PHASE-SPECIFIC CYTOTOXICITY IN VIVO OF HYDROXYUREA ON MURINE FIBROSARCOMA PULMONARY NODULES

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Summary.—The cytotoxic effects in vivo of hydroxyurea (HU) on murine fibrosarcoma (FSa) cells grown as pulmonary tumours were determined. Tumour cells from 13-day-old nodules were made into suspension and separated on the basis of cell size by centrifugal elutriation. Flow microfluorometry (FMF) was used to determine the cell-cycle parameters and the relative synchrony of the separated populations, as well as the degree of contamination by normal diploid cells in each of the tumour-cell populations. HU cytotoxicity was tested by administering both a single 1 mg/g i.p. dose into mice that had been injected i.v. 20 min earlier with known numbers of synchronized viable FSa cells, and i.p. doses of 1 mg/g each into mice bearing 13-day-old pulmonary nodules. In the latter experiments, animals were killed 1 h after the last dose, and the tumour nodules were excised and made into a single-cell suspension and elutriated. Known numbers of cells from each fraction were injected into recipient mice to determine survival. In both sets of experiments, cell killing by HU correlated with the percentage of S-phase cells. The treatment of 13-day-old pulmonary nodules with 3 doses of HU also depleted the (G2+M) phase tumour cells and increased the heterogeneity between tumour subpopulations, as determined by FMF analysis.

Many of the chemical agents currently used in cancer therapy have phase-specific cytotoxicity. To evaluate these agents better before clinical trials, it would be advantageous to characterize their effectiveness on synchronized target cells within living animals. Recently, an in vivo method of studying the cell-cycle phase specific effects of a variety of chemotherapeutic agents in vivo was described (Grdina et al., 1979, 1980). Target tumour populations, enriched with cells in either G1, S or G2+M phase by centrifugal elutriation, were injected i.v. into mice 20 min before the i.v., i.p., or s.c. administration of the drug to be tested. With the appropriate controls, the phase-specific cytotoxicity of any test agent could be determined (Grdina et al., 1979). A limitation of this procedure, however, was the need for the inherent heterogeneity of the tumour-cell populations derived from solid tumours to be reduced for cell separation by centrifugal elutriation to be effective (Anderson et al., 1969; Grdina et al., 1978b). This was accomplished by a 48h in vitro incubation (Grdina et al., 1978a). To avoid this limitation, the method of centrifugal elutriation has been applied to separate murine fibrosarcoma (FSa) cells from pulmonary tumour nodules grown in C3H mice. Hydroxyurea (HU), because of its well characterized S-phase-specific cytotoxicity (Kim et al., 1967; Sinclair, 1967), was chosen to demonstrate the applicability of this procedure to characterizing the in vivo effectiveness of phasespecific chemotherapeutic agents on advanced metastatic disease.

MATERIALS AND METHODS

Mice and tumour.—Female C3Hf/Kam mice, 10–12 weeks old, from our specific-
pathogen-free breeding colony and a methy- 
cholanthrene-induced fibrosarcoma were used 
(Suit & Suchato, 1967). Tumours, 6th- 
generation isotransplants, were made into 
viable single-cell suspensions and injected 
into untreated recipient mice to produce 
100–150 pulmonary nodules (Grdina et al. 
1978b). After 13 days, tumour-bearing 
animals were killed, either immediately 
or after treatment with multiple doses of 
HU. Tumour cells derived from these 
pulmonary nodules were used in all the 
experiments.

_Tumour-cell suspension._—Single-cell sus-
pensions were prepared by mincing and 
trypsinizing colonies of tumour cells from 
excised lungs (Grdina et al., 1979). Because 
no advantage was found from the careful 
excision of individual tumour nodules from 
surrounding lung tissue, cell suspensions 
were routinely made from entire lung lobes 
containing tumour tissue. Briefly, lung lobes, 
separated and removed from the surrounding 
viscera, were carefully minced with ophthal-
mic scissors. The mince, containing normal 
and tumour tissue, was added to a beaker 
containing 0·025% trypsin in Solution A 
(8:0 g NaCl, 0·4 g KCl, 1·0 g glucose and 
0·35 g NaHCO₃ in a litre of water) and 
stirred on a magnetic stirrer for 20 min at 
room temperature. DNase (crude Deoxyribo-
nuclease 1 from beef pancreas; Sigma 
Chemical Co., St Louis, MO) was also added 
to the mixture to achieve a final concentra-
tion of 0·1 mg/ml. After stirring, the beaker 
was allowed to stand undisturbed for 5 min. 
Highly enriched undigested tumour tissue 
settled to the bottom of the beaker, whereas 
most of the lung tissue floated to the top. 
While exercising care to avoid collecting 
floating lung tissue, the upper two-thirds 
of the suspension was removed and mixed 
with an equal volume of a modified McCoy’s 
5A growth medium supplemented with 20% 
foetal calf serum (Humphrey et al., 1970). 
The stirring and collection procedure was 
repeated × 3. Each resultant suspension 
was passed through a stainless-steel mesh 
(200 wires/inch) and centrifuged at 225 g 
for 5 min. The supernatants were discarded, 
and the pellets were resuspended in McCoy’s 
5A supplemented with 5% FCS containing 
DNase at a final concentration of 0·1 mg/ml. 
Also included was 5 mM 2-naphthol 6-8 di-
sulphonic acid to reduce cell clumping 
(Shortman, 1973). After centrifugal elutria-
tion, cell viability was routinely > 95% as 
determined by phase-contrast microscopy.

_Separation by centrifugal elutriation._— 
FSa cells derived from pulmonary nodules 
were separated under sterile conditions by 
centrifugal elutriation using the same pro-
cedure described for their separation from 
either solid tumours or tissue culture (Grdina 
et al., 1978a, 1979). With the rotor speed set 
at 1525 rev/min, 2–3 × 10⁸ cells, suspended 
in 20 ml of medium, were introduced into the 
elutriator chamber at a flow rate of 5·4 
ml/min. The rotor speed was held constant 
throughout the separation, and the flow 
rates were varied by equal increments from 
5·4 to 27·4 ml/min. Routinely, 12 fractions 
(F), each of 50 ml, were collected and stored 
at 4°C. Cells collected in each fraction were 
counted by haemacytometer and by Coulter 
Counter (model ZB1; Coulter Electronics, 
Hialeah, FL), and their volume distributions 
determined with a multichannel analyser 
(Channelyzer II; Coulter Electronics). The 
modal volume was designated as that 
corresponding to the modal channel number 
of the volume distribution of each sample 
(Grdina et al., 1978a). F 1 containing small 
cells and cellular debris, and F 11 and 12, 
containing a heterogeneous mixture of cells, 
were discarded.

_Flow microfluorometry._—The DNA content 
of individual cells in suspension was deter-
bined by FMF using an ICP 11 flow cyto-
meter (Phywe Co., Göttingen, Germany). 
cells were fixed in 70% ethanol and then 
stained with 50 mg/ml thymamycin (Mi-
thracin; Pfizer and Co., Inc., New York, 
NY) in solution with MgCl₂ (7·5 mM) and 
12·5% aqueous ethanol (Grdina et al., 
1978a). The resultant histograms of DNA 
fluorescence were computer-analysed (John-
ston et al., 1978). Because cell suspensions 
were derived directly from FSa lung nodules, 
a considerable number of normal diploid 
lung cells were present in all the elutriator 
fractions. Since FSa cells are heteroploid 
(i.e., 60–70 chromosomes) and contain ~1·8 
as much DNA as normal diploid cells 
(Grdina et al., 1977), an estimate of the normal 
cell contamination in each of the tumour-
cell suspensions can be made by determining 
the area under the G₁ normal diploid peak 
and dividing it by the area under the total 
FMF histogram (i.e., the area under both the 
tumour and normal peaks) (Grdina et al., 
1978a). While the relative proportions of
normal cells in the S and G$_2$+M phases containing FSa tumour-cell suspensions are uncertain (i.e., fluorescence from these cells would be detected at the same channels as those from G$_1$ tumour cells), they are considered to be sufficiently rare in this experimental system to be ignored in these calculations. FMF histograms of lung cells from tumour-free animals indicate that less than 2% of the cells are in S or G$_2$+M phases (unpublished data). Likewise, few if any diploid cells appear to be in the S phase in suspensions from pulmonary nodules (i.e., low background fluorescence in Channels 60 to 80, see Figs. 2 & 6). Therefore all cell counts were adjusted after FMF analysis to represent only tumour-cell numbers.

Lung colony assay.—The colony-forming efficiency (CFE) of FSa cells was determined in a lung colony assay. Recipient mice, with their hind legs shielded, were whole-body-irradiated with 10 Gy 24 h before use. These mice were then injected with known numbers of viable FSa cells, corrected for normal-cell contamination, from each of the elutriator fractions and the unseparated control (USC) population. Each aliquot of cells also contained 2 x 10$^6$ heavily irradiated (HIR; 100 Gy) FSa cells. HIR cells were not separated by centrifugal elutriation. Thirteen days after the mice were killed, their lungs were removed, and the lobes separated and fixed in Bouin’s solution, and tumour colonies counted.

HU testing in vivo.—HU (manufactured by Ben Venue Laboratories, Bedford, OH) was obtained from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD. Stock solutions were made up at 100 mg/ml in sterile water. Two procedures, designated A and B, were followed to test HU’s effectiveness in vivo. At least 3 replicate experiments were performed using each procedure. In procedure A, FSa cells grown as pulmonary nodules were harvested, made into suspension, and separated by centrifugal elutriation before drug treatment according to the method described earlier (Grdina et al., 1979). Twenty minutes after the injection of viable synchronized FSa cells, 10 of the 20 animals in each group were injected i.p. with a single dose of HU at 1-0 mg/g body wt. Thirteen days later the animals were killed, and the resulting colonies were counted. In Procedure B, mice bearing 13-day-old pulmonary nodules were injected i.p. each hour with 1 mg/g HU for 3 h (i.e., total dose 3 mg/g). One hour after the last injection, the animals were killed and their lungs removed. Suspensions of tumour cells were made and separated by centrifugal elutriation. The CFE of these cells was determined in a lung-colony assay with preconditioned mice.

RESULTS

The recovery of cells from centrifugal elutriation was routinely > 90% and the viability of these cells, determined by phase-contrast microscopy, was > 95%. Fig. 1 shows a representative sedimentation profile of the relationship between modal cell volume and the number of cells recovered in each elutriator fraction. Analysis of the DNA histograms for each separated population enabled the distribution of tumour cells with respect to DNA content to be determined (Fig. 2). The average sedimentation velocity of

![Diagram](image-url)

**Fig. 1.**—Separation of FSa tumour cells from lung metastasis by centrifugal elutriation. The percentage of total cells (● — ●), the FMF corrected percentage of tumour cells only (○ — ○), and the modal cell volume (▲—▲) are plotted as a function of sedimentation velocity and fraction number.
IN VIVO CYTOTOXICITY OF HYDROXYUREA

90
60
30
0
-30
-60
-90

J90
60
30
0
90
60
30
0
90
60
30
0

Fig. 2—Representative DNA histograms obtained by FMF analysis of an unseparated lung and tumour-cell suspension (USC) and fractions of cells separated from that suspension by centrifugal elutriation (F2−F10). Normal diploid G1 = G1(N), tumour G1 = G1(T); and tumour G2 = G2 + M(T).

the cells was 11.4 mm/h/g, and their average modal volume was 1250 μm³. The percentage of normal diploid G1 cells and tumour G1, S and (G2 + M) cells, and the coefficients of variation (CV) of the tumour G1 fluorescence peaks, as calculated from the histograms in Fig. 2, are presented in Table I. In contrast to results with tumour cells cultured in vitro for 48 h (Grdina et al., 1979) normal cell populations were observed in all elutriator fractions, with the largest percentage of normal diploid cells in F2.

The CFE of recovered cells, both uncorrected and corrected for normal diploid cell contamination by FMF analysis, is presented in Fig. 3. The average modal volumes of cells collected in F2 and 3 were <800 μm³. The CFE of tumour cells collected in these fractions, even after FMF correction for diploid cells, was significantly less than for cells in the other fractions.

Fig. 3.—The colony-forming efficiency (CFE) of F5a cells separated by centrifugal elutriation from lung metastasis. The CFE uncorrected (△—△) and corrected (○—○) for contaminating normal diploid cells (as determined by FMF analysis) is plotted as a function of fraction number. Each vertical bar represents ± s.e.
The cell-killing of a single dose of HU on FSa cells lodged in the lungs of test animals (i.e., Procedure A) is presented in Fig. 4. The CFE of both the control and treated populations was routinely corrected for normal-diploid-cell contamination. Cell killing with HU was most evident for FSa cells collected in F 6 and 7. These fractions contained the greatest concentrations of cells with S-phase DNA content. These data are consistent with the results using cultured FSa cells (Grdina et al., 1979).

The cytotoxicity of 3 equal in vivo doses of HU at hourly intervals to FSa lung-nodule cells (i.e., Procedure B) is shown in Fig. 5. Animals were killed 1 h after the last dose, lungs were removed and made into a single-cell suspension, and cells were separated by centrifugal elutriation. DNA histograms describing the separated tumour populations following HU treatment are presented in Fig. 6. Whereas the fluorescence peaks representing normal diploid G₁ cells remained unperturbed and comparable to those of untreated populations (Fig. 2), those representing HU-treated G₁ and (G₂+M) tumour cells are broader and more heterogeneous. Nevertheless, cell killing again correlated with the percentage of cells in S (see Fig. 5). A significant reduction in the percentage of cells with (G₂+M) DNA content was also found after this treatment, which is not surprising since the total treatment time was 4 h, and the duration of G₂+M FSa cells in vivo is only 2.8 h (Grdina, 1982). The percentages of normal diploid G₁ and tumour G₁, S, and (G₂+M) cells, and the CVs of the G₁ tumour fluorescence peaks are contained in Table II.

**DISCUSSION**

Although centrifugal elutriation has been successfully used to separate and isolate populations of FSa tumour cells enriched in G₁, S or (G₂+M) phases following growth in vitro (Grdina et al., 1978b, 1979) it has not been effective for synchronizing FSa cells derived directly from solid tumours growing in vivo (Grdina et al., 1977, 1978b). The cellular parameter that is exploited using this procedure is cell volume, because the sedimentation rate of a cell is proportional to the two-thirds power of its volume (Glick et al., 1971). Cell size, under conditions of uniform growth, is known to increase during the division cycle (Anderson et al., 1969). Thus, after exponential growth in vitro, the relationship between cell size and DNA content is readily exploitable for FSa tumour cells. In contrast, cells growing in solid tumours are exposed to a variety of physiological conditions, including variations in the availability of nutrients, O₂ tension and pH. Consequently tumour cells of various sizes can have

**Table I.—Distribution of untreated cells in various phases of the cell cycle after centrifugal elutriation (Determined by FMF analysis)**

| Fraction Number | G₁(N)* | G₁(T)† | S(T) | G₂+M(T) | CV of G₁(T) peak |
|-----------------|--------|--------|------|---------|-----------------|
| USC             | 21     | 65     | 18   | 17      | 4.1             |
| 2               | 46     | 91     | 9    | 0       | 3.2             |
| 3               | 26     | 86     | 11   | 3       | 1.4             |
| 4               | 14     | 81     | 16   | 3       | 3.8             |
| 5               | 13     | 73     | 24   | 3       | 4.0             |
| 6               | 10     | 27     | 43   | 30      | 4.4             |
| 7               | 15     | 17     | 37   | 46      | 4.5             |
| 8               | 15     | 16     | 19   | 65      | 4.6             |
| 9               | 14     | 12     | 17   | 71      | 6.0             |
| 10              | 12     | 18     | 16   | 66      | 5.8             |

* G₁ of normal diploid cells.
† G₂ of tumour cells.
similar DNA contents, making separation of cell populations on the basis of cell size ineffective (Sigdestad & Grdina, 1981). Although FSa cell populations from pulmonary nodules are also more heterogeneous than those from exponentially growing cultures in vitro, they are considerably less so than those from solid tumours, which is not surprising since the variations in the microenvironment in solid tumours are, presumably, considerably greater than those in the smaller lung-tumour nodules. Biological parameters that appear to reflect this "intermediate" situation include a modal cell volume of 1250 μm³ and an average sedimentation velocity of 11.4 mm/h/g for FSa cells from pulmonary nodules, compared to modal volumes of 980 and 1620 μm³ and sedimentation velocities of 10.7 and 15.1 mm/h/g for FSa cells from solid tumours and in vitro cultures, respectively (Grdina et al., 1978a). These differences cannot be explained solely by variations in the cell-cycle distribution of each of these populations; since FSa cell suspensions from pulmonary nodules and solid tumours exhibit similar DNA histograms by FMF analysis (Grdina et al., 1977, 1978a).

Little if any variation in CFE or contamination with normal cells was
Fig. 6.—Representative DNA histograms obtained by FMF analysis of an unseparated tumour-cell suspension (USC) and fractions of cells separated from that suspension by centrifugal elutriation (F2–F10) following in vivo exposure to 3 doses of HU.

TABLE II.—Distribution of HU-Treated (3/1|mg|g) cells in various phases of the cell cycle after centrifugal elutriation (Determined by FMF analysis)

| Fraction number | \(G_1(N)\) | \(G_1(T)\) | \(S(T)\) | \(G_2+M(T)\) | \(CV\) of \(G_1(T)\) peak |
|-----------------|--------|--------|--------|------------|------------------|
| USC             | 36     | 73     | 22     | 5          | 6.2              |
| 2               | 71     | 94     | 6      | 0          | 5.7              |
| 3               | 59     | 86     | 11     | 3          | 6.0              |
| 4               | 13     | 84     | 11     | 5          | 5.2              |
| 5               | 14     | 77     | 20     | 3          | 5.3              |
| 6               | 15     | 65     | 27     | 8          | 5.8              |
| 7               | 18     | 52     | 34     | 14         | 7.4              |
| 8               | 22     | 37     | 48     | 15         | 6.3              |
| 9               | 28     | 29     | 55     | 16         | 7.1              |
| 10              | 24     | 17     | 56     | 17         | 8.0              |
malignant cells in tumour-cell suspensions. Studies on the effect(s) of host cells on tumour growth and kinetics might be facilitated in this way.

FSa cells, synchronized by centrifugal elutriation following growth as pulmonary nodules and injected into recipient mice (i.e., Procedure A), responded to a single i.p. dose of HU in a manner similar to that described for FSa cells separated from exponential cultures (Grdina et al., 1979). HU was administered 20 min after tumour-cell injection in this procedure, because at this time >95% of the injected cells are retained in the lungs (Grdina et al., 1978b). As described in an earlier report, cell killing by HU was strongly correlated with the percentage of cells in S phase, as determined by FMF analysis (Fig. 4).

The use of Procedure A for assessing the cycle-dependence of cytotoxic agents in vivo is predicated upon direct cell killing of target tumour cells. A phenomenon that could preclude the usefulness of this method would be a drug-mediated effect on the host response to the injected tumour cells that might selectively (i.e., as a function of their position within the division cycle), affect their clonogenic capacity. Two extremely effective agents that are known to enhance CFE of injected tumour cells are radiation and cyclophosphamide. However, using mice pre-conditioned with either of these agents, both lung-retention patterns of 125I-labelled FSa cells and the CFE of unlabelled cells were found not to vary with either cell size or position within the division cycle (Grdina et al., 1978b). Thus, under these conditions Procedure A remains an effective method for characterizing the relative in vivo phase specificity of cytotoxic agents such as HU. The usefulness of this procedure is confirmed by the close agreement between data acquired in this manner and those derived from established in vitro methods (Grdina et al., 1980; Mehn et al., 1980).

The S-phase-specific cytotoxicity of HU to FSa cells was also demonstrable after in situ treatment of 13-day-old tumour lung nodules (i.e., Procedure B; Fig. 6). Cell killing was again correlated with the fraction of cells in S phase. No reduction in cell recovery was evident, either in the preparation of cell suspensions or by centrifugal elutriation. Because the treatment time exceeded the duration of $G_2 + M$ (Grdina, 1982) it was not surprising that the proportion of cells in this phase was diminished. FMF analysis was made difficult, however, by the bimodal nature of the fluorescence peaks describing the DNA distributions of the tumour populations (Fig. 6). This heterogeneity may have been due to a differential effect of HU on either the progression and killing and/or the stainability by mithramycin of cells in at least 2 distinct classes (i.e., with respect to DNA content) of FSa cells. Because of the relatively short exposure, it is unlikely that HU altered the DNA content of FSa cells. Rather, it is more likely that HU acted somehow to affect the stainability of a class of FSa cells by mithramycin. This phenomenon has been described in Chinese hamster ovary cells treated with bromodeoxyuridine (Swartzendruber, 1977). It is also interesting that the fluorescence peak describing the normal-diploid cells from each elutriator fraction was not affected by this treatment.

In conclusion, the phase-specific cytotoxicity of HU, administered in either a single- or multiple-dose schedule, on FSa cells from pulmonary nodules was described. In addition to measuring cytotoxicity, this procedure can be used to monitor and characterize perturbations in cell kinetics, both during and after therapeutic treatment. Target tumour systems can be single cells lodged in the lungs as well as pulmonary nodules of various ages and sizes. In this manner, various therapeutic modalities used either alone or in combination can be routinely and rapidly tested and characterized under in vivo conditions.

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