Eur J Cancer Clin Oncol, Vol. 18, No. 10, pp. 973–978, 1982.
Printed in Great Britain.

0277-5379/82/100973-06$03.00/0

DNA Content Distribution of *in Vivo* and *in Vitro* Lines of Lewis Lung Carcinoma*†

G. STARACE,‡ G. BADARACCO,§ C. GRECO,§‖ A. SACCHI§¶ and G. ZUPI§

‡*Consiglio Nazionale delle Ricerche c/o CNEN-Casaccia, 00100 Roma,* §*Istituto "Regina Elena" per lo Studio e la Cura dei Tumori, 291 v. le Regina Elena, 00161 Roma, Italy and* ¶*Istituto "Regina Elena" (Lab. Biofisica)*

Abstract—*The kinetic features of Lewis lung carcinoma (3LL) and of two* in vitro *and two* in vivo *derivative lines were studied by flow cytometry. The* in vitro *lines C108 and BC215 show the same tetraploid DNA content and a very similar cell cycle structure, characterized by a prevalent S fraction. Also, the* in vivo *lines, the original 3LL and M1087, show a tetraploid DNA content, while the BM21548 is characterized by a hyperdiploid DNA mode and a broader distribution of DNA values. No difference in the modal DNA value is found between each primary tumor and the corresponding lung metastases for all the* in vivo *lines. In addition, an increase in the G1 component corresponding to a decrease in the S fraction is observed during the tumor growth. These kinetic features were related to some malignant properties of 3LL lines, such as growth pattern and metastatic potential. Our findings indicate that a direct correlation is not always possible to establish.*

INTRODUCTION

THE AVAILABILITY of different tumoral lines isolated from the same parent tumor represents a useful tool for cancer chemotherapy studies [1]. In fact, such model systems are very suitable for reproducing the heterogeneous nature of both experimental and human tumors [2, 3]. As previously reported [4, 5], variant lines from a Lewis lung carcinoma (3LL) were selected in our laboratory either *in vitro* or *in vivo*. These tumor subpopulations differ from each other in such biological parameters as clonogenic ability, tumorigenicity, metastatic potential as well as therapeutic response to antineoplastic agents [6–8].

In the present work the cell cycle structure of both *in vivo* and *in vitro* lines of 3LL was studied by determining the relative DNA content distribution and analysing the cell fraction in the various cycle phases. This was in order to (i) ascertain whether the heterogeneity observed in some biological characteristics of these tumor lines could also extend to such kinetic parameters; (ii) verify whether these kinetic features, in particular the modal DNA content, could be related to some malignant properties of 3LL lines (such as growth rate and metastatic ability); (iii) gain, through the analysis of the cell cycle structure of this model system, helpful information to the therapeutic control of tumors which express multiclonal properties.

Accepted 18 May 1982.

*Presented in part at the 5th International Symposium on Flow Cytometry (Bracciano-Roma, 2–5 December 1980).

†This work has been supported in part by Grants No. 81.01326.96 and No. 78.02847.96 from Progetto Finalizzato "Controllo della Crescita Neoplastica", CNR, Italy.

‖To whom correspondence and requests for reprints should be addressed at: Regina Elena Institute for Cancer, 291 v. le Regina Elena, 00161 Roma, Italy.

MATERIALS AND METHODS

3LL lines

Throughout *in vivo* studies M1087 and BM21548 lines (at the 65th and 50th passage in animals respectively) were analysed and compared with the original 3LL line. These tumor lines routinely transplanted intramuscularly into C57B1/6 mice maintain both the macroscopic features and the histologic type consistent with those of the parent line. For the *in vitro* studies C108 and BC215 lines derived from lung nodules of a 3LL tumor were used. Cells were grown in monolayers adherent to the plastic surface of 25-mm^2 Falcon flasks in Waymouth's medium supplemented with 15% calf serum. Monolayers were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. When these experiments were performed, the C108 line was in the 218th passage and BC215 in the 129th passage.

The procedure we followed to isolate and select either the *in vivo* or the *in vitro* lines of 3LL in our laboratory, their main biological characteristics and their response to some antineoplastic agents have all been previously reported [4–8].

Preparation of cell samples

In vivo *lines.* Tumor biopsies were removed from all the 3LL lines at various stages of the growth (i.e. days 8, 15, 23 after implantation of 5×10^5 viable tumor cells). Single cell suspensions of both the primary tumor and lung metastases were prepared as follows: non-necrotic tissue was rinsed in Dulbecco's phosphate-buffered saline (PBS), Ca^{2+} and Mg^{2+}-free (Gibco), pH 7.4, finely minced with scissors and then filtered through a triple layer of gauze. In some instances, when cell clumps were observed in DNA distribution histograms, the measurements were repeated after digestion with 0.5% pepsin hydrochloride (Serva) at room temperature for 10 min.

In vitro *lines.* The analysis of DNA distribution was carried out on cells in the exponential phase of growth. Cells were harvested using 0.025% tripsin at 37°C for 5 min and after centrifugation the pellet was resuspended in Dulbecco's PBS, Ca^{2+} and Mg^{2+}-free, pH 7.4.

The microscopic monitoring of tumor cell suspensions assured a satisfactory level of cellular dispersion. Sometimes cells were fixed in cold ethanol at a final concentration of 70% and measured within one week. The DNA distribution patterns of fixed cells were quite comparable to those of cells measured immediately after harvesting.

Flow cytometry (FCM)

DNA fluorochromation was accomplished with a staining solution containing: 10 μg/ml mithramycin (kind gift from Dr. C. Forte-Pfitzer, Italy), 10 μg/ml propidium iodide (Calbiochem Behring Co., U.S.A.), 15 mM magnesium chloride, 0.1% Nonidet, Tris–HCl, pH 7.5. After 1 hr at 4°C the sample was measured in a ICP22 pulse cytophotometer (PHYWE) using a mercury 100 W lamp, excitation filter BG12 3 mm coupled with a step filter K590. Signals were processed by a multichannel analyser (Laben). For each sample, 20×10^3–40×10^3 cells were measured and DNA histograms were analysed by computer fitting a polynomial function to the S phase, according to the method of Dean [9]. The location of the diploid peak was established by measuring a sample of whole blood from normal C57Bl/6 mice. Normal cells, always found to a varying amount in biopsies, served as a standard reference for the ploidy identification of the tumor cells in the *in vivo* samples. Ploidy was expressed as DNA index (DI), which represents the relative DNA content of tumor G1/G0 cells in comparison to the content of diploid normal cells. The calculated coefficient of variation (c.v.) of both the normal and the tumor G1/G0 peak ranged between 2.5 and 3.5%. When present, the background was subtracted using the method of Beck [10].

RESULTS

3LL in vivo *lines*

DNA histograms of both primary tumors and relative lung metastases of the *in vivo* lines are illustrated in Fig. 1 and the fraction of cells in the cycle phases is reported in Table 1.

In all the histograms the first peak corresponds to the normal value, 2C, and this is due to the normal cells (leukocytes, fibroblasts, etc) present in the tumor. The amount of cells under this peak varies according to the tumor age, being relatively higher in the early growth stages and in lung metastases. By comparing the data referring to the M1087 line and to the original one, the following observations were made:

(i) At the tumor stages considered the two lines show a very similar structure of the cell cycle. In particular, for the original line the percentage of cells in the various cycle phases is in good agreement with data reported by Crissman *et al.* [11] on the same tumor.

(ii) The two lines exhibit a tetraploid DNA content (DI = 2.0) which is the same in both the primary tumor and in lung metastases. Moreover, as reported in Fig. 2, the proliferative pattern of the metastatic cell population seems to be related to the pattern of the primary tumor. In fact, a linear correlation between G1/G0 and S fractions of the primary tumor and those of the corresponding lung metastases appears to hold ($0.05 > P > 0.02$).

(iii) Finally, as expected, in both the lines the G1/G0 component increases according to the tumor age (from early to late stages of the growth), while the S fraction correspondingly decreases.

The DNA content distribution of the BM21548 line is illustrated in the panels G–I of Fig. 1. As evident, this line differs from the others in that the modal DNA value is hyperdiploid (DI = 1.35). Moreover, a broader distribution of DNA extending to a peak located at four times the modal DNA value is observed.

Table 1. In vivo *3LL lines. Fraction of cells in the cycle phases, at different stages of growth*

Tumor Stage	Day	Original line G1/G0	S	G2/M	M1087 line G1/G0	S	G2/M
Early	8	37	55	8	38	55	7
Intermediate	15	43	52	5	43	50	7
Late	23	59	35	6	54	39	7
Late metastases	23	51	41	8	52	37	11

The values concerning primary tumor and lung metastases at late stages of the growth (23rd day) were calculated on the same host.

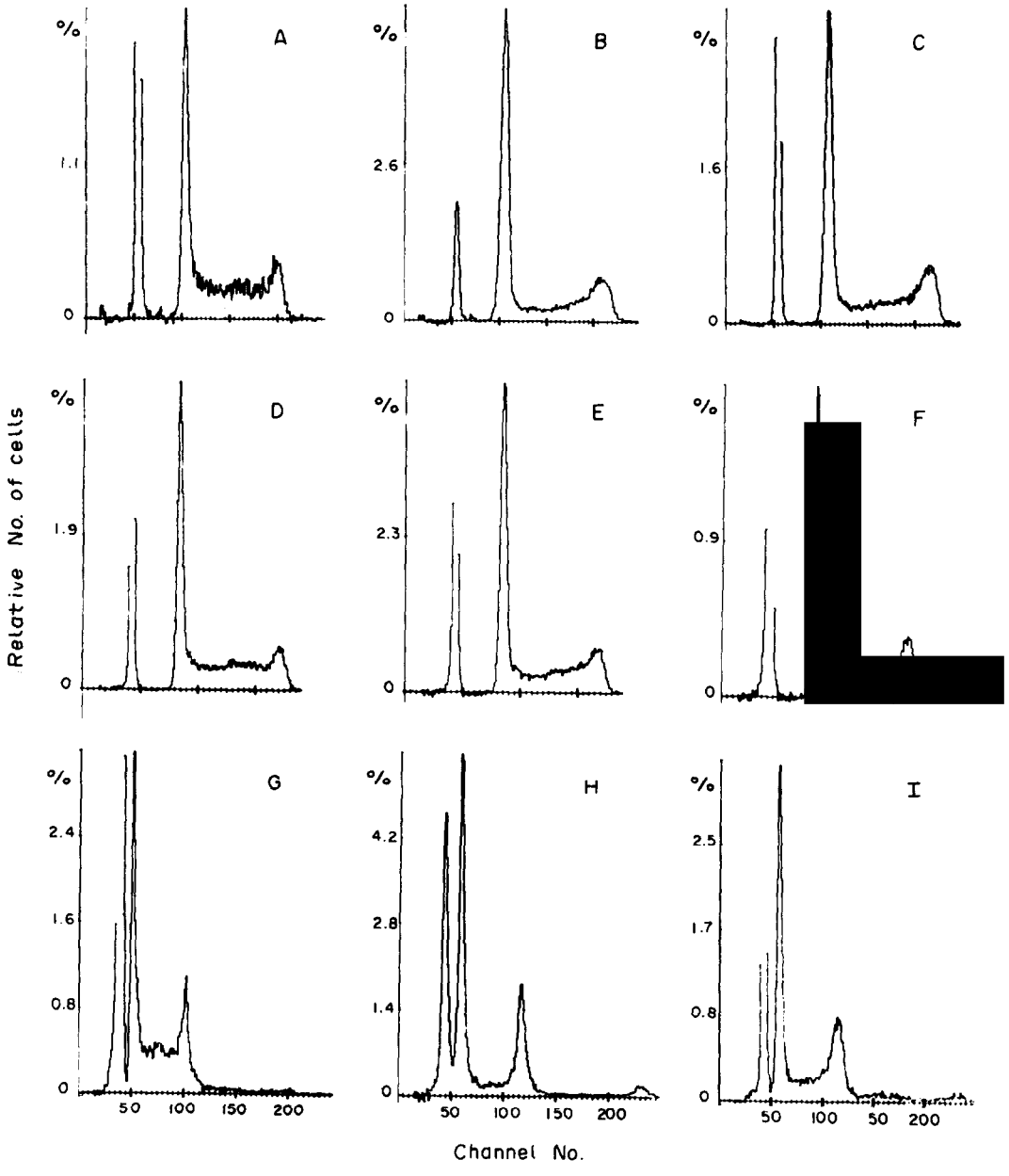

Fig. 1. *DNA histograms of* in vivo *3LL lines. Upper panels = original line (A–B–C); middle panels = M1087 line (D–E–F); lower panels = BM21548 line (G–H–I). A, D, G = primary tumor at day 8 after implantation of* 5×10^5 *viable cells; B, E, H = primary tumor at day 23 after implantation of* 5×10^5 *viable cells; C, F, I = lung metastases corresponding to primary tumors B, E, H, respectively. In all histograms the first peak on the left represents 2C cells; the difference in its location depends on different instrumental settings. The fraction of diploid cells relative to total population is:* 61% (*A*), 18% (*B*), 32% (*C*), 50% (*D*), 38% (*E*), 75% (*F*), 56% (*G*), 29% (*H*), 52% (*I*).

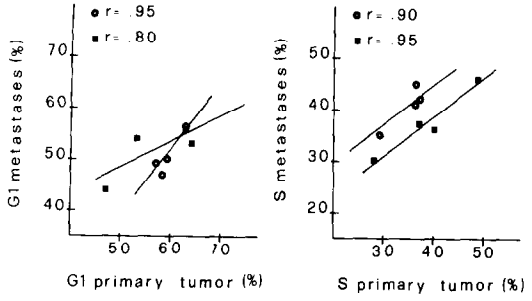

Fig. 2. Fraction of G1/G0 and S cells in the original line (●) and in M 1087 line (■). Metastases versus primary tumor in the same animal. The level of significance of correlation coefficient (r) *is*: $P < 0.05$. (*G1/G0 original line*), 0.20 (*G1/G0 M1087 line*), 0.10 (*S original line*), 0.05 (*S M1087 line*).

In this line the fraction of cells beyond the G2 tumor peak accounts for about 10% of the whole tumor DNA distribution, while in the other lines for less than 1%. This holds true even when the samples are measured after pepsin digestion in order to minimize artifacts due to cell clumping.

On the basis of these observations the presence in the BM21548 line of a second cell population with a multiple DNA content (DI = 2.70) could not be excluded. Consequently, the calculation of cell fraction in the cycle phases is impossible.

Moreover, it is interesting to note that also in this line both primary tumor and lung metastases exhibit a similar DNA distribution and the same DNA index.

3LL in vitro *lines*

The DNA content distributions of the lines named C108 and BC215 were analysed in the exponential phase of the growth. A quite similar pattern is observed in both lines during this phase. A representative DNA content histogram at day 5 of growth is shown in Fig. 3, panels A and B, for the C108 and BC215 lines respectively. As can be seen, a similar DNA distribution is found in the two *in vitro* lines, mainly as regards the high S phase compartment. The DNA index, determined as previously described, has the same value for both C108 and BC215 lines (DI = 2.0).

DISCUSSION

One of the purposes of the present study was to examine whether there is any correlation between DNA content distribution, as evaluated by FCM analysis, and some biological properties elicited by 3LL lines (such as growth rate and metastatic potential). In the *in vivo* lines, the original 3LL and M1087, no positive correlation can be established between the cell fractions in the cycle phases and growth rate. In fact, as can be seen from Tables 1 and 2, a quite similar cell cycle structure is found in the two lines in spite of the differences previously observed in their growth curve [7, 12]. In particular, in the early stage the original line grows faster than the M1087 ($Td = 2.2$ days vs 3.0 days respectively), whereas in the late stage the growth of the original line is considerably slowed down in comparison with that of the M1087 line ($Td = 11.6$ days vs 6.0 days respectively). It is therefore reasonable to assume that in this case the differences in the growth rate should be ascribed to factors like cell loss or vascularization rather than to strictly temporal or cell cycle-dependent parameters. As regards the *in vitro* lines C108 and BC215, the similarity in the cell cycle structure reflects the similarity of the growth characteristics shown in the two lines [12, and Table 2 in this paper]. In this work we found that the tumor lines

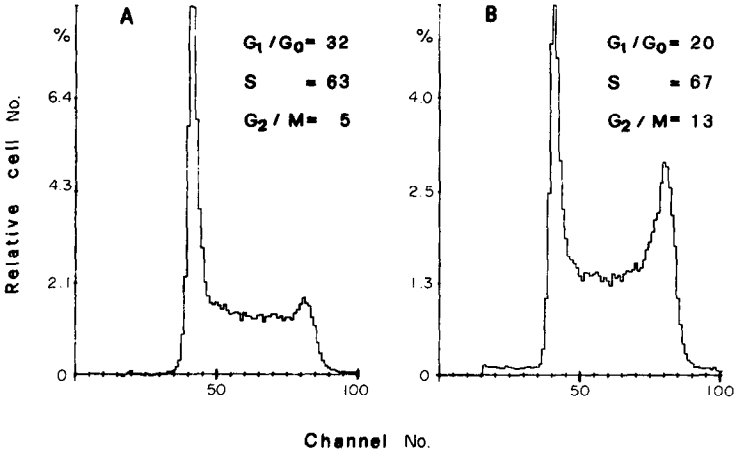

Fig. 3. DNA histograms of in vitro *3LL lines. C108 line (panel A), BC215 line (panel B) at day 5 of growth.*

Table 2. Biological properties of the in vivo *and* in vitro *lines of Lewis lung carcinoma*

	Mean No. colonies/lung ± S.E. Spontaneous metastases*	Artificial metastases†	Doubling time (hr) E	L‡	DNA index	Fractions of cells in S E	I	L	M§
In vivo lines									
Original line	38.0 ± 2.0	19.0 ± 2.0	52.8	278.4	2.0	55	52	35	41
M1087 line	69.0 ± 9.0	50.0 ± 8.0	72.0	144.0	2.0	55	50	39	37
BM21548 line	8.0 ± 2.5	4.4 ± 1.4	48.0	216.0	1.35		n.e.		
In vitro lines									
C108 line	—	4.0 ± 0.5	16–17	—	2.0	n.e.	63	n.e.	—
BC215 line	—	0.3 ± 0.1	16–17	—	2.0	n.e.	67	n.e.	—

*Lung colonies evaluated 21 days after i.m. implantation of 2.5×10^5 viable tumor cells [8, 14].
†Lung colonies evaluated 19 days after i.v. injection of 2.0×10^5 viable tumor cells [6, 8, 14].
‡E: early stages of growth (8th day) for the *in vivo* lines or exponential phase (5th day) for the *in vitro* lines. L: late stages of growth (15th day).
§E, I, L: early, intermediate, late stages of growth, respectively. M: metastases.

derived from 3LL are heterogeneous in their DNA content. Experimental studies by other laboratories have demonstrated that a positive correlation between DNA content and other cellular properties (such as malignancy) often exists, even if the origin of this correlation has not been clarified. We reported in previous work [6–8, 13] that a marked heterogeneity in metastatic potential (both in terms of artificial lung colony-forming ability and spontaneous metastatic ability) is expressed by the various lines of 3LL. Data reported in the present study indicate that for the *in vitro* lines such heterogeneity is not correlated with the DNA content. In fact, the C108 line, which exhibits a metastasizing ability ten times greater than that of the BC215 line [6, 14 and Table 2 in this paper], shows, on the other hand, the same tetraploid DNA content. On the contrary, if we consider the *in vivo* lines, the higher DNA content of M1087 line compared with that of BM21548 (DI = 2.0 vs 1.35 respectively) corresponds to a higher metastasizing ability of the former line in respect to the latter [7, 8, 14, and Table 2 in this paper].

Such a positive correlation between increased DNA and more malignant characteristics was also described by Suzuki *et al.* [15] on different clones of a mouse fibrosarcoma. Moreover, it is interesting to note that in all the *in vivo* lines there is a strict correlation between the DNA distribution patterns of the primary tumor cells and cells from the corresponding metastases, as well as an identical DNA content. These results are in agreement with data reported by various authors on some human tumors [16, 17]. The biological and clinical significance of such correlations have still to be exploited; they might be interpreted to reflect similar patterns of cellular proliferation in both tumor sites.

As previously mentioned, the present work was designed as part of a study of the relationship between the cell cycle structure of our model system (*in vitro–in vivo* 3LL) and drug-induced cell kinetic perturbations. The similarity in cell cycle structure between the two *in vivo* lines (the original 3LL and M1087) and the two *in vitro* ones (C108 and BC215) does not seem to account for the different chemosensitivity and/or resistance to various drugs shown by these tumor lines [7, 8, 14]. On the other hand, because of the great interest of this kind of proliferative study, we intend to continue and extend our researches for a more detailed analysis of possible cycle-specific effects of drug regimens on this tumor model system.

Acknowledgements—The authors are indebted to Prof. Carlo Nervi (Istituto Medico e di Ricerca Scientifica, Roma, Italy) and to Dr. Francesco Mauro (Lab. Dosimetria e Biofisica, CNEN-Casaccia, Roma, Italy) for the use of the flow cytometer for DNA content analysis performed in the present study. Thanks are also due to Dr. Marcello Spanò and to Dr. Emilia Romiti for their useful support and advice.

REFERENCES

1. HEPPNER GH, DEXTER DL, DENUCCI T, MILLER FR, CALABRESI P. Heterogeneity in drug sensitivity among tumor cell subpopulations of a single mammary tumor. *Cancer Res* 1978, **38**, 3758–3763.

2. FIDLER IJ. Tumor heterogeneity and the biology of cancer invasion and metastasis. *Cancer Res* 1978, **38**, 2651–2660.
3. BARRANCO SC, HO DHW, DREWINKO B, ROMSDHAL MM, HUMPHREY RM. Differential sensitivities of human melanoma cells grown *in vitro* to arabinosylcytosine. *Cancer Res* 1972, **32**, 2733–2736.
4. SACCHI A, CORSI A, CAPUTO M, ZUPI G. *In vitro* and *in vivo* selection of two Lewis lung carcinoma cell lines. *Tumori* 1979, **65**, 657–664.
5. ZUPI G, MAURO F, SACCHI A. Cloning *in vitro* and *in vivo* of Lewis lung carcinoma: properties and characteristics. *Br J Cancer* 1980, **41** (Suppl. IV), 309–310.
6. CAPUTO M, CORSI A, SACCHI A, ZUPI G. Drug response of two different *in vitro* tumor lines derived from lung metastases of 3LL. In: HELLMAN K, HILGARD P, ECCLES S, eds. *Metastasis: Clinical and Experimental Aspects.* The Hague, Martinus Nijhoff, 1980, Vol. 4, 396–400.
7. GRECO C, CALABRESI F, CAPTUO M, CORSI A, SACCHI A, ZUPI G. Adriamycin effect on Lewis lung carcinoma: comparison between the original line and its derivative subline. *Eur J Cancer* 1980, **16**, 1251–1255.
8. GRECO C, CALABRESI F, CAPUTO M, SACCHI A, ZUPI G. Effect of bleomycin scheduling on lung metastases of 3LL and a subline of different metastatic potential. In: HELLMAN K, HILGARD P, ECCLES S, eds. *Metastasis: Clinical and Experimental Aspects.* The Hague, Martinus Nijhoff, 1980, Vol. 4, 406–410.
9. DEAN PN. A simplified method of DNA distribution analysis. *Cell Tissue Kinet* 1980, **13**, 299–308.
10. BECK HP. Evaluation of flow cytometric data of human tumours. *Cell Tissue Kinet* 1980, **13**, 173–181.
11. CRISSMAN HA, KISSNAE RJ, OKA MS, TOBEY RA, STEINKAMP JA. Flow microfluorometric approaches to cell kinetics. In: DREWINKO B, HUMPHREY RM, eds. *Growth Kinetics and Biochemical Regulation of Normal and Malignant Cells.* Baltimore, Wilkins & Wilkins Co. 1977, 143–156.
12. BADARACCO G, GRECO C, STARACE G. Cell proliferation kinetics of the intramuscularly implanted Lewis lung carcinoma. *Tumori* 1980, **66**, 15–21.
13. ZUPI G, GRECO C, CAPUTO M, CORSI A, SACCHI A. *In vitro* and *in vivo* model of Lewis lung carcinoma: a rational approach to experimental chemotherapy. *Chemioter Oncol* 1979, **4**, 270–275.
14. SACCHI A, CALABRESI F, GRECO C, ZUPI G. Different metastatic potential of *in vitro* and *in vivo* lines selected from Lewis lug carcinoma: correlation with response to different bleomycin schedulings. *Invasion and Metastasis* In press.
15. SUZUKI N, WILLIAMS M, HUNTER NM, WITHERS HR. Malignant properties and DNA content of daughter clones from a mouse fibrosarcoma: differentiation between malignant properties. *Br J Cancer* 1980, **42**, 765–771.
16. AUER GU, ARRHENIUS E, GRANBERG PO, FOX CH. Comparison of DNA distributions in primarily human breast cancers and their metastases. *Eur J Cancer* 1980, **16**, 273–278.
17. BARLOGIE B, DREWINKO B, SCHUMANN J *et al.* Cellular DNA content as a marker of neoplasia in man. *Am J Med* 1980, **69**, 195–203.