged AM, since quantification of the relative contributions of AM and metabolites to total drug fluorescence by a scanning fluorescence technique [18, 19] indicated that 100% fluorescence either in primary tumor or in metastases was accounted for by the unmetabolized compound at any time.

The areas under the AM concentration versus time curve (AUC) were calculated by trapezoidal integration.

(4) *Mathematical analysis*

The experimental correlation laws of AM effects versus doses, peak concentrations or total drug exposures (AUC) were studied using a logic computer (Honeywell) by a regression analysis program. Calculations are based on least squares curve fits of the data after appropriate linear transformation. The initial values for parameters were calculated using an analog computer (Pharmacokinetics simulation center—Comdyna).

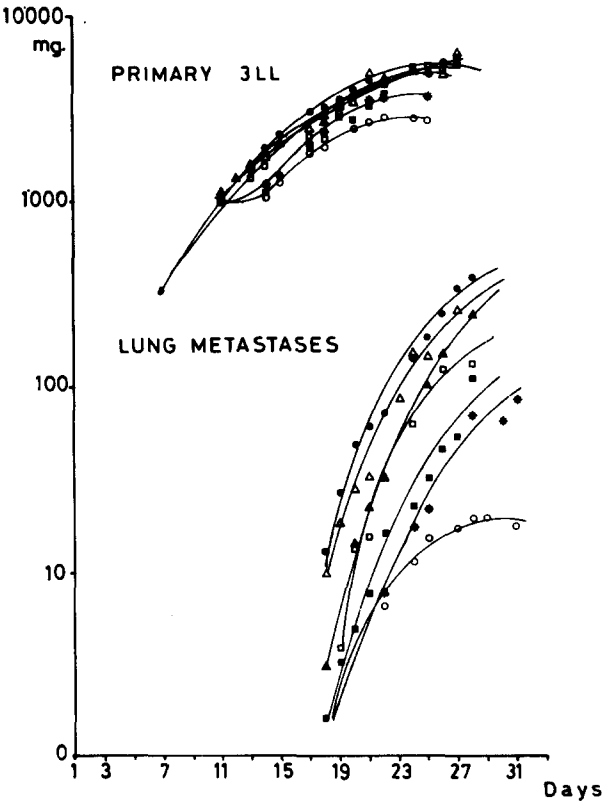

Fig. 1. Growth curves (mg *of tumor*) *of primary Lewis Lung carcinoma and of its lung metastases in* C57Bl/6 *mice given graded doses of adriamycin injected i.v.* 11 days *after intramuscular tumor implantation. Each curve is fitted on a mean of* 10 *mice per point in AM treated groups and* 30 *mice per point in the control groups. Time is given on the abscissae and on tumor or metastases weight on the ordinates. The top curves refer to primary tumors, the bottom curves to metastases.* ●, *Controls;* △, 1.875 mg/kg; ▲, 3.75 mg/kg; □, 5.625 mg/kg; ■, 7.5 mg/kg; *, 11.25 mg/kg; ○, 15 mg/kg.

RESULTS

The intramuscular Lewis Lung carcinoma in C57Bl/6 mice after implantation of 2×10^5 cells shows a growth pattern very similar to its pulmonary metastases, but this pattern was differently modified by AM therapy (Fig. 1). Graded doses of AM given i.v. 11 days after tumor implantation resulted in dose-dependent inhibition of growth (compared to controls) which was very slight for the primary tumor and much more pronounced on a percentage basis for the lung nodules, indicating that the secondary neoplasms respond to a greater extent to this chemotherapeutic treatment.

A dose of 3.75 mg/kg, which did not modify the growth of the primary 3LL, reduced metastatic weight to approximately 80%, and after 15 mg/kg the weight of the primary tumor was reduced to about 50% and that of the lung metastases to less than 10% of the control weight. This difference in response to AM of primary 3LL and its pulmonary metastases is even more evident considering the ratio between mean tumor or metastases weights of treated over untreated animals (Fig. 2).

The percentage effect on intramuscular and lung tumors after graded AM doses is reported in Table 1. Doses higher than 3.75 mg/kg AM reduced metastatic tumor weight by more than 50% whereas less than 50% of the primary tumor was affected even at the highest dose employed.

The levels of AM (Table 2) reached in primary tumor and in pulmonary metastases of i.m. 3LL-bearing mice after i.v. drug treatment on day 11 or 25 after tumor transplant were measured. As drug distribution in the intramuscular 3LL is not modified with tumor growth, only the results obtained on day 25, when AM levels at the metastatic site could also be measured, are reported. Since metastases have no necrosis, the presence of

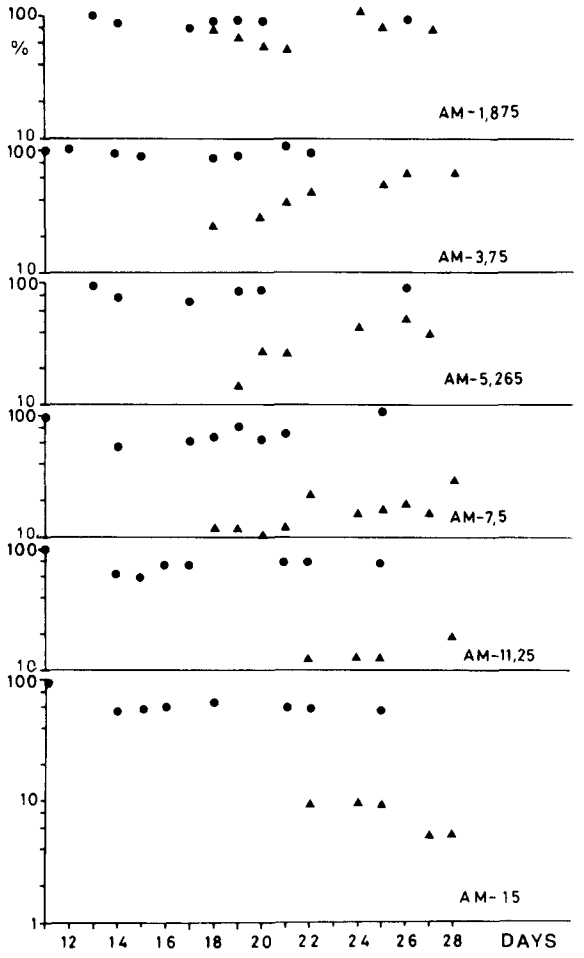

Fig. 2. Time course of % Size treated (t)/Size controls (t) *ratio for primary* 3LL *tumor and metastatic lung nodules under the influence of graded AM doses* (1.875–15 mg/kg i.v.). *Each value is the mean of* 10 *ratios of the tumor size in each individual treated animal to the mean tumor size of all controls.* ●, *Intramuscular* 3LL; ▲, *lung metastases.*

which in the primary tumor explains the much lower drug concentrations per g of total tumor tissue [20], only the drug levels in the viable part of the i.m. 3LL tumor were considered. The drug concentration, in terms of peak level or area under the concentration versus time curve (AUC), was 3–5 times higher in metastases than in the viable part of the primary 3LL, indicating that drug availability at the metastatic site is much greater than at the intramuscular site.

In order to investigate whether the different response elicited by AM on primary 3LL and its lung nodules were related to the different exposure of the two tissues to the drug, we studied the experimental correlation law for AM of (a) effects versus doses, (b) effects versus peak concentrations, (c) effects versus total drug exposures (AUC), where the effects were calculated as described in Materials and Methods (section 2).

Among the curves considered, neither the straight line nor the exponential Ae^{ax} fitted the experimental data ($r^2=0.54$–0.85 and $r^2=0.67$–0.74, respectively). Other curves, the potency Ax^B and the hyperbola

$$\frac{1}{A+Bx},$$

which gave a good fit of the results ($r^2=0.85$–0.99), did not agree with the condition of having 100% cell survival with the dose 0 (i.e. the tumor size of treated mice equal to tumor size of controls). In fact, for $x \to 0$, $Ax^B \to \infty$ (being $B<0$) and the fitted

Table 1. Percentage of AM effect and statistical variablity on intramuscular 3LL and its lung metastases

Group	AM (mg/kg i.v.)	Effect (%)	S.E.	5% confidence intervals
i.m. 3LL	1.875	81	7.2	65–97
	3.75	71	6	57–85
	5.625	72	6.3	58–86
	7.5	61	9.4	39–83
	11.25	59	7.7	42–76
	15.0	56	8.4	38–74
Lung metastases	1.875	54	3.8	45–63
	3.75	23	5	12–34
	5.625	14	1.9	10–18
	7.5	12	2.2	7–17
	11.25	11	1.1	9–13
	15.0	9	1.3	6–12

The smallest $\frac{\text{Size treated } (t)}{\text{Size controls } (t)}$ ratio reported in Fig. 2 is taken as the effect of treatment, evaluated as % of control. Variance calculated as indicated under Materials and Methods (section 2).

Table 2. Levels of adriamycin in intramuscular Lewis Lung carcinoma and its pulmonary metastases after i.v. injection of graded doses

Treatment (mg/kg i.v.)	i.m. 3LL Peak μg/g	i.m. 3LL AUC (μg/g) × min	Lung metastases Peak μg/g	Lung metastases AUC (μg/g) × min
1.875	<0.5	<100	2.2±0.01	1978±31
3.75	1.1±0.1	1168±141	5.6±0.5	3099±263
5.625	1.4±0.1	1488±101	8.2±0.01	4683±178
7.5	2.0±0.4	1975±84	9.0±0.8	7154±962
11.25	2.2±0.1	2870±280	14.2±1.2	11,287±1517
15	4.0±0.8	3922±229	20.1±2.4	15,042±1285

Four animals per point. Adriamycin was administered on day 25 after tumor transplantation. Only the viable part of the primary tumor after discarding the necrotic area was utilized for measuring drug levels.

hyperbola shows a starting point around 40 with the data available for metastases. These curves can be fitted only on the experimental points and no extrapolation beyond these limits is possible.

The best equation law ($r^2 = 0.89$) describing the whole relation between effect and the 3 variables, dose, peak and AUC, was found to be the biexponential descending curve ($Ae^{6\alpha x} + Be^{6\beta x}$) reported in Figs. 3–5. The parameters of this curve for both tumor and metastases are illustrated in Table 3. Analysis of the values A and B, whose sum represents the value of Co (concentration of AM at time 0) supports the hypothesis that the starting point Co of the curve at dose 0 is close to 100 or within its variability interval, for the intramuscular and the lung tumor curves.

Fitting with the biexponential function means that variations of a unit in the dose, peak or AUC values cause the effect to increase much more for low values than for high ones.

If the independent variable is represented by the dose, it can be seen in Fig. 3 that the same AM dose has striking different effects on primary and metastatic tumor cells, the ED_{50} (dose which causes 50% of effect) on primary tumor being 17 mg/kg as compared to a 10 times lower dose of 1.6 mg/kg on metastases.

Figures 4 and 5 report the concentration–response curves, where the independent variables considered are the peak levels or the AUC. When the drug effect is related to these variables, differences in the response to AM of primary 3LL and its metastases are definitely

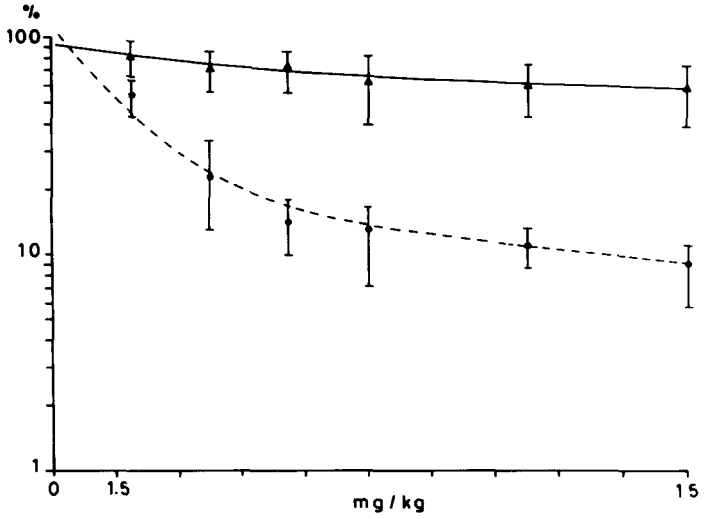

Fig. 3. Biexponential dose–response curve for AM in intramuscular 3LL *and its lung metastases. AM doses* (mg/kg i.v.) *are given on the abscissae and % effect is given on the ordinates (see Table* 1*). For each point* 5% *confidence intervals are reported.* ●, *intramuscular* 3LL; ▲, *lung metastases.*

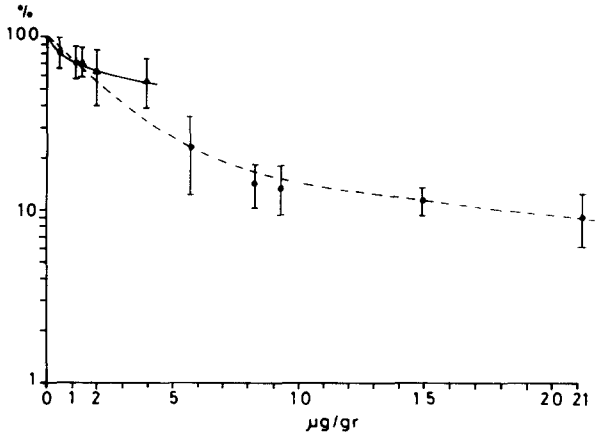

Fig. 4. Biexponential peak concentration–response curve for AM in intramuscular 3LL and its lung metastases. AM peak levels (µg/g) in tumor or metastases are given on the abscissae and % effect is given on the ordinates (see Table 1). For each point 5% confidence intervals are reported. ●, Intramuscular 3LL; ▲, lung metastases.

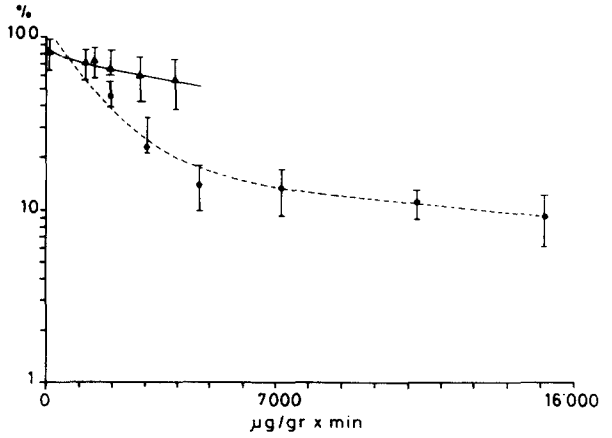

Fig. 5. Biexponential AUC–response curve for AM in intramuscular 3LL and its lung metastases. AM AUC values [(µg/g) × min] in tumor or metastases are given on the abscissae and % effect is given on the ordinates (see Table 1). For each point 5% confidence intervals are reported. ●, Intramuscular 3LL; ▲, lung metastases.

smaller or even non-existent at certain drug concentrations. For levels below 4 µg/g neoplastic tissue (primary or metastatic) the effect of AM on the i.m. 3LL is superimposable on that observed on the lung nodules, within 5% confidence intervals.

The same is evident with drug exposure below 2000 (µg/g) × min. At higher drug concentrations the two curves of primary and metastatic 3LL, either for peak or AUC values, both follow a biexponential law, but differ in entity: metastatic tumor nodules respond to the same AM concentration clearly better than the primary tumor cells from which they are derived. The corresponding ED_{50} values, calculated on these concentration–response curves, are of a different order of magnitude from those of the dose–response curve. The ED_{50} is only 2–3 times higher in primary 3LL than in its lung metastases; the peak level in primary tumor is 5 µg/g as compared to 2.2 µg/g in metastases and the AUC is 5000 (µg/g) × min as opposed to 1500 (µg/g) × min in metastases.

DISCUSSION

The different response to AM of intramuscular and metastatic lung tumors of the Lewis Lung carcinoma in C57B1/6 mice presents further evidence that secondary neoplasms are more sensitive to chemotherapy. In our conditions a 10-fold drug dose was required for the primary tumor as compared to its metastases to reach the same effect of 50% inhibition of growth, and doses with no activity on the i.m. tumor appear active on the lung nodules.

Table 3. Parameters relative to the biexponential fitting of the curves of % effect versus dose, peak concentration or AUC of AM and statistical variability (standard error)

Effect on tumor versus	A	α	B	β	r^2	A (S.E)	α(S.E.)	B (S.E.)	β(S.E.)
Dose	27.31	0.245	66.66	0.011	0.87	1.4	0.09	1.02	0.001
Peak	25.6	2.064	74.63	0.079	0.83	1.02	0.026	1.11	0.04
AUC	10.92	0.002	77.44	0.00009	0.75	2.18	0.001	1.1	0.00003
Effect on metastases versus									
Dose	93.29	0.633	18.4	0.047	0.98	1.34	0.12	1.02	0.002
Peak	85.46	0.418	18.26	0.035	0.99	1.08	0.02	1.03	0.002
AUC	98.15	0.0007	17.41	0.00004	0.93	1.75	0.0002	1.03	0.000001

These parameters were calculated by the peeling method on a logic computer, according to the equation $y = Ae^{-\alpha x} + Be^{-\beta x}$.

The superior response to chemotherapy, as well as to γ irradiation [21] and to non-specific immunotherapy [22], of metastases compared to the primary tumor was based on comparison of the fractions of neoplastic tissue 'lost' with therapy in the two neoplastic implants, and the inhibitory activity was assessed relative to untreated tumors of the same type and site.

In an attempt to clarify the reasons for the greater sensitivity of secondary tumors to chemotherapy, a series of studies were undertaken, utilizing mainly the 3LL tumor system (which was devised for studying the relationship between the growth of a local tumor and its metastases) and various factors were analysed as determinants of drug sensitivity.

The role of the higher proliferative rate and shorter doubling time of metastatic neoplasms was considered by De Wys [3], who correlated the growth rate of 3LL to the cell kill in its pulmonary metastases after cyclophosphamide treatment. Greater effectiveness of cyclophosphamide on small 3LL lung nodules was also observed by Steel and Adams [4] who did not, however, draw any conclusion about the possible reasons. The possibility that the response to therapy might be a function of tumor size, and that no change in sensitivity to cyclophosphamide due to a different proliferative rate needs to be postulated was suggested by Hill and Stanley [23].

Selection of tumor cells during the process of metastasis formation was suggested by Fidler *et al.* [24] and this could be a further factor accounting for the superior response. In this regard Tropé [25] observed that subcutaneous neoplasms obtained from transplantation of metastatic 3LL cells after 3 or 5 passages become as sensitive as metastases to the same cytostatic drugs *in vitro*.

The possibility that differences may exist between the intrinsic sensitivity to chemotherapy or primary and secondary tumor cells is supported by a report from this laboratory describing the superior response to AM *in vitro* of 3LL cell suspensions from pulmonary tumors as compared to cells derived from the intramuscular implant [26]; under the same experimental conditions the uptake of AM was, however, similar in the primary and secondary 3LL cells.

The finding that the cytotoxicity of AM *in vitro* is not directly related to intracellular drug levels, as observed by other authors [17, 27–29] on different cell lines treated with AM or daunomycin, is not in contrast with the obvious contention that a therapeutic advantage is gained from higher drug concentration at the target cell site within the limits of acceptable host toxicity [30, 31]. In this regard it has been reported by Wilkott *et al.* [31] that the rate of reduction in viability of proliferating cultured L1210 cell populations depends on the concentration of AM to which they are exposed but sensitivity of L1210 and P388 cultured cells to this compound varies at low and high doses.

As to the levels of AM achieved *in vivo* at the primary and metastatic tumor site, the capacity to take up the compound being comparable in the two cell populations, the reported better drug availability in secondary neoplasms [15] should result in higher intracellular concentrations contributing to greater activity on metastases.

Which fraction of tumor mass is destroyed by equal AM concentrations at the two sites, i.e., what is the specific activity of AM on primary and secondary tumor cell populations *in vivo*, is the question we tried to answer in our study by constructing concentration–response curves that relate percentage effect to drug availability in terms of peak levels of AM or time course of its accumulation (AUC values) at the primary 3LL or metastatic sites. If the actual drug concentrations rather than the total dose given to the animal are related to drug effect, the differences in sensitivity to AM of intramuscular implant and its lung nodules decreased or even disappeared at concentrations below 4 μg/g or 2000 (μg/g) $\times$ min in the neoplastic tissue, thus explaining the importance of the amount of drug at the target in determining the cytotoxicity of this agent.

At higher concentrations, however, metastatic nodules responded to AM clearly better than primary 3LL, the corresponding ED_{50} derived from the biexponential relation, either with peak levels or AUC values, being 2–3 times higher for the intramuscular tumor than for the pulmonary nodules. This finding supports the idea that other factors contribute to the superior response, such as proliferative rate or size of neoplastic tissue or intrinsic sensitivity due to *in vivo* cell selection.

The lack of linear relationship and the existence of a biexponential correlation between the variables of effect and dose, peak or AUC, either for intramuscular or pulmonary 3LL, indicates that when drug amounts are increased the effect does not increase proportionally but, with variations of a unit in the dose, peak or AUC values, it increases much more for low values than for high

values of the variable considered. In particular, beyond peak levels or AUC values of approximately 8 μg/g and 5000 (μg/g) × min, only slight increases in drug effect are observed on either primary or metastatic tumors.

In planning the chemotherapy of metastasizing tumors, these findings may represent an example of what information helps in adjusting drug dosages to achieve optimal concentrations at the desired target, without useless increases in drug doses.

Whether the laws described regulating the relationship between AM concentration and response in the 3LL tumor model are representative of a more general situation and apply to other antitumoral drugs and other tumors remains to be established experimentally.

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