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Effects of DTIC, DM-COOK and ICRF-159 on the number of circulating Lewis lung carcinoma cells detected by flow cytometry

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Circulating tumor cells can be detected by means of flow cytometry in the blood of mice bearing i.m. Lewis lung carcinoma. This technique can be applied in the case of aneuploid tumors and does not require either concentration of nucleated cells or other processing of the blood samples. It offers the advantages of simplicity and speed and allows quantitative measurement of the number of circulating tumor cells. It can be applied to the study of the effects of drug treatment on the number of circulating tumor cells, for those drugs which do not cause alterations in the nuclear DNA content of normal diploid blood cells. The number of circulating tumor cells determined by flow cytometry is markedly reduced by treatment with ICRF-159, by dimethyltriazene DM-COOK, and also by its clinically used analog, DTIC.

Introduction

Several techniques have been employed for detecting circulating tumor cells in animals bearing solid metastasizing tumors. Both indirect methods, involving bioassay of blood samples *in vivo* for assessment of the presence of viable tumor cells, and direct morphological identification of tumor cells present in blood or lymph samples, have been used [1]. When the direct determination of tumor cells is performed, the nucleated cells in the blood are concentrated in order to make it possible to identify tumor cells in a sample of reasonably small size, since the average concentration of circulating tumor cells in blood is exceedingly low [21]. The methods usually employed for the concentration of nucleated cells consist in the removal of the bulk of the red blood cells by centrifugation [22], accelerated sedimentation induced by agents such as dextran [1], or the lysis of red blood cells by substances such as streptolysin, eventually followed by collection of the cells on a Millipore filter [7, 16]. These procedures also have drawbacks, since their use can modify the morphology of tumor cells, and they are mainly of a qualitative nature since they do not allow an easy or precise quantitative evaluation of the number of tumor cells circulating *in vivo* [4, 7, 21]. Flow cytometry has been widely employed for cytodiagnostic [8, 17, 18, 29] as well as for cell kinetic investigations [2, 3, 6, 19]. It has also been used for quantitating the number of established pulmonary

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(micro)metastases in mice bearing B16 melanoma [11], as well as for studying the properties of B16 melanoma lines with differing metastatic potentials [14].

The aim of the present investigation has been the use of flow cytometry to measure the number of circulating tumor cells in the blood of mice bearing solid tumors. Using this technique, large cell populations can be quickly analysed, since more than 1000 cells can be examined per second.

The method of Krishan was chosen, since it involves the use of whole unprocessed blood samples [13]. Mice bearing intramuscular Lewis lung carcinoma were used since the tumor line is tetraploid and the tumor cells can therefore be discriminated from normal nucleated blood cells on the basis of their relative DNA content [27].

The effect of selective antimetastatic agents on the number of circulating tumor cells has also been examined. The drugs used were (±) 1,2-di(3,5-dioxopiperazin-1-yl) propane (ICRF-159), the dimethyltriazene *p*-(3,3-dimethyl-1-triazeno) benzoic acid potassium salt (DM-COOK) and its clinically used analog 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC). These substances selectively reduce metastasis formation in mice, with a mechanism unrelated to their cytotoxicity for tumor cells [9, 10]. Indirect evidence suggests that these agents prevent the entrance of tumor cells into the blood stream [10, 22] but for ICRF-159, direct microscopic examination of blood smears indicated that it reduced the number of circulating tumor cells [22].

Materials and methods

Tumor transplantation

The tumor used in this investigation was a Lewis lung carcinoma line (M1087) selected for its high potential to metastasize spontaneously from i.m. implants [20, 30]. The tumor line was maintained in syngeneic C57BL/6 female mice and propagated in female BD2F1 hybrids. The animals, weighing 18–20 g, were purchased from Charles River, Calco, Como, Italy and the tumor was maintained by implanting 10^6 viable tumor cells i.m. into the calf of the left hind leg. A single cell suspension was prepared by mechanical dissociation of the tumor tissue as already described [10], using donors similarly inoculated 2 weeks before. The tumor inoculum for the experiments reported below consisted of 10^6 viable tumor cells implanted i.m. in the left hind leg.

Drug treatment

DM-COOK was generously supplied by Dr. L. Lassiani (Institute of Pharmaceutical Chemistry, University of Trieste); DTIC and ICRF-159 were kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, USA, and cyclophosphamide was a gift from Schering SpA, Milan. The treatment was given i.p. daily with the schedules indicated in table 1, starting 24 hours after tumor implantation.

The drugs were administered in volumes of 0·1 ml/10 g as freshly prepared solutions in water (cyclophosphamide) or 0·1 N $NaHCO_3$ (DM-COOK), or suspensions in 0·9 per cent NaCl containing 1 per cent sodium carboxymethylcellulose (ICRF-159 and DTIC). The controls for each treated group received the same volume of vehicle used for drug administration.

Table 1. Number of signals in the region 4n–8n detected in blood samples of mice undergoing different treatments.

Tumor	Drug treatment	Dose (mg/kg/day)	Tetraploid signals
+	—	—	1183 ± 305
—	—	—	399 ± 95
—	DM-COOK	50	556 ± 216
—	DTIC	60	458 ± 115
—	ICRF-159	25	703 ± 31

BD2F1 mice implanted i.m. with Lewis lung carcinoma and animals not bearing tumors, treated daily for 11 consecutive days, starting on day 1 after tumor transplantation for mice bearing tumors, were sacrificed 24 hours after the last drug administration and their blood was analysed as described in the experimental section. The data are expressed as the number of signals 4n–8n per 10^5 diploid signals: each value is the mean ± S.E. obtained using groups of 3–10 mice.

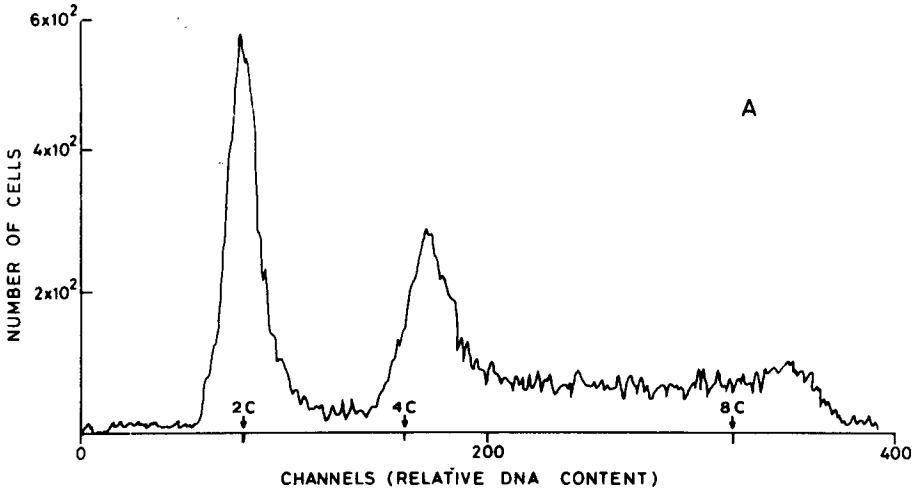
Measurement of the number of circulating tumor cells

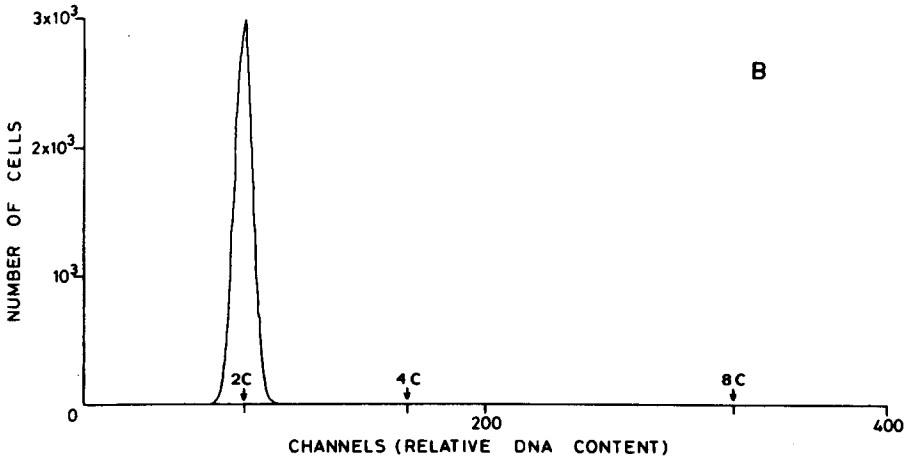
This determination was performed by flow cytometric measurement of peripheral blood by staining with propidium iodide as described by Krishan [13] for the analysis of the mammalian cell cycle. Samples (0·3 ml) of whole blood were obtained by intracardiac puncture from the right ventricle of mice whose thorax had been opened and who were anesthetized with ethyl urethane (1·5 g/kg). The samples were mixed with 3 ml of sodium citrate solution in water (0·1 per cent W/V), containing 50 μg/ml propidium iodide (Cal-Biochem, USA). After 15 min, the stained samples were measured by means of a Cytofluorograph 4800 A (Bio-Physics System Inc., USA). A 488 nm argon ion laser-line at 10 mW was used for excitation, and the fluorescence emission above 610 nm measured. A multichannel pulse height analyser (Spectrascope Modular 8000, Laben, Italy) was used to produce and store the DNA histogram distributions.

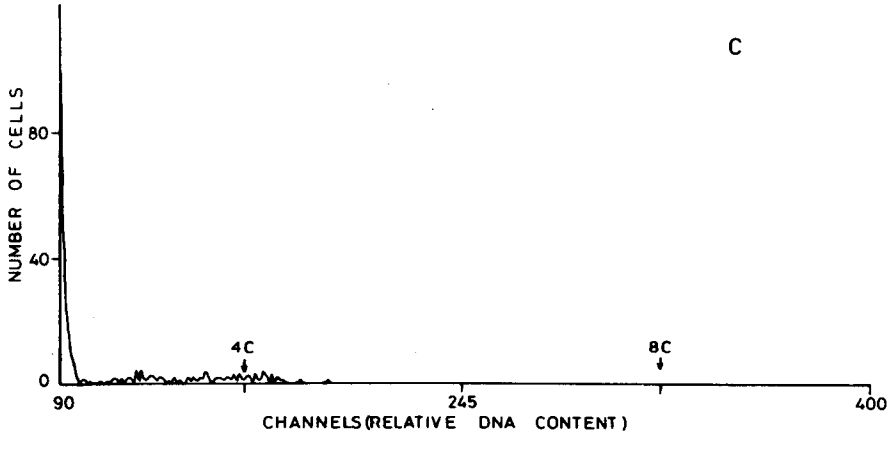
The number of signals corresponding to the tetraploid population (in the region 4n–8n), counted in a sample providing approximately 10^5 signals of normal diploid blood cells, was determined in each sample. The number of tumor cells was calculated for untreated controls and for each drug-treated group as the difference between the average values obtained in mice bearing or not bearing tumors, undergoing the same drug treatment.

Results and discussion

The results illustrated in the figure and reported in the tables are single representative experiments: repeated determinations (at least six different evaluations for each value presented) have provided substantially identical results. The data illustrated in the figure (Panel A) were obtained by examining a tumor cell suspension prepared by gentle mechanical dissociation of the primary i.m. tumor, and confirm that the Lewis lung carcinoma line presently used is tetraploid, as previously reported by other authors [27]. Blood samples obtained from untreated mice not bearing tumors show the presence of normal diploid nucleated blood cells, and the virtual absence of tetraploid signals (Panels B, C). On the other hand, blood


6x10²
4x10²
2x10²
NUMBER OF CELLS
A
2C
4C
8C
0
200
400
CHANNELS (RELATIVE DNA CONTENT)


3x10³
2x10³
10³
NUMBER OF CELLS
B
2C
4C
8C
0
200
400
CHANNELS (RELATIVE DNA CONTENT)


80
40
0
NUMBER OF CELLS
C
4C
8C
90
245
400
CHANNELS (RELATIVE DNA CONTENT)

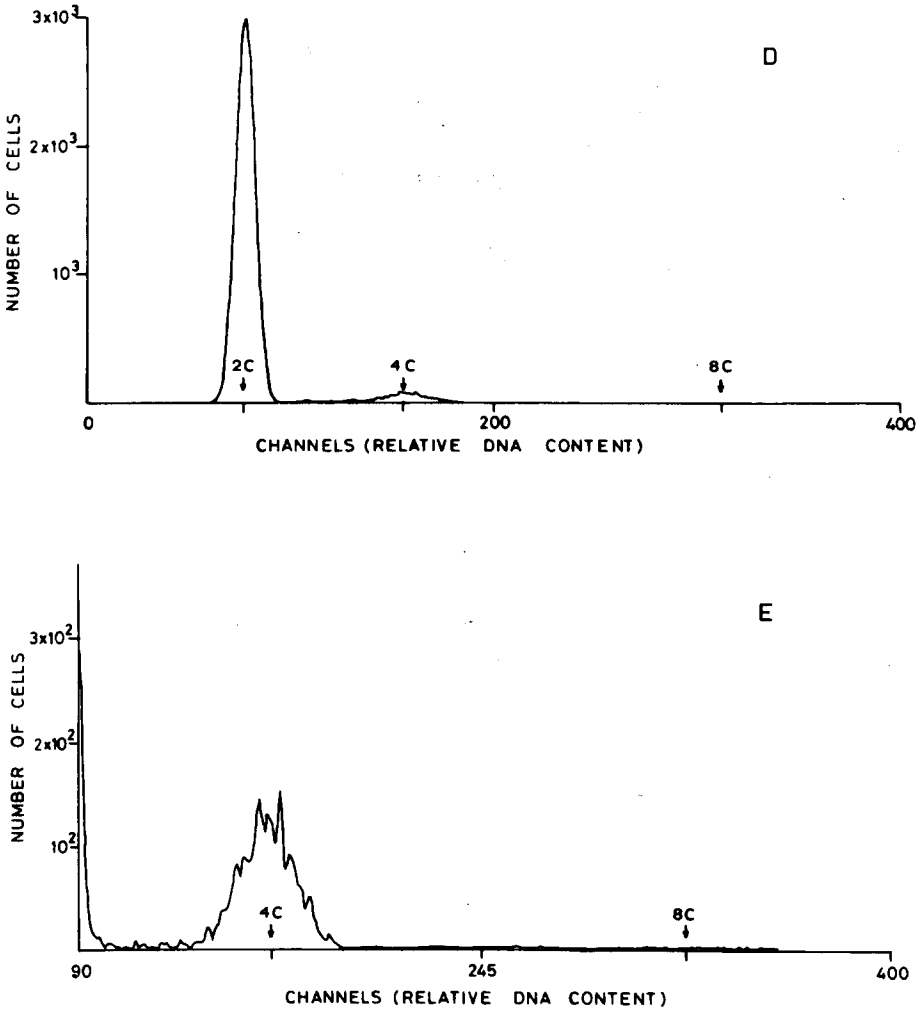


Figure 1. DNA distribution after propidium iodide staining of a cell population obtained by gentle mechanical dissociation of intramuscular primary tumor of Lewis lung carcinoma (Panel A), or of blood samples obtained from nontumor-bearing (Panels B, C) and tumor-bearing (Panels D, E) mice implanted i.m. with Lewis lung carcinoma.

samples obtained from untreated animals bearing tumors provide tetraploid signals corresponding to those obtained from tumor cells of the primary tumor (Panels D, E), which indicate the presence of circulating tumor cells. The small number of signals in the region 4n–8n which was detected in blood samples obtained from untreated mice not bearing tumors presumably originated from the aggregation of normal diploid nucleated blood cells. These basal value 4n–8n signals in animals not bearing tumors were increased by drug treatment, but still were smaller than the number of these signals counted in the blood of untreated mice bearing tumors (table 1). These signals presumably originate from single normal blood cells having nuclear alterations induced by the drug treatment. This possibility is supported by

(unpublished) experiments with cyclophosphamide, a bifunctional alkylating agent whose cytotoxic mechanism of action is based mainly on the formation of cross-links in the DNA of the target cells. Treatment with this drug markedly enhances the number of 4n–8n signals in animals with or without tumors. The alternative possibility, that drug treatment promotes the formation of cell aggregates, is unlikely because DM-COOK, DTIC and ICRF-159 do not cause aggregation of platelets, small lymphocytes or (L1210 or P388) tumor cells. Moreover treatment *in vitro* with DM-COOK does not cause aggregation of polymorphonuclear leukocytes of the guinea pig (unpublished results).

Considering that the Lewis lung carcinoma line M1087 is tetraploid, and that the basal value of 4n–8n signals obtained in blood samples of animals not bearing tumors is constantly smaller than that of animals with tumors (table 1), it is highly probable that the difference of the values in mice with and without tumors undergoing the same drug treatment can be taken as the number of circulating tumor cells. A significant number of circulating tumor cells can be detected in untreated control animals bearing tumors and this number does not vary greatly when the determinations are made at different times (8, 12 or 15 days) after tumor implantation (table 2).

To examine the effects of drug treatment, equitoxic doses were used for each compound and were equal to the 0·05 per cent lethal dose as previously determined with the method of Litchfield and Wilcoxon [15] using BD2F1 mice treated daily for 14 consecutive days. These dosages are markedly effective in reducing the formation of spontaneous pulmonary metastases in mice bearing solid metastasizing tumors [5, 9, 10, 23, 24]. Since equitoxic dosages were employed, the observed effects of the drugs after their administration can be compared as to their inhibitory potency. The differences in the number of 4n–8n signals counted in mice with and without tumors

Table 2. Effects of DM-COOK, DTIC and ICRF-159 on the number of circulating tumor cells in BD2F1 mice bearing Lewis lung carcinoma.

Compound	Dose (mg/kg/day)	Number of tumor cells (per cent of controls, at stated days after i.m. tumor implantation		
		8	12	15
Controls		100±42 (940±394)†	100±41 (783±319)†	100±44 (837±372)†
DM-COOK	50	0±30	0±32	17±26
DTIC	60	28±52	29±35	21±13
ICRF-159	25	10±38	22±24	24±32

† Actual finding obtained in control groups (number of tumor cells per 10^5 diploid blood cells).

Groups of 4–10 BD2F1 mice implanted i.m. with Lewis lung carcinoma, were treated daily starting 24 hours after tumor implantation. Sacrifice and the determination of circulating tumor cells was performed on the day indicated; the last drug administration was performed 24 hours before sacrifice. Each value reported in the table has been calculated as described in the experimental section, and is expressed as the mean per cent ratio (±S.E.) for each treated group as compared with the relevant controls.

undergoing the same drug treatment is significantly reduced by the three drugs tested, at the three time-periods after tumor implantation examined. This reduction indicates that the number of circulating tumor cells is lowered by the treatment, since the theoretical possibility that the drugs tested reduce tumor cell ploidy, thus lowering the number of 4n–8n signals, is unlikely, at least for ICRF-159 and DTIC. In fact, ICRF-159 has been found to increase the ploidy of EMT6 tumor cells, and DTIC has been shown to increase the mean DNA content of L1210 tumor cells [25, 28]. The most pronounced effects were observed with DM-COOK, which causes the complete disappearance of tumor cells by days 8 and 12 (table 2). Similar degrees of reduction in the number of circulating tumor cells are reproducibly and consistently obtained in separate and repeated experiments, although a relatively high individual variability of the data obtained from single mice is observed, as indicated by the relatively large standard errors of the mean values in tables 1 and 2. This variability has to be interpreted in terms of the variable numbers of circulating tumor cells rather than as a consequence of the inherent sensitivity of the method employed. However, the observed reductions caused by drug treatment are so large that the variability encountered does not affect their significance. Since the data reported in table 2 are expressed as the number of circulating tumor cells per 10^5 normal diploid blood cells, reductions in the number of leukocytes caused by drug treatment have to be taken into consideration. In the presence of leukocytopenia, the observed number of circulating tumor cells expressed per volume of blood is smaller than that referred to 10^5 normal diploid blood cells.

Thus, the reduction in the number of circulating tumor cells caused by drug treatment is presumably even more pronounced for DTIC and ICRF-159, but not for DM-COOK which does not reduce leukocyte counts in therapeutic regimens involving similar treatment schedules [23]. It would seem that in further experiments the number of circulating tumor cells ought perhaps to be referred to the volume of the blood samples rather than to the number of diploid normal blood cells, in spite of greater technical difficulties.

The data obtained here support previous observations by Salsbury *et al.* [22] on the reduction of the number of circulating tumor cells caused by ICRF-159, as determined with direct microscopic examinations of blood smears. These authors found, in control animals, the presence of significant numbers of circulating tumor cells to appear at day 10 after tumor implantation, peaking on day 11 and markedly dropping from day 12 onwards. The data reported here, on the other hand, indicate the presence of a comparable number of circulating tumor cells on days 8, 12 and 15. The early detection of circulating tumor cells appears to agree with the results provided by surgical experiments, which indicate the systemic presence of metastasizing tumor cells in mice bearing i.m. Lewis lung carcinoma having the primary tumor removed at various times between 5 and 9 days after implantation [12, 24]. They also show that DM-COOK, as suggested on the basis of indirect evidence, pronouncedly reduces the number of circulating tumor cells. Furthermore, DTIC [26] also markedly reduces the number of circulating tumor cells, suggesting that the antimetastatic action evinced in animals [9] might be involved in its clinical antitumor activity. Additionally, flow cytometry, which appears from the literature to have been used so far for the detection of already established pulmonary (micro)metastases, appears to be of value also for the detection of circulating aneuploid tumor cells, offering some advantages over the other techniques applied for the same purpose. The effects of drug treatment on the number of circulating

tumor cells can be easily determined provided the agents do not markedly alter the DNA content of normal and tumor cells.

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