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Toxicity of 5-fluorouracil for aerobic and hypoxic cells in two murine tumours*

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Summary. Chronically hypoxic cells in solid tumours are reported to be more slowly proliferating than aerobic cells situated closer to blood vessels. Cycle-active drugs such as 5-fluorouracil (5-FU) might be expected to have greater activity against rapidly proliferating aerobic cells. The effect of 5-FU was therefore assessed against aerobic and hypoxic cells in murine tumours; this was done by giving the drug alone or 1 h before irradiation of the tumour under aerobic or hypoxic conditions. Aerobic and hypoxic cells appeared to have equal sensitivity to 5-FU in the KHT and 16/C tumours, and the effect of a single dose of 5-FU was additive to that of radiation.

Introduction

Many solid tumours are known to contain a subpopulation of hypoxic cells which are situated distant from functional blood vessels and which may have a low rate of cell proliferation [9, 15, 16]. Some anti-cancer drugs might be expected to show less toxicity for hypoxic than for aerobic cells in solid tumours. This may occur if drugs have limited penetration in tissue or if their toxicity is strongly dependent on the proliferative state of the target cells. Penetration of labelled or fluorescent drugs into tissue has been studied using spheroids, and experimental results suggest poor penetration of adriamycin, methotrexate and vinblastine, but uniform penetration of 5-fluorouracil [11, 14, 20]. All these drugs show greater toxicity for proliferating cells [17].

The effects of drugs against aerobic and hypoxic cells in experimental tumours have been assessed indirectly by comparing the effects of drug when used alone and when used with radiation. Thus, the effect of treatment on an unselected population of cells is compared with drug effects on a relatively pure population of hypoxic cells which survives radiation treatment. This type of experiment has shown selective killing of aerobic cells by the unstable drugs nitrogen mustard and BCNU, and by adriamycin, which penetrates tissue poorly; for cyclophosphamide selective toxicity for aerobic cells was found in only one of three tumours [4, 7, 8, 18]. Experiments reported below were designed to assess the relative toxicity of 5-fluorouracil (5-FU) and methotrexate for aerobic and hypoxic cells in murine tumours.

Materials and methods

The KHT sarcoma and the 16/C mammary carcinoma, which have been maintained by serial transplantation in syngeneic C3H mice (with periodic re-establishment from frozen stock), were used in most of the experiments. For generation of tumours a suspension containing about 10^5 cells was injected into the left hind leg of recipient mice. Palpable tumours appeared about 1 week later, and tumour size was estimated by passing the tumour-bearing leg through a series of holes of different diameters drilled in a lucite strip. This estimate of diameter has been calibrated previously against weight of the excised tumour, and estimates of tumour diameter by trained observers are reproducible to ± 0.5 mm.

Tumours were treated when the diameter of the tumour-bearing leg was in the range of 8–9 mm (tumour weight ~ 0.3 g), and response was assessed by growth delay. Treatment groups of six to nine mice were coded with ear tags to prevent observer bias in assessment of tumour size. The endpoint of response was the time from treatment for the tumour to grow to 1 g (equivalent to a diameter of 12.5 mm).

Standard clinical formulations of 5-FU (Roche) or methotrexate (Lederle) were diluted in sterile saline and injected i. p. into mice, using an injection volume of 0.01 ml/g body weight. Controls were injected with saline. Some groups of mice received 15 Gy X-irradiation to their tumour 1 h after the injection of drug. Radiation was delivered to tumours in awake, air-breathing mice, or to hypoxic tumours in mice that were anaesthetized with tribromoethanol. Hypoxia was induced by applying a clamp across the tumour-bearing leg for 5 min before and during irradiation [13]. The radiation source was a double-headed 100 kVp X-ray unit, which delivered a tumour dose at a rate of 11.4 Gy/min.

Because the KHT and 16/C tumours showed little or no response to methotrexate, the Lewis lung tumour was obtained from the NIH tumour bank, Frederick, Md, USA. This tumour has been reported to be sensitive to methotrexate in NIH screening assays [6]. Tolerated doses of methotrexate did not cause growth delay of this tumour in our laboratory. A subline of the Lewis lung tumour was

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therefore selected by sequential growth in syngeneic C57 mice, and in culture, which allowed the use of higher doses of methotrexate with assessment of response by tumour excision and colony formation. In this assay, the mice were killed 24 h after treatment, and a suspension of single cells was prepared by stirring for 30 min in 0.05% trypsin solution in saline. Serial dilutions of cells were plated in petri dishes in alpha medium +10% fetal calf serum. Stained colonies were counted about 10 days later.

Results

The relationship between the time for tumours to grow to a size of 1 g in test animals and in controls is plotted as a function of the dose of 5-FU for the KHT (Fig. 1) and 16/C tumours (Fig. 2). Growth delay increased up to a dose of 100 mg/kg body weight, but there was no increase in effect at 150 mg/kg. Doses of 100 or 150 mg/kg caused a mean growth delay of about 3 days for the KHT tumour and about 5 days for the 16/C tumour. In other experiments 5-FU was given to mice bearing the KHT tumour by three i. p. injections at 4-h intervals using doses of 20, 30, 40 or 50 mg/kg body weight per injection; this schedule was less effective than a single dose and led to growth delay in the range of 1–2 days. Toxicity increased with dose of 5-FU, and at 150 mg/kg body weight 5 of 66 animals (7.5%) died of toxicity; surviving animals had a mean weight loss of 2.6±0.4 g or 9% of their initial weight. Because of toxicity, doses of 5-FU greater than 150 mg/kg body weight were not used in these experiments.

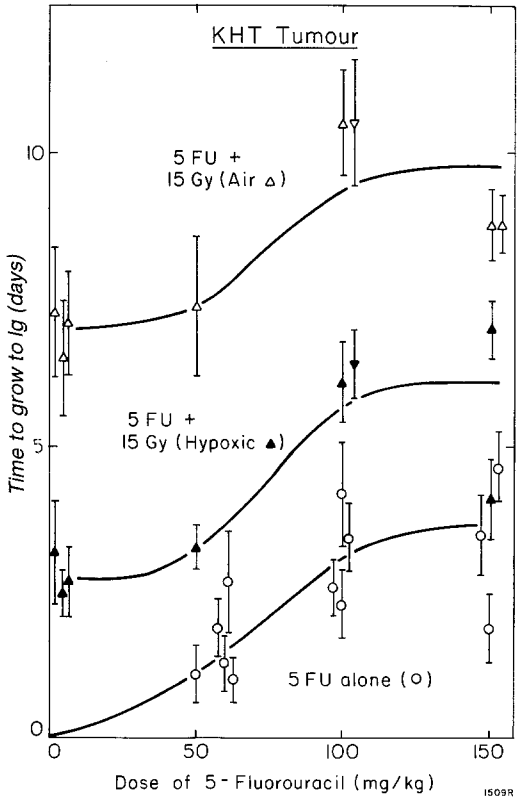

Fig. 1. Relationship between time for KHT tumours to grow to 1 g and dose of 5-FU. The drug was given alone (○), 0.5 h before (△) or after aerobic radiation (▽), or 0.5 h before (▲) or after radiation given under hypoxic conditions (▼). Means and their standard errors are indicated for groups of 6–9 tumours, and replicate results at the same dose level are from independent experiments

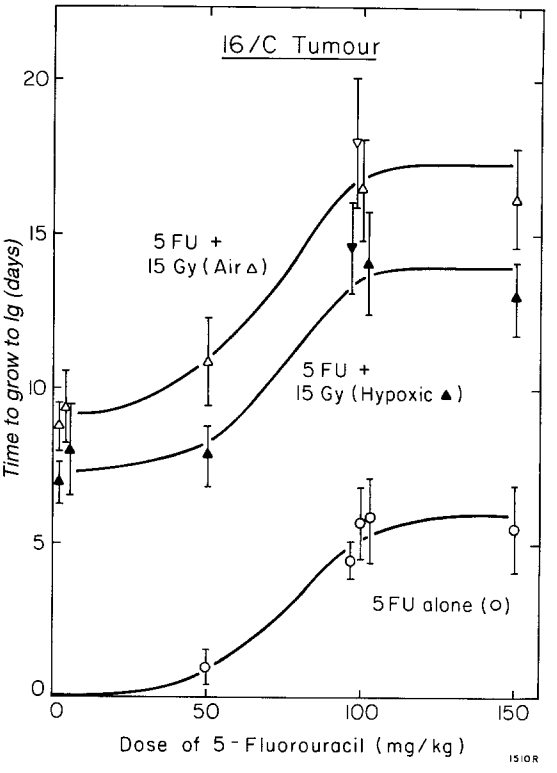

Fig. 2. Relationship between time for 16/C tumours to grow to 1 g and dose of 5-FU. The drug was given alone (○), 0.5 h before (△) or after aerobic radiation (▽), or 0.5 h before (▲) or after radiation given under hypoxic conditions (▼). Means and their standard errors are indicated for groups of 6–9 tumours, and replicate results at the same dose level are from independent experiments

As expected, radiation alone was more effective under aerobic conditions than under hypoxic conditions: the difference in growth delay was about 4 days for the KHT tumour and about 2 days for the 16/C tumour (Fig. 1 and 2, Table 1). A dose-response curve relating growth delay of the 16/C tumour to dose of radiation given under aerobic or hypoxic conditions has been published previously [18] and shows that the difference in growth delay is independent of radiation dose above about 10 Gy. The difference in growth delay observed under aerobic and hypoxic conditions is a measure of the proportion of aerobic cells in each tumour that is protected by application of the clamp to cause hypoxia during irradiation. If a drug were selectively toxic for the aerobic cell population, then the *difference* in effect between aerobic and hypoxic radiation administered after drug treatment would presumably be smaller, since there would then be a smaller proportion of aerobic cells to be protected by occluding the blood supply to the tumour. This was found previously for adriamycin [18]. In contrast, the difference in growth delay of KHT and 16/C tumours treated with aerobic or hypoxic radiation is independent of whether 5-FU was given prior to radiation, within the limits of experimental variation (Table 1). There was a trend for this difference to *increase* after 5-FU treatment of the 16/C tumour, but this result was not significant. There is no evidence that 5-FU is selectively toxic to aerobic or hypoxic cells in these two tumours.

Table 1. Differences in growth delay[a] for tumours treated with 15 Gy aerobic radiation compared with 15 Gy hypoxic radiation, under control conditions or at 1 h after various doses of 5-FU

Dose of 5-FU (mg/kg)	Difference in growth delay (mean ± SEM)	
	KHT tumour	16/C tumour
0	4.1 ± 1.3	1.8 ± 0.9
	4.0 ± 1.1	1.4 ± 1.8
	4.4 ± 0.7	
50	4.1 ± 1.3	3.1 ± 1.6
100	4.4 ± 0.9	2.4 ± 2.4
	4.1 ± 1.1[b]	3.4 ± 2.6[b]
150	1.8 ± 0.8[c]	3.2 ± 1.9
	4.7 ± 0.9	

[a] Growth delay is measured as the time interval from day of treatment (tumour weight ~ 0.3 g) to the day on which tumours attain a weight of lg

[b] For these groups, 5-FU was given 1 h after radiation

[c] Only for this group of animals did results differ significantly from concurrent controls which did not receive 5-FU. Since this result was not reproducible it is probably due to biological variation

The data shown in Fig. 1 and 2 also include experimental groups in which 5-FU was given 1 h after irradiation. Growth delay was similar to that observed when the drug was given before irradiation, so that single doses of 5-FU do not appear to sensitize the tumour cells to subsequent radiation. When given at 1 h before or after radiation treatment (under either aerobic or hypoxic conditions), the effects of 5-FU appear to be simply additive.

A variety of doses and schedules of methotrexate were used to treat the KHT tumour (single doses up to 120 mg/kg, and fractionated doses of 40 mg/kg × 3 and 25 mg/kg × 6). Growth delay varied from none to 3 days, with a mean of 1.5 days, and showed no clear relationship with dose. Methotrexate had no effect at all on growth of the 16/C tumour. In replicate experiments methotrexate gave no additional growth delay when used with radiation to treat the KHT tumour. Although this result might suggest that the limited effects of methotrexate are directed against the same (i. e. aerobic) population as is preferentially killed by radiation, the limited efficacy of the drug does not allow definitive conclusions.

Mice bearing the Lewis lung tumour received higher doses of methotrexate (3 injections of 100 mg/kg at 3-h intervals) given alone, or with irradiation of their tumour given under aerobic or hypoxic conditions at 1 h after the last injection of methotrexate. This dose of methotrexate would have been lethal to the mice, but they were killed after 24 h, before lethal effects of the drug were manifest. A radiation survival curve, with and without methotrexate, is shown in Fig. 3. Methotrexate had no detectable effect on the tumour, even at this high dose.

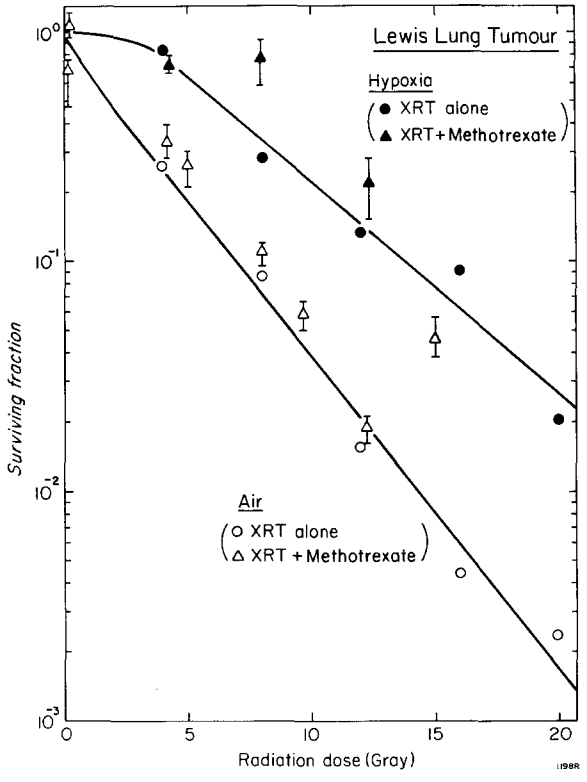

Fig. 3. Radiation survival curve for the Lewis lung tumour irradiated under aerobic *(open symbols)* or hypoxic conditions *(closed symbols)*, either alone (○, ●) or 1 h after the last of three doses of 100 mg/kg methotrexate (△, ▲). Mean and range of cell survival are indicated for triplicate dishes. Regression lines were fitted to data over the dose range of 4–20 Gy

Discussion

The present results indicate no selectivity of 5-FU for aerobic or hypoxic cells in two murine tumours. This result is surprising, since although 5-FU penetrates well into tissue [11] it has much greater toxicity for proliferating cells. A possible explanation is that the nutrient-deficient environment in hypoxic regions of the tumour renders cells more susceptible to the drug, even though studies in other tumours have indicated that chronically hypoxic cells have a low rate of proliferation. Alternatively, many of the cells which survive radiation treatment in these tumours might be acutely hypoxic due to rapid changes in blood flow, rather than chronically hypoxic (i.e. diffusion-limited) slowly proliferating cells.

The anti-tumour effects of 5-FU in the current experiments appear to be additive with those of radiation. When different schedules of 5-FU and radiation have been studied in animals, therapeutic outcome has been found to depend on schedule, and some schedules appear to give better results than the use of either agent alone in higher dosage [10, 19]. These effects may occur because treatment with one agent causes partial synchrony in the surviving population, but their relevance to human cancer is uncertain in view of the wide heterogeneity in cell cycle times of human tumours [17]. Chronic exposure of cells to 5-FU in tissue culture has been reported to increase the lethal effects of radiation [1]. This effect seems to occur even if 5-FU is given after radiation, and continuous infusion of 5-FU has given encouraging results when used in the clinical treatment of squamous cell carcinomas [3]. Thus, the results now obtained with single injections of 5-FU might not be obtained with chronic administration of the drug.

The methods used in the current experiments to determine drug effects against aerobic and hypoxic cells are indirect. They are based on comparisons of tumour response

to radiation under four conditions (aerobic or hypoxic, drug-treated or control), and because of biological variation they are subject to considerable error; thus, they cannot detect small degrees of drug selectivity for aerobic and hypoxic cells. Also, the endpoint of regrowth delay cannot distinguish between lethal and non-lethal effects on tumour cells. Recently a method has been described which allows drug effects to be studied at different depths from the surface of spheroids or at different distances from tumour blood vessels [2, 5]. This method is based on the establishment of a concentration gradient of the fluorescent stain Hoechst 33342, which in some spheroids and tumours can be achieved at a dose that does not kill cells. Cells from drug-treated spheroids or tumours can then be separated by flow cytometry, and survival curves can be generated for cells at different distances from the spheroid surface or from tumour blood vessels. A study of Chinese hamster V79 spheroids demonstrated that the inner cells were slightly more resistant to the effects of 5-FU. A much larger, and unexpected, effect was the greater resistance (by a factor of ≈ 7 in cell survival) in cells treated in intact spheroids than in cells treated after dissociation of spheroids [5]. 5-Fluorouracil has been reported to show good penetration of other types of spheroid [11], and the small increase in cell survival at increasing depth probably relates to a reduced rate of cell proliferation. The reason why cells in solid tissue appear to be more resistant to the drug than single cells remains unknown.

In vivo experiments using the Hoechst stain have shown selective toxicity of adriamycin for aerobic cells in a murine tumour, in agreement with results yielded by the present indirect methods [2, 18]. Limitations of the newer method are that the fluorescent stain may interact with therapeutic agents to increase toxicity [12], and changes in the distribution of patent blood vessels might occur between the time of drug administration and injection of the fluorescent dye. The new technique should be used to complement the present methods in studying regional effects of anti-cancer drugs, since variations in the concentration or activity of drugs within solid tumours may limit their effectiveness.

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