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Development of MeCCNU-Resistance in Clonally Derived Lines of Lewis Lung Carcinoma

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Abstract—*The development of resistance to MeCCNU in four clonal lines of Lewis lung carcinoma derived from lung colonies has been examined. Treatment of the clones with* 15 mg/kg *MeCCNU once in each* in vivo *passage resulted in a rapid reduction in tumour responsiveness to this drug. This shows that MeCCNU resistance can arise at rates which may be applicable in spontaneous tumours and their metastases.*

Karyotypic heterogeneity was evident very early after isolation of the clones and this has potentially important implications for the experimental use of clones and for the features of metastatic disease. Karyotype analysis of one of the clones at each passage suggested that development of resistance was associated with the selection of a single sub-population of tumour cells but the kinetics of resistance development cannot be totally explained in terms of this simple selection.

INTRODUCTION

THE DEVELOPMENT of drug resistance in tumours is a process which is still far from being understood, despite its importance in cancer therapy. Many studies have examined drug-resistant tumour lines but rarely have the kinetics of resistance development been examined during *in vivo* treatment. In previous studies from this laboratory the development of resistance to MeCCNU has been examined in the Lewis lung carcinoma [1] and resistance to melphalan, *cis*-platinum and cyclosphosphamide has been followed in the MT carcinoma [2]. In these studies differences in the kinetics of resistance development suggested that the underlying process may be different in these two tumour systems. The development of resistance to MeCCNU in LL carcinoma may be due to the selection of a pre-existing highly drug-resistant population of tumour cells, although some anomalies were evident during repeated treatments [1]. However, a simple model like this, in which a tumour is seen to be composed of two subpopulations, one sensitive and one resistant, would appear to be inadequate to explain the development of resistance to melphalan, *cis*-platinum and cyclophosphamide in the MT carcinoma [2].

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In the present study we have investigated the development of resistance to MeCCNU in lines of the LL carcinoma derived from individual lung colonies. This was performed in order to determine whether resistance development in the wild-type tumour could be due to the selection of variants that are produced at a rate which may be applicable in spontaneous tumours.

MATERIALS AND METHODS

Animals and tumours

The LL carcinoma was carried routinely by implantation of tumour brei into the gastrocnemius muscles of female C57Bl/Cbi mice. Mice were obtained from the Institute of Cancer Research Breeding Centre and were used when they were 8–10 weeks old at which time they weighed 20–24 g.

Preparation of tumour cell suspensions

Tumour cell suspensions were prepared as previously described [3]. Tumour tissue was removed aseptically, chopped finely using crossed scalpels and washed once in PBSA (phosphate-buffered saline, Dulbecco 'A', Oxoid Ltd). The tissue was incubated for 10 min at 37°C in PBSA containing 0.2% trypsin (Bacto-trypsin, Difco Laboratories) and 0.05 mg/ml deoxyribonuclease I (DNase, Sigma Chemical Co). After this time the enzymes were replaced with a fresh trypsin/DNase mixture

and this was incubated for a further 20 min at 37°C with continuous agitation. The suspension was then filtered through 35 μm polyester mesh, the cells were washed once in Hams F12 culture medium with antibiotics and 17% donor calf serum (Flow Laboratories), and finally suspended in culture medium.

Drug treatment

MeCCNU (methyl-chloroethyl cyclohexyl nitrosourea), obtained from the National Cancer Institute, U.S.A., was dissolved at a concentration of 20 mg/ml in dimethyl sulphoxide and stored until needed at −20°C. Just before use this was diluted to a concentration of 1 mg/ml in 5% Tween-80 in PBSA and further dilution was done in PBSA. These solvents alone were not cytotoxic at the levels used. MeCCNU was injected intraperitoneally into non-anaesthetized mice.

For *in vitro* MeCCNU treatment, 5 ml aliquots of a single-cell suspension at a concentration of 1–4 × 10^5 cells per ml were incubated at 37°C for 1.5–2.5 hr. The cells were then incubated with MeCCNU for 1 hr, with continuous gentle agitation, the drug-containing medium removed and the cells resuspended in culture medium. The colony forming ability was assayed as described below.

Estimation of growth delay

A calibration curve technique was used to estimate tumour growth delay following MeCCNU treatment. Tumours were treated when they were 0.1–0.25 g and tumour size was measured three times per week by passing the tumour-bearing legs through holes of known diameter in a perspex disc. The size of the tumour-bearing leg was taken as the size of the smallest hole through which the leg would pass without any resistance. This leg diameter was converted to a tumour weight using a calibration curve [4].

A minimum of six tumours were used in each group and the time for each tumour to reach four times its weight at the time of treatment ($T4\times$) was calculated. Growth delay (GD) was then calculated as: GD = median $T4\times$ treated group − median $T4\times$ untreated group.

Karyotype analysis

Cells were prepared for karyotype analyis using standard techniques. Tumour cells were plated onto plastic and incubated overnight at 37°C in a water saturated atmosphere of 90% nitrogen, 5% oxygen and 5% carbon dioxide. After a 2-hr exposure to 0.4 μg/ml Colcemid (Gibco Diagnostics) the tumour cells were harvested, incubated for 10 min in 0.75 M potassium chloride and then fixed in two changes of fixative (3 parts methanol plus 1 part glacial acetic acid). Cells were dropped onto glass microscope slides, allowed to dry for 48 hr at room temperature and then stained with giemsa stain (BDH Chemicals Ltd).

In vitro *clonogenic cell survival assay*

The survival of tumour cells after *in vitro* MeCCNU treatment was assessed using the double-layer soft agar clonogenic assay developed by Courtenay [5]. Results were expressed as surviving fraction (SF) where:

$$SF = \frac{\text{plating efficiency of treated cells}}{\text{plating efficiency of control cells}}.$$

RESULTS

The development of MeCCNU resistance

The isolation of the clonal lines from lung colonies and the subsequent development of resistance to MeCCNU is outlined in Fig. 1. Four lines (LLC1–LLC4) were derived from individual lung colonies which had formed following intravenous injection of cells of LL carcinoma with 10^6 heavily irradiated cells and 10^6 plastic microspheres. This method has been used previously to isolate lines which have been assumed to be clonal on the basis of existing evidence [6–8]. Whole lung colonies were implanted subcutaneously into mice and allowed to grow to approx. 1 g. Cell suspensions were made from each line and these were analysed for karyotype and injected into mice for the estimation of growth delay following 15 mg/kg MeCCNU. The first MeCCNU treated tumour to reach four times its volume at the time of treatment was disaggregated and used for the next passage and karyotype analysis. This was repeated at each passage.

Figure 2 shows the $T4\times$ for the four lines of the LL carcinoma for tumours from each passage which were either untreated or treated with 15 mg/kg MeCCNU. No cures were achieved but it can be seen that this dose of MeCCNU had a substantial effect on the growth of all the lines at the beginning of the series of experiments with for example a growth delay of 13.9 days in LLC1 in the first passage. However after three treatments with 15 mg/kg MeCCNU all four lines were almost completely non-responsive to MeCCNU. Thus resistance developed very quickly in each case. Resistance in LLC4 was confirmed by clonogenic cell survival following *in vitro* MeCCNU treatment (Fig. 3). D_{10} values (dose required to reduce survival by one decade) of 26.3 and 2.9 μg/ml were seen for LLC4 and wild-type LL carcinoma, respectively.

Karyotype analysis of LLC4 during development of MeCCNU resistance

The chromosomes of cells from LLC4 were examined at each passage. As with the wild-type LL carcinoma [9] the majority of the cells in the first

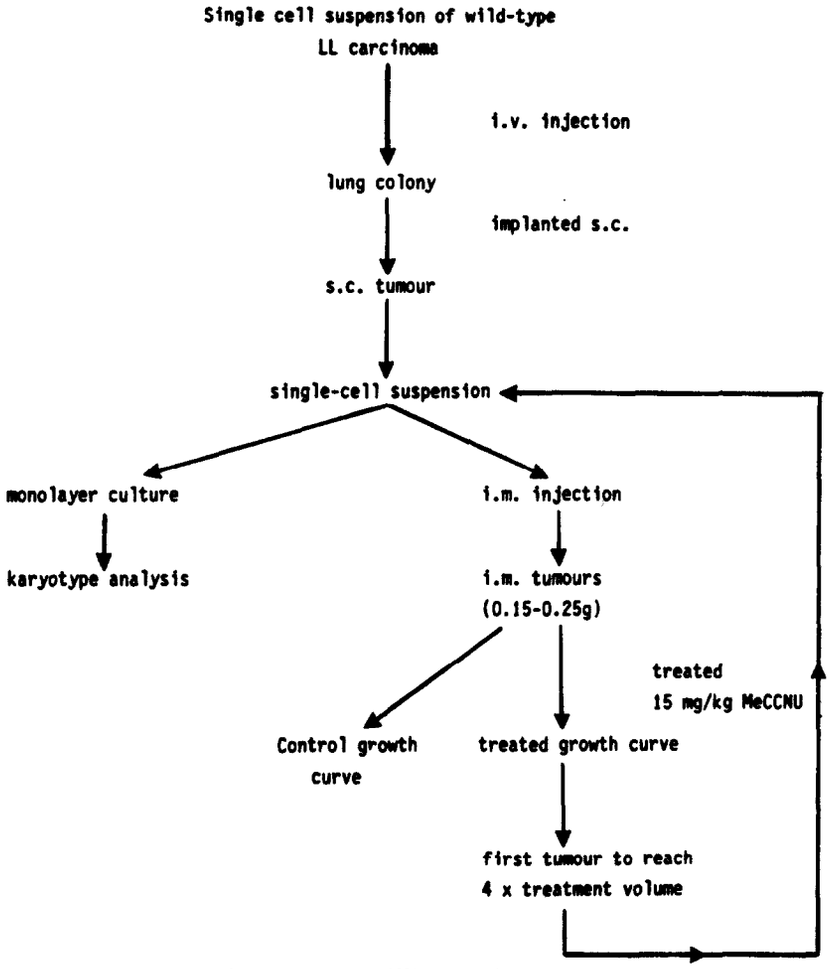

Fig. 1. Protocol for the development of resistance to MeCCNU in lines of the Lewis lung carcinoma derived from lung colonies.

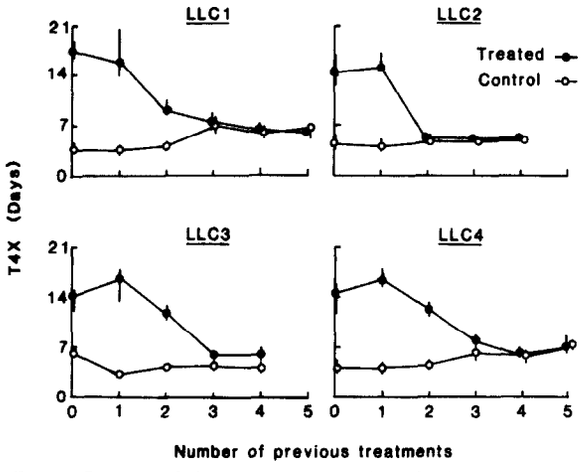

Fig. 2. Time to reach four times treatment volume for clonal lines of LL carcinoma treated with MeCCNU during the development of resistance to MeCCNU. At each passage the clones were untreated or treated with 15 mg/kg *MeCCNU and the growth of tumours was measured.*

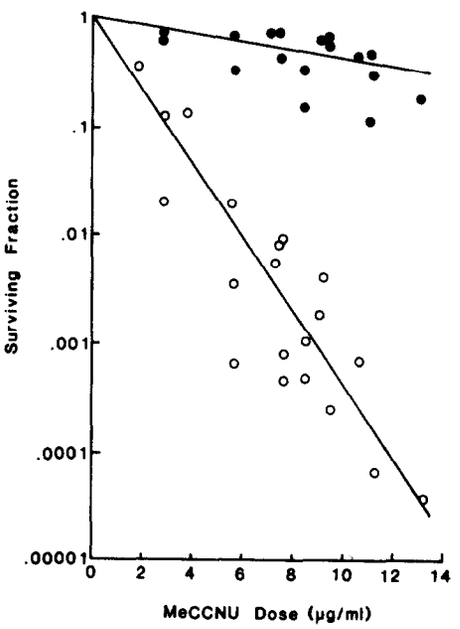

Fig. 3. In vitro *MeCCNU dose-survival curve for wild-type LL carcinoma* (○) *and LLC4* (●). *Tumour cell suspensions were incubated for* 1 hr *at* 37°C *in the presence of MeCCNU and plated in soft agar to assess clonogenic cell survival.*

passage of LLC4, i.e before treatment, contained three marker chromosomes, two metacentric and one sub-metacentric, which were easily distinguishable from the other chromosomes which were all acrocentric. The number of chromosomes per cell was generally lower in this passage than the modal value of 69 seen for wild-type LL carcinoma. It is evident, however, that the distribution of chromosome numbers is very wide for LLC4 even at this

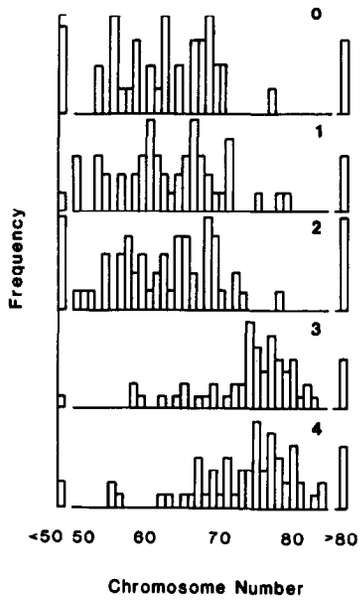

Fig. 4. Chromosome number distributions for LLC4 during the development of resistance to MeCCNU. Tumour cell suspensions were prepared at every passage and giemsa-stained metaphase spreads were analysed. The number of in vivo *MeCCNU treatments is indicated for each profile.*

early stage in the history of this line. This rapid development of large variation in chromosome number in lines derived from lung nodules was also seen in the other three lines and we have made similar observations in sub-lines from other tumours (data not shown).

The chromosomal makeup of LLC4 cells changed significantly during the repeated MeCCNU treatment (Fig. 4). After three treatments there was a significant increase in the number of chromosomes per cell with 78% of the cells having more than 70 chromosomes. In addition the proportion of cells with three marker chromosomes decreased progressively during the development of resistance. Thus before treatment 86% of cells had all three marker chromosomes whereas the figure was 88%, 72%, 18% and 0% after one, two, three and four MeCCNU treatments, respectively. The missing marker chromosome in each case was the same one, one of the metacentric markers. This did not occur when LLC4 was passaged five times without treatment (mode = 68, 97% of cells with all three marker chromosomes). The loss of one of the marker chromosomes is not uniquely associated with MeCCNU-resistance in the LL carcinoma as the other three lines did not show this loss (data not shown).

DISCUSSION

In this paper we have been able to demonstrate that repeated treatment of clonal lines derived from four independent lung colonies from LL carcinoma, with MeCCNU, very quickly reduced the response of the lines to this drug. These results are consistent with previously published results with the wild-type LL carcinoma [1] because in both the sensitivity of the cell populations was lost after just three treatments. The data presented therefore provide evidence that the MeCCNU-resistant cells seen in the wild-type tumour are not a consequence of the long term passage of this tumour.

Clonogenic cell survival assays on LL carcinoma suggested that a sub-population of cells (approx. 1 in 10^5 cells) were about 10 times more resistant to MeCCNU than the majority of cells [10]. Under some conditions the development of MeCCNU resistance by repeated drug treatment was consistent with the selection of this sub-population [1]. The karyotype data presented here support this since examination of the karyotype of LLC4 at points during the development of drug resistance suggest that resistance was associated with the selection of a sub-population of cells with a higher chromosome number and with only two marker chromosomes. These karyotypic features would appear not to be directly related to the MeCCNU resistance as we have not seen them in other MeCCNU resistant lines.

However, the kinetics of resistance development would appear not to be consistent with models involving the selection of totally resistant sub-populations of tumour cells. These clones of the LL carcinoma showed initial sensitivities to MeCCNU which were very similar to the wild-type LL carcinoma [1]. On the basis of this and the known clonogenic cell survival of wild-type LL following MeCCNU treatment [1, 10] we would expect 15 mg/kg MeCCNU to kill over seven decades of the sensitive cells in the clones. Therefore, if the emergence of a drug resistant tumour was due to the selection of a pre-existing population of tumour cells which were totally resistant to the drug [11, 12] it would have been expected that a single treatment would have been almost sufficient to completely eliminate the sensitive population if we assume that a 0.1 g tumour contains approx. 10^8 cells.

Even though resistance did develop rapidly in these clones this process was not as rapid as these theoretical predictions. Some of the possible reasons for this have been discussed previously [1, 2, 10, 13]. One of the major considerations in this regard is the fact that cells refractory to the common anticancer agents are never completely resistant. This leads to the possibility that a certain proportion of "resistant" cells will be killed with each treatment [14], thus slowing down the emergence of a resistant tumour. This phenomenon may have played some role in the current experiments but the sensitivity of the original tumour and the final degree of resistance achieved suggests that this is not the primary "slowing" mechanism. What it is that delays resistance development, however, is not known.

The instability and heterogeneity of the karyotype in tumour cell populations is a common finding [15–17] but the data presented here show that this variation can arise very rapidly in expanding populations. This suggests that using clonal populations to analyse the properties of cells in the original tumour may be misleading since by the time the clone has grown to a useful size it may have diversified substantially. This is consistent with studies of other cellular characteristics [8, 18–20]. In addition to the experimental problems which this creates it may be an important cause of variation within small metastatic deposits.

The data presented here show that the rapid development of MeCCNU resistance previously reported in LL carcinoma [1] can also occur in clonal populations. Thus the analysis of this phenomenon may be directly applicable to drug resistance devlopment in both spontaneous tumours and their metastases.

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