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Effect of the Dosing Interval on Myelotoxicity and Survival in Mice Treated by Cytarabine

Zvia Agur, Ruth Arnon and Bilha Schechter

Many antineoplastic drugs are cell-cycle-phase-specific. These drugs are often highly toxic to the host, as they have the potential to impair replication, not only in the cancer cells, but also in the normal tissues. Using mathematical models it has been shown how selectivity of these drugs can be increased by exploiting the relatively large variability in cell-cycle parameters of the neoplasia. These models predict that toxicity to the host of cell-cycle-phase-specific drugs can be minimised if the dosing interval is an integer multiple of the average inter-mitotic interval of the susceptible host cells. Experimental evidence supporting this prediction is presented in this work. Our results show that a constant duration of the dosing interval yields higher survival rates in mice treated by cytarabine, as compared with random dosing intervals. Minimal myelotoxicity is exerted when the dosing interval is an exact multiple of the inter-mitotic time of bone marrow stem cells and erythroid progenitors (i.e. 7 h). Survival is significantly lower in mice treated every 8 h, or its multiple, as compared with that of mice treated at a 7 h or 10 h dosing interval.
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INTRODUCTION

IN THE heyday of cell kinetics, scientists attempted to improve the rationale of cancer chemotherapy by showing that synchronisation of dosage with cell division phase could allow greater tumour cell kill, while minimising the damage to the normal tissues. To prove the theory they used highly transplanted murine tumour cells, where there was quite a narrow distribution of inter-mitotic intervals, so that beneficial effects could be shown. However, as spontaneous tumours that arise in patients have a very large distribution of inter-mitotic intervals [1], the experimental results could not be reproduced in clinical trials. The inevitable conclusion from this experience is that variation in cell-cycle duration should be taken into account in models of cell dynamics. However, rather than drawing this conclusion and improving the theory, the quantitative approach to cancer chemotherapy has been almost completely abandoned [2, 3].

In previous work a mathematical model of cell population dynamics has been developed, incorporating realistic assumptions about the variation in cell-cycle parameters of host and cancer cells [4–6]. This work suggests that the differences in the distribution (average or variation) of the inter-mitotic interval between normal and cancer cells can be exploited for increasing treatment selectivity. Based on this model the Z-method was put forward, suggesting that host toxicity of cell-cycle-phase-specific drugs may be reduced without hampering efficacy, by using short, high dose, drug pulses; the dosing interval should be an integer multiple of the inter-mitotic interval in the most susceptible host cells [7]. The mathematical proof of this assertion is presented elsewhere [8], but its essence can be captured if we consider a normal cell population whose average inter-mitotic interval is 7 h. When the cells are not naturally synchronised, only a small fraction of this population will be in the S-phase of the cell-cycle (the susceptible phase for many anticancer drugs) at, say, time 0 h, and their daughter cells at around 7 h, 14 h, 21 h, etc. Now, if a cell-cycle-phase-specific drug is applied exactly at 0, 7, 14, 21 h etc, as the Z-method suggests, it will effectively eliminate only this small fraction of the cell population. In another population, e.g. cancer, where cells have a large variation in the inter-mitotic interval [1], a much larger fraction will be eliminated by this protocol. This will be the case also if the cells' inter-mitotic interval has a limited variation but its average differs from the dosing interval. In theory the method will not be effective only in the unlikely case in which the host susceptible cells and the cancer cells have exactly the same distribution in the inter-mitotic interval.

We have previously tested the efficacy of the Z-method *in vitro* and *in vivo*. *In vitro* results show that cytarabine is least toxic when applied in a fixed dosing interval, similar to the inter-mitotic interval of the treated cells [6]. In the *in vivo* experiments mice were treated by short duration zidovudine protocols and toxicity was measured by spleen weight, differential peripheral blood cell measurements, and the proportion of bone marrow (BM) cells in the S-phase gate of the DNA-content distributions. Results support the model's predictions in showing that zidovudine dosing interval per se affects the drug's toxicity to murine BM and that this drug is least toxic when the dosing interval is similar to the average BM inter-mitotic interval [9].

The present work investigates the effect of cytarabine dosing interval on treatment efficacy. Cytarabine is an S-phase-specific cytotoxic agent, with a half-life of about 20 min in the serum of mice [10, 11]. This drug causes severe depletion to murine bone marrow pluripotent stem cells, commited cells and erythrocyte progenitors [12, 13] whose cycle time, as measured from growth curves and from flow cytometry data is roughly 7 h [14–16]. Our results, showing out that a smaller BM toxicity is related to a dosing interval of 7 h, indicate the need for a careful estimation of cell-cycle parameters in the human BM.

MATERIALS AND METHODS

Drug

Cytarabine was obtained from Sigma and dissolved in 0.01 mol/l phosphate buffer containing 0.15 mol/l NaCl, pH

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7.3 (PBS) at the working concentration immediately before use. Doses were as indicated for the individual experiments. Control mice were injected with PBS.

In vivo *experiments*

Unless specified otherwise $C_3H.H_eJ$ mice, 6–10 weeks old, were given intraperitoneal inoculations of 10^4 38C–13B lymphoma cells [17], and treatment with periodic intraperitoneal injections of cytarabine was initiated 1 or 2 days following tumour inoculation. All protocols involved interval drug dosing with a drug-free episode between successive dosings. Dosing intervals in a given protocol were either fully periodic or random. Fully periodic protocols were applied in intervals of 7 h or an exact multiple of 7 h (denoted × 7 h), or every 8 h or 16 h (× 8 h), or every 10 h or 20 h (× 10 h), or every 11.5 h, or every 13 h. Random protocols involved dosing intervals generated by the random number generator of the IBM/NAG routine. The individual and total drug doses and the number of injections were identical, while the total treatment period was roughly the same for all mice in a given experiment. Treatment initiation and termination, as well as blood collection, were performed simultaneously in all mice in a given experiment. In order to neutralise the possible effect of circadian rhythms on cellular sensitivity and cytarabine pharmacokinetics, the number of night/day injections and the timing of blood tests were roughly similar for all mice in a given experiment.

Toxicity measurement

Peripheral blood assay was performed by collecting samples from the tail vein of mice approximately 30 h following termination of treatment. The effect of cytarabine on peripheral blood was evaluated by determining various blood parameters, such as white blood cell (WBC) count, or by differential blood counts, employing light scattering at different angles (Technicon H * 1™ [18]). Mice were observed for survival for 2 months. SAS/GLM pairwise *t* test was employed for measuring significance of differences in peripheral blood counts while the χ^2 test was employed for measuring significance of differences in survival and nuclear lobularity cytograms (NLC).

Bone marrow analysis

Mice were treated with cytarabine, as described above, and BM aspirates were obtained 48 h after the last injection, simultaneously from all mice. Propidium iodide labeled cells were analysed for DNA content distribution and cell size, using the fluorescence-activated cell sorter (FACS; Becton Dickinson). In addition, viscosity vs. cell size FACS BM analysis (right angle/forward light scatter), was performed for detecting differential damage to different BM compartments. SAS/GLM pairwise *t* test was employed for measuring significance of differences between schedules in the BM analysis.

RESULTS

Effect of dosing interval on myelotoxicity

The first three experiments were aimed at checking the effect of the dosing interval on host toxicity alone. In each experiment several groups of healthy mice were given the same number and same total dose of intraperitoneal cytarabine injections over the same period of time. The groups differed in the dosing interval alone, which was either random or fully periodic of × 7 h, or × 8 h, or × 10 h, etc. Since these were short duration experiments, it was expected that meaningful toxic effects will be manifested in WBC counts, rather than in red blood cells

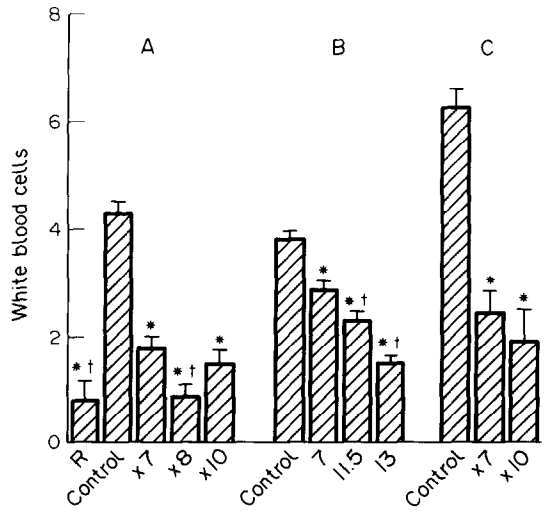

Fig. 1. WBC counts of mice treated by different cytarabine protocols. (a) Five groups of mice (10 mice in each group) received within 5 days seven intraperitoneal injections of 0.75 mg cytarabine each (225 mg/kg total dose) every 7 h or 14 h (× 7), every 8 h or 16 h (× 8), every 10 h or 20 h (× 10) or at random intervals (R). Control mice received seven intraperitoneal injections of PBS. (b) Four groups of mice (10 mice in each group) received five intraperitoneal injections of 0.7 mg cytarabine each (156 mg/kg total dose) at intervals of 7 h (7), 11.5 h (11.5) and 13 h (13). In this experiment treatment at 7 h and 11.5 h intervals began 15 and 6.5 h, respectively following the initiation of treatment at 13 h interval. Control mice received seven intraperitoneal injections of PBS. (c) Three groups of mice (10 mice in each group) received seven intraperitoneal injections of 0.75 mg cytarabine each (225 mg/kg total dose) according every 7 h or 14 h (× 7) or every 10 h or 20 h (× 10). Control mice were treated by 7 injections of PBS. Means marked by * are significantly different from the control in the SAS/GLM pairwise *t* test (significance level is $P < 0.01$). Means marked by † are significantly different from the × 7 h protocol group in the SAS/GLM pairwise *t* test (significance level is $P < 0.01$). Note that the discrepancy between group A (or B) and C in WBC counts is due to different scaling of the haematological analysers.

(RBC) counts, due to a relatively short turnover time of the former. Indeed, WBC counts showed lower toxicity in mice treated by cytarabine exactly every × 7 h, as compared to all other tested protocols (Fig. 1). Note, however, that the difference between WBC counts of mice treated by this protocol and those treated every × 10 h was not statistically significant.

In the experiment presented in Tables 1 and 2 healthy mice were given 6 × 1.15 mg cytarabine injections, every × 7 h,or every × 10 h, during a period of 3 days. From the samples presented in Table 1 it appears that the average loss of body weight was higher in mice treated every × 10 h, as compared with mice treated every × 7 h. Moreover, there was a statistically significant reduction in both treated groups in spleen weights and the counts of WBC, platelets, and mean platelet volume (MPV), but this reduction (and also that in RBC counts, not shown) was significantly larger in mice treated every × 10 h. An instructive result appears in the NLC of the Technicon H * 1™ peripheral blood analysis of this experiment. This cytogram displays granulocytes according to their size and nuclear lobularity [18]. Interestingly, 100% of the mice treated every × 10 h, displayed an atypical NLC, as compared with 0% in the control group, and 36% of the mice treated every × 7 h. The atypical NLC is characterised by a visually distinct cluster of cells with increased lobularity and reduced size (Fig. 2). Statistical analysis shows that mice with atypical cytograms have significantly lower

Table 1. Effect of the dosing interval on cytarabine toxicity

Dosing interval	Body weight* (%)	Spleen weight (g)	PLT ($10^3/\mu l$)	MPV (fl)	AT NLC (%)
Control	− 2	77.58 (8.0)	713 (174)	6.28 (0.52)	0
× 7 h	− 8.6	60.06 (14.3)†	408.2 (150)†	4.93 (0.42)†	36
× 10 h	−15.3	48.8 (5.2)†§	279.6 (98)†‡	4.37 (0.44)†‡	100†

Mice were given 6 × 1.15 mg cytarabine injections (total dose 319 mg/kg) at different dosing intervals. Last injection was done simultaneously in all mice. Differential blood measurements were performed by Technicon H*1™ haematology analyser.
Mean (S.D.).
* Total group changes in body weight; statistical analysis was not performed.
† Means are significantly different from the control ($P < 0.01$).
‡ Means are significantly different from those of the group treated every × 7 h ($P < 0.05$).
§ Means are significantly different from those of the group treated every × 7 h ($P < 0.005$).

Table 2. Effect of cytarabine on DNA content distribution of BM cells

Dosing interval	Cell-cycle compartment		
	G_0/G_1	S	G_2 plus M
Control	64.83 (3.88)	25.76 (3.74)	11.6 (4.3)
× 7 h	82.36 (4.3) *	15.21 (4.25)†	4.21 (1.25)*
× 10 h	88.19 (2.92)*‡	9.8 (2.59)*‡	2.91 (83)*§

Entries are the group average percentages (S.E.) of BM cells, categorised into three cell-cycle compartments according to FACS evaluation of DNA content of individual cells (for experimental details see Table 1).
* Results are significantly different from control ($P < 0.0001$).
† Results are significantly different from control ($P < 0.0002$).
‡ Results are significantly different from the group treated every × 7 h ($P < 0.002$).
§ Results are significantly different from the group treated every × 7 h ($P < 0.02$).

WBC, platelet and MPV, as well as a higher lobularity index (LI) ($P < 0.01$). It may be concluded that an atypical lobularity cytogram, characterised by a significant number of small highly nucleated cells, is correlated with increased blood toxicity, and that such an effect is significantly more prevalent in mice treated every × 10 h. In zidovudine experiments of the same nature an atypical NLC is also shown to be significantly more prevalent in mice treated every × 10 h. Here such cytograms are characterised by a distinct cluster of cells with reduced lobularity and increased size (Fig. 2) and are correlated with decreased WBC, platelet, MPV, LI and mean red blood cell volume [9].

To check the dosing interval effect on the BM, FACS analyses were performed on BM aspirates of mice treated as described above. The sensitivity of this method was used to assess our assumption that differences in cytarabine toxicity should be realised in different proportions of BM cells found in the S-phase gate of the FACS analysis, either due to an S-phase arrest, or due to a higher cell mortality: recent evidence shows that the DNA content of cells in the process of dying is in the range characteristic of S-phase cells, even though they are not incorporating precursors into DNA (R. Schimke, personal communication). The compartmental FACS analysis (Table 2) points out a significantly higher proportion of cells in the S-phase gate in mice treated at a × 10 h dosing interval, as compared with those treated at a × 7 h dosing interval and the control. It seems plausible that this higher proportion of S-phase cells, characterising the × 10 h dosing interval, reflects not only a higher S-phase arrest but also a higher cell mortality. An additional analysis of BM cell distribution according to size and viscosity (not shown) indicates that cytarabine treatment causes a large damage to granulocytes; damage is larger in mice treated every × 10 h, but the difference between the two treated groups is not statistically significant.

In conclusion, the above experiments in cytarabine treated healthy mice indicate that a × 10 h dosing interval is associated with a higher degree of BM and peripheral blood toxicity.

Effect of dosing interval on survival and tumour elimination

The next step was to check the dosing interval effect on treatment efficacy. To this end mice were inoculated with 38C–13B lymphoma cells and treatment with various cytarabine protocols was initiated 2 days later. The average inter-mitotic interval of this lymphoma cell line was previously determined

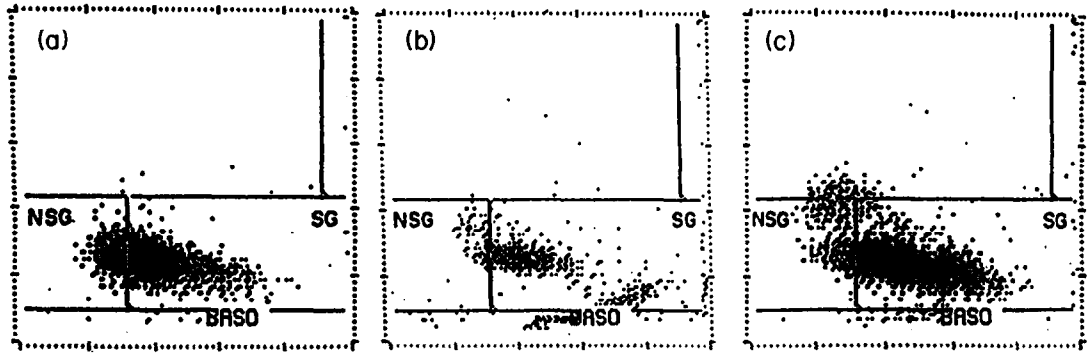

Fig. 2. Nuclear lobularity cytograms of treated mice. Results obtained when the effluent of the lobularity method was analysed by the differential light scattering cytometer of the Technicon H * 1™ system. Computer analysis discriminates segmented (S) from nonsegmented nuclei (NS); ordinate represents nucleus size and abscissa the segmentation level. (a) A typical nuclear lobularity cytogram of a mouse treated with cytarabine using a protocol of 7 h or 14 h dosing intervals; this cytogram is similar to a typical cytogram of a normal mouse [18]. (b) A nuclear lobularity cytogram of a mouse treated every 10 h or 20 h. (c) A nuclear lobularity cytogram of a mouse treated by zidovudine every 10 h [9].

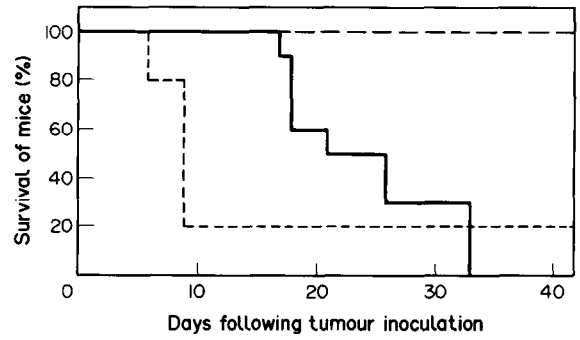

Fig. 3. Effect of cytarabine dosing interval on mice survival during 40 days following intraperitoneal inoculation of 10^4 38C–13B lymphoma cells. Two groups of mice (5 mice in each group) received, between day 2 and day 4, five intraperitoneal injections of 2 mg cytarabine each (322 mg/kg total dose) at 14–21–14–7 h intervals (———) or 10.5–21–8.5–19 h intervals (----). Control mice received five intraperitoneal injections of PBS (——).

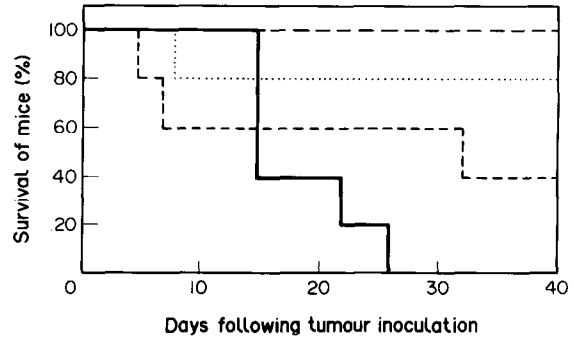

Fig. 4. Effect of cytarabine dosing interval on mice survival during 40 days following intraperitoneal inoculation of 10^4 38C–13B lymphoma cells. Five groups of mice (10 mice in each group) received, between day 2 and day 4, seven intraperitoneal injections of 0.75 mg cytarabine each (225 mg/kg total dose). Survival of mice treated at × 7 h intervals (———); survival of mice treated at × 10 h intervals (···); survival of mice treated at random intervals R (----). Control mice received seven intraperitoneal injections of PBS (———).

to be 12 h and it was shown that a dosing interval of 12 h, applied *in vitro*, is less toxic to these cells than other dosing intervals [6]. In the present work we showed that a dosing interval significantly different from 12 h effectively eliminated these cells.

In the first experiment we checked if a fully periodic drug protocol is more effective, with respect to tumour elimination and mice survival, than a protocol of the same total dose and total treatment duration, but random dosing intervals. In this experiment one group of mice received, during a total period of 56 h, five intraperitoneal administrations of 2.0 mg cytarabine each, with dosing intervals of 7, 14 or 21 h (× 7; see legend to Fig. 3 for details of schedule). Another group received five injections of the same dose of cytarabine at random intervals; the total treatment period was 59 h. The control group was given five intraperitoneal injections of PBS. As is shown in Fig. 3, a protocol involving dosing intervals of exactly 7 h or its multiple was well tolerated by the mice, resulting in 100% survival. In contrast, only 20% of the mice treated at random intervals survived, with death occuring between days 6 and 9 due to the toxic effects of the drug. The difference in survival between mice treated every × 7 h and those treated at random intervals is statistically significant ($P < 0.05$). Death of mice in the control group, as a result of the tumour, occurred between days 17 and 33.

In further experiments we compared treatment efficacy, with respect to host toxicity and tumour elimination of different fully periodic drug protocols. In the experiment described in Fig. 4, treatment by seven applications of 0.75 mg cytarabine per mouse, per injection, at dosing intervals of × 7 h, resulted in 100% long term survival. A group of mice receiving periodic treatments of the same amount of drug but at × 10 h dosing intervals had a survival rate of 80%, with toxic death occurring on days 7 to 12 following tumour inoculation. Survival of mice treated every × 10 h was still higher than that of the group treated by random intervals, where 60% of the mice died between days 6 and 7. Control mice died between day 15 and 26. Another experiment of a similar nature (Fig. 5) showed 88% long term survival of mice receiving the drug every × 7 h. In a group of mice receiving periodic treatments of the same amount of drug but at × 8 h dosing intervals only 42% survived, toxic death occuring on days 7 to 12 following tumour inoculation. However, the difference between the two groups was not statistically significant. Survival in the group treated every × 8 h was still higher than in the group treated at random intervals, where 72% of the mice died between days 6 and 7. In this experiment mice treated at × 10 h dosing intervals manifested 100% long term survival. The survival of the latter group was significantly larger than that of the group treated every × 8 h ($P < 0.01$). Control mice died between days 10 and 24.

The results of these experiments support our previous results in showing the superiority, with respect to host survival, of fully periodic drug regimens. Moreover, these results suggest that certain frequencies of drug administration are more effective than other frequencies; a dosing interval of × 8 h was inferior to other periodic protocols, but the group treated at × 10 h intervals did not differ in survival from the one treated at × 7 h intervals; both protocols were well tolerated by the mice. Since our peripheral blood and BM analyses consistently showed higher damage to mice treated every × 10 h, it may be speculated that this damage of the × 10 h protocol will affect survival only in a long duration treatment.

DISCUSSION

The ability to predict cell survival in a multidose drug schedule is the first step in the design of effective treatment protocols.

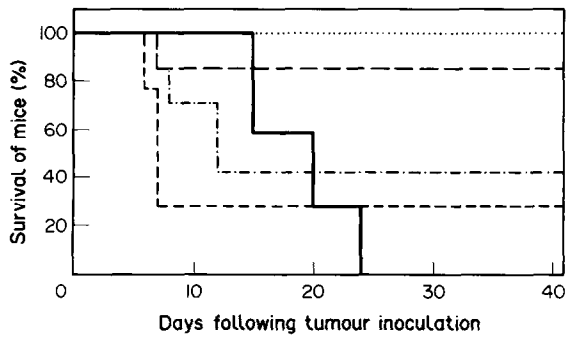

Fig. 5. Four groups of mice (10 mice in each group) were treated by the protocols described in the legend to Fig. 4. Survival of mice treated at × 7 h intervals (———); survival of mice treated at × 8 h intervals (-·-); survival of mice treated at × 10 h intervals (···); survival of mice treated at random intervals (----). Control mice received seven intraperitoneal injections of PBS (——).

However, even for a relatively well characterised drug, such as cytarabine, the current level of knowledge is insufficient to predict optimal therapeutic schedules. It has been noted that the cytokinetic characteristics of the population, the dose and the dosing interval, determine the proportion of cells killed by multiple doses of cytarabine [10, 19] and host tolerance [20]. However, only recently a mathematical method has been provided, which enables a logical and systematic prediction of treatment efficacy for cell-cycle-phase-specific drugs, such as cytarabine. This method defines the exact relation between the drug pharmacokinetics, the dynamic properties of a given cell or viral population and the prospects of its elimination [6–8].

Using this method it has been suggested that drug selectivity can be increased by manipulating the duration of the dosing interval. Thus a "resonance" effect can be created for the normal cells, minimising their mortality, and a lack of "resonance" for the cancer cells, whose frequency of replication differs from that of the normal cells. According to this theory continuous drug infusion should be less selective than certain protocols of interval drug dosing. For this reason we have focused attention in this work on protocols involving strictly positive drug-free intervals. Moreover, the above method suggests that minimal toxicity is exerted when the dosing interval is equal to or an integer multiple of the average inter-mitotic interval of the susceptible host cells. The aim of the present study was to verify this prediction: Our results show that in a short duration treatment, a dosing interval of exactly 7 h or its multiple is less damaging to murine BM, peripheral blood, as well as body and spleen weight, than other treatment protocols. This effect is strictly associated with the basic drug periodicity, since the total drug dose, the treatment duration and circadian effects were similar for all protocols. If the average inter-mitotic interval of murine BM progenitors is indeed 7 h, as is indicated in the literature (see above), then the present results support our prediction that a protocol whose dosing interval is similar to the average inter-mitotic interval of susceptible host cells, is less toxic to these cells than other periodic treatments.

In order to show that the effects reported here were due to direct BM toxicity, a DNA content distribution analysis was performed on BM aspirates of mice treated at different dosing intervals. Results of these experiments show a clear direct effect of cytarabine on the BM granulocytes, and a complete elimination of the BM S-phase compartment when the drug doses were very high (results not shown). It should be noted, though, that the possibility that mice mortality was due to other toxic effects, e.g. gastrointestinal toxicity [19], cannot be ruled out.

Further experiments are warranted for examining our conclusions in greater detail. Thus, the putative effects of factors, such as the circadian rhythms [21], or drug effect on cell-cycle synchronisation and on shortening cycle duration [22], can be incorporated in our model. Note, however, that significant differences between protocols in our experiments, imply that in a short duration treatment such effects are not a dominant factor in determining treatment efficacy.

The implications of our results for chemotherapy should be interpreted with some caution, bearing in mind that the average inter-mitotic interval of BM cells (about 24 h [23]) and cytarabine half-life are longer in humans. Our theoretical results [8, 6] suggest that treatment efficacy is affected by the relation between the dosing interval and the inter-mitotic interval of the susceptible cells. Based on the observation, reported here, that a 1 h difference in cytarabine dosing interval affects murine BM toxicity, we expect a 3–4 h difference in this interval to have a similar effect on the human BM. A theoretical analysis of interval drug dosing in human patients is provided elsewhere [8, 24]. This and previous studies [25] suggest that a single daily dosing of cell-cycle phase specific drugs whose half-life is 1–3 h (e.g. cytarabine or zidovudine) will be less toxic to human BM than schedules involving the same total dose divided into several daily dosings. However, the latter schedules are expected to be somewhat faster in virus or neoplasia elimination. The potential clinical importance of these predictions indicates the need for further investigation.

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Immunohistochemical and Biochemical Characterisation of the Expression of a Human Embryonal Carcinoma Cell Proteoglycan Antigen in Human Germ Cell Tumours and other Tissues

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In the embryonal carcinoma (EC) cell line GCT 27, monoclonal antibody GCTM-2 recognises an epitope on a 200 kD pericellular matrix keratan sulphate proteoglycan. Immunohistochemical analyses demonstrated staining of tissue sections from 21 out of 22 human non-seminomatous germ cell tumours, and from 22 out of 28 sections of seminomas. In normal human fetal tissues gut epithelium and muscle stained strongly, and certain other epithelia stained moderately. In adult tissues, the distribution of the epitope was similar, but staining intensity was weaker. Neoplastic tissues showed reactivity with embryonal rhabdomyosarcoma and colorectal carcinoma, but no other non-germ cell tumours. Immunofluorescence microscopy showed that GCTM-2 also stained cell lines from human colorectal carcinoma, embryonal rhabdomyosarcoma and choriocarcinoma. In contrast to EC cells the epitope in these other cell types required permeabilisation of the cells to be visualised, and the protein bands in immunoblots lacked extensive modification with keratan sulphate and were smaller. Thus, GCTM-2 reacts with an epitope which has a previously unrecognised tissue distribution; its expression as a pericellular matrix proteoglycan is predominantly a characteristic of human EC cells.
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INTRODUCTION

THE SERUM markers α-fetoprotein and human chorionic gonadotropin are useful in monitoring patients with testicular non-seminomatous germ cell tumours (NSGCT) [1]. These secreted polypeptides are products of differentiated yolk sac cells and trophoblastic lineage respectively, which appear in a proportion of testicular teratomas [2]. Despite the utility of these markers, it would be desirable for cell biological studies and for certain clinical applications to define markers expressed on embryonal carcinoma stem cells themselves, rather than on their differentiated derivatives. A preliminary study has suggested that the GCTM-2 antigen merits investigation as one such clinical marker [3].

A number of cell surface antigens defined by monoclonal antibodies have been described in association with embryonal carcinoma. Many of these monoclonals react with carbohydrate antigens and are of the IgM class [4]. Monoclonal antibody GCTM-2 was shown to recognise a pericellular matrix EC proteoglycan which was susceptible to degradation by keratanase but not other glycohydrolases or lyases [5]. More recent studies on the purified GCTM-2 antigen confirmed that it is a keratan sulphate proteoglycan. The further evidence supporting this conclusion included aminoacid and sugar analyses of the antigen; the reactivity of the antigen with Alcian blue dye; high affinity of the antigen for anion exchange resins; metabolic labelling of

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