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Response of Cell Populations in Tumor Cords to a Single Dose of Cyclophosphamide or Radiation*

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Abstract—*We have examined tumor cords of hepatoma 3924A in rats treated by 15 Gy of X-rays or 150 mg/kg of cyclophosphamide. Parameters measured were: cord thickness, cellular density, [^{3}H]-TdR LI, MI and proportion of necrotic cells within the cord. With cyclophosphamide, a drop in cell density of 40% and in LI of 60% did not cause shrinkage of the cords. During the recovery phase, increase in cell number coincided with changes in the kinetics of the cell population adjacent to the capillary. For X-rays an approximately 10% decrease in cord thickness was associated with a rapid reduction in cell density and a relatively poor proliferative response of the cells adjacent to the vessel. There was little evidence for the sparing of remote cells with either agent. It did not appear that the progression of cells across the cord would be interrupted sufficiently for any 'spared' remote cells to participate in the therapeutic response.*

INTRODUCTION

THE NEOPLASTIC cells of a number of human and animal tumors grow as 'cords' that separate blood vessels from areas of necrosis. Thomlinson and Gray [1] showed for a series of human lung tumors that the thickness of tumor cords correlated well with the expected diffusion length of oxygen out from the subtending capillary. The histologically intact cells most remote from the vessel have been considered as candidates for the source of hypoxic radioresistance that can be demonstrated by radiobiological assay, e.g. in the inflection of curves of tumor growth delay after irradiation of air-breathing hosts [2]. Two sets of qualitative observations on dead cells in irradiated tumor cords suggested that there might indeed be 'sparing' of remote cells [3, 4].

Tannock [5] found for a murine mammary carcinoma that the population of cells remote from the capillary had a growth fraction only half that of the population adjacent to the blood supply. Therefore a greater proportion of the remote cells might also be expected to be resistant to the action of cell cycle-active cytotoxic drugs.

Using the rat hepatoma 3924A, we have examined the cellular kinetic and histological response of cells within tumor cords to single doses of X-irradiation and the alkylating agent cyclophosphamide, whose action is held to be greater against cells that are in cycle [6]. The dose of each agent was chosen on the basis of previously published growth delay curves for single doses [7, 8] as being the approximate point at which the curves inflected to a shallower slope.

MATERIALS AND METHODS

Animals and tumor

Female rats of the inbred ACI strain (Laboratory Supply Co., Indianapolis, IN) were used at a weight of 120–140 g. The rats were caged individually in a room lighted from 08.00 to 20.00 hr and were provided with rat chow and water *ad libitum*. Implants of the Morris hepatoma 3924A were made by trocar injection subcutaneously into the dorsal flank of the rats. Tumor size was measured with calipers and

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animals were treated when their tumor reached a mean diameter of 15 mm.

Radiation

Tumors were irradiated with 250 kV X-rays filtered by 0.5 mm of Cu and 1 mm of Al (15mA GEC Maximar III unit). A single dose of 15 Gy was delivered at a dose-rate of 2.45 Gy/min. The body of the ether-anesthetised host was shielded by lead.

Drug

Cyclophosphamide (CY; Mead-Johnson, Evansville, IN) was prepared in sterile saline and given by intraperitoneal injection as a single dose of 150 mg/kg body weight ($\frac{2}{3}$ the approximate $LD_{10/30}$ dose in these rats).

Experimental

Between 12 hr and 2 weeks after CY or X-rays, and 1 hr prior to killing, 3 treated or control rats were injected with 50 μCi of tritiated thymidine ([^{3}H]-TdR, sp. act. 3 Ci/mmol; Schwarz-Mann, Orangeburg, NY). Tumors were excised and fixed in neutral formalin. Autoradiographs of 5 μm tumor sections were prepared and the cell nuclei were stained by the Feulgen reaction.

Tumor cords were scored which were cut through in longitudinal section and for which the capillary lining and the row of pyknotic cells adjacent to the necrosis were approximately parallel (a photomicrograph is given in ref. [9]). Cord thickness was measured between the capillary endothelium and the first pyknotic cell at the viable/necrotic interface. The tumor cord was divided arbitrarily into 2 zones of 200 μm (parallel to the vessel) by 62.5 μm (at right angles to the vessel). One zone was adjacent to the capillary ($zone_{adj}$), while the other contained those cells remote from the capillary and adjoining the necrotic area ($zone_{rem}$). Parameters measured for each zone were: (1) [^{3}H]-TdR labelling index (LI); (2) mitotic indices for intact and aberrant (pyknotic or multipolar) mitoses (MI); (3) cellular density (CD), i.e. the number of histologically intact tumor cells within the zone; (4) necrotic index (NI), i.e. the ratio of number of necrotic cells (severely pyknotic or karyorrhectic) to all cells (intact plus necrotic) *within* the cord. The value of each parameter was calculated for an individual cord, then means and standard errors were obtained for 30 cords from 3 tumors (in total, 1500–2000 cells per datum point per zone).

RESULTS

Groups of 10 animals were irradiated with 15 Gy of X-rays or injected with 150 mg/kg of CY and the delay in tumor growth to 4 times initial volume was measured with respect to a group of 10 untreated controls (time to 4 vol. = 4.1 ± 0.7 days). The growth delay after X-rays was 18.4 ± 1.5 and after CY was 12.8 ± 0.5 days. During the delay period the tumors did not regress but remained at, or slightly above, treatment size.

Cord thickness

Mean cord thickness remained constant (120, 124 μm) in groups of control tumors whose volume differed 9-fold (Table 1). Cord thickness did not decrease below control levels after treatment with CY. The cords in irradiated tumors showed some evidence of shrinkage, but by no more than 10% (Fig. 1A,C).

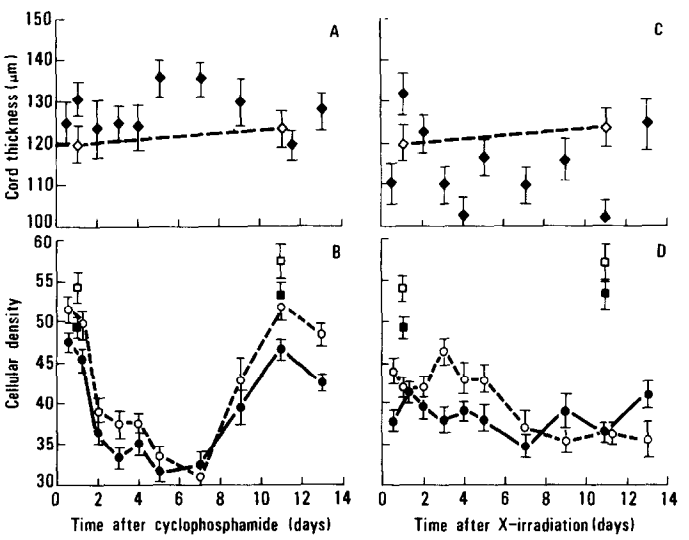

Fig. 1. (A,C) Radial thickness of tumor cords in hepatoma 3924A at different intervals after a single dose of 150 mg/kg of CY (◆, 1A) or 15 Gy of X-rays (◆, 1C). Untreated controls (◇, dashed lines). All errors as ± 1S.E. (B,D) Cellular density in $zone_{adj}$ (●, solid line) and $zone_{rem}$ (○, dashed line) after CY (1B) or X-rays (1D). Untreated controls (■, $zone_{adj}$; □, $zone_{rem}$).

Cellular density

Mean values for CD in control tumors (Table 1) were in reasonable agreement with previous measurements (58.9_{adj}, 57.5_{rem}; ref. [9]). From the values for CD and cord thickness it was calculated that 6–7 layers of tumor cells spanned the cord.

Table 1. Values of histological and cellular kinetic parameters in cords of untreated 3924A tumors of different size

Parameter	Mean ± 1S.E.	
Tumor diameter (mm)	16.5 ± 2.6	34.5 ± 5.9
Cord radius (μm)	120 ± 4	124 ± 4
First row Cell density	46.3 ± 2.3	59.8 ± 2.5
Last row	47.5 ± 1.7	59.8 ± 2.9
ADJ Cell density	48.9 ± 1.0	53.3 ± 1.5
REM	53.7 ± 1.3	57.6 ± 2.0
First row Percentage labelling index	44.6 ± 5.3	42.0 ± 2.8
Last row	11.6 ± 3.4	4.2 ± 1.6
ADJ Percentage labelling index	37.0 ± 2.0	31.0 ± 1.8
REM	17.0 ± 1.6	9.8 ± 1.8
ADJ Percentage mitotic index	1.60 ± 0.43	2.00 ± 0.51
REM	0.80 ± 0.26	0.56 ± 0.22
ADJ Necrotic cells	1.1 ± 0.3	1.4 ± 0.2
REM	1.8 ± 0.2	2.5 ± 0.5

Between 1 and 2 days after CY a sharp drop occurred in the number of intact cells per zone. The major recovery phase began at 7 days and was complete by 11 days. The radiation response was markedly different, with a reduction in CD at 12 hr and no recovery even by 13 days. For both agents the patterns of response in $zone_{adj}$ and $zone_{rem}$ were similar (Fig. 1B,D).

The division of a cord into 2 compartments is, of course, artificial. However, we have analysed separately the characteristics of the row of cells immediately adjacent to the capillary and the last complete row before the necrotic zone (Table 1; Fig. 2). In terms of oxygenation, growth fraction and drug availability, these would be expected to be respectively the cells most and least sensitive to radiation and chemotherapy. From Fig. 2 it is apparent that the two-zone analysis reflects reasonably well the behaviour of these two 'extreme' cases.

Labelling and mitotic indices

Taking the mean of the 2 control groups (Table 1), the relationship between LI and MI in the 2 zones was the same (19:1 in $zone_{adj}$, 20:1 in $zone_{rem}$). The ratio of LI_{adj}/LI_{rem} was 2.5:1 and MI_{adj}/MI_{rem} was 2.6:1.

In CY-treated tumors the initial response was a suppression of mitosis (12-hr point, Fig. 3B). The subsequent wave of mitosis coincided with a rapid fall in the number of intact interphase cells (Fig.

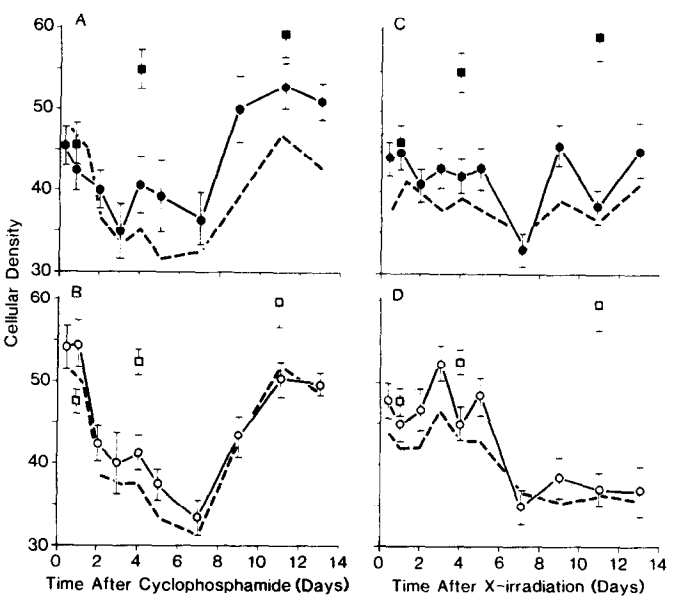

Fig. 2. (A,C) Cellular density of the row of tumor cells adjoining the blood vessel (●, solid line) compared to the mean CD of $zone_{adj}$ (dashed line) after CY (2A) or X-rays (2C). (B,D) Cellular density of the last row of cells before the necrotic zone (○, solid line) compared to the mean CD of $zone_{rem}$ (dashed line) after CY (2B) or X-rays (2D). Untreated controls, first row (■), last row (□).

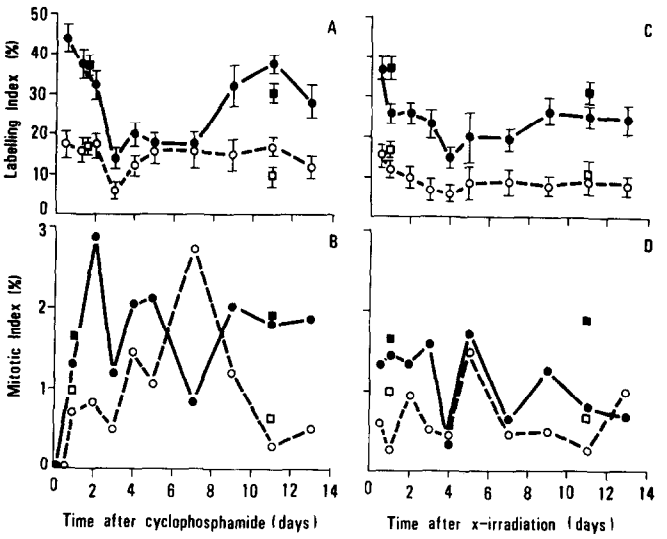

Fig. 3. (A,C) One-hour [^{3}H]-TdR labelling index at different intervals after CY (3A) or X-rays (3C). Symbols as for Fig. 1 (B,D). (B,D) Mitotic index for histologically intact mitotic figures in cords treated by CY (3B) or X-rays (3D). Errors have been omitted for clarity but were approximately 25% of the mean for zone$_{adj}$ and 40% of the mean for zone$_{rem}$. Symbols as for Fig. 1 (B,D).

1B). Although LI_{rem} was restored to pretreatment level by 4–5 days, appreciable repopulation of the cord did not occur until after 7 days, coincident with the increase in LI_{adj} (Figs 1B, 3A). Cords irradiated with 15 Gy of X-rays showed no initial (12 hr) major suppression of intact mitotic figures. Recovery in LI and MI was not so marked after X-rays as after CY (Fig. 3A, C). Even by 13 days, LI_{adj}, MI_{adj} and LI_{rem} remained lower than in controls, and little cellular repopulation occurred within this period (Fig. 1D). The LIs for the first and last rows of cells have been measured separately. The values for controls were approximately 30% higher (zone$_{adj}$) and 30% lower (zone$_{rem}$) than the average for their zone (Table 1). Nevertheless, as for CD, the pattern of temporal response to treatment was essentially the same as shown in Fig. 3 (A,C).

Necrotic cells

Pyknotic and karyorrhectic nuclei were present in zone$_{adj}$ of untreated tumors, comprising 1 cell per 50 interphase cells. Within zone$_{rem}$ the proportion was about 2 in 50 (Table 1).

After CY there was little additional cell death until the recovery of mitosis between 1 and 2 days (Fig. 4A). The subsequent failure to produce viable daughter cells would largely account for the concurrent fall in CD. The number of necrotic cells did not return to control levels immediately, either because further deaths occurred (note the persistence of aberrant mitoses; Fig. 4B) or because liquefaction of necrotic cells was slow, or both. Dead cells appeared more rapidly in irradiated cords in which mitosis was not initially so completely suppressed (Fig. 4C,D; Fig. 3D). For both agents the proportion of dead cells in zone$_{rem}$ at early intervals was the same as, or greater than, that in zone$_{adj}$.

DISCUSSION

Tannock [10] calculated the expected oxygen diffusion radius (R_O, venous) for 5 tumors in rats, based on rates of oxygen consumption by the tumors, and obtained values between 100 and 330 μm. The measured thickness of tumor cords in hepatoma 3924A was 120 μm, in agreement with this calculation and with previously measured values for cord thickness in rodents (cited in ref. [10]).

Durand and Sutherland [11] demonstrated that hypoxic cells in multicellular spheroids entered a G_1-like state and it has been shown for tumor cords of murine mammary carcinomas that the LI fell as the cord was traversed (cf. Fig. 3, Table 1), with a proportionate fall in the growth fraction, from 100 to about 50% [5, 12]. Rapidly proliferating populations of mammalian cells are up to 15 times more sensitive to CY than slowly- or non-proliferating populations [6]. On kinetic grounds, therefore, a greater proportion of the cells in zone$_{rem}$ of 3924A cords might be more resistant to the action of CY, even assuming that a significant proportion of the drug is not metabolised by intervening cells.

Lacking a clonogenic assay for the different cell populations in tumor cords (*in vivo*; see below), evidence for or against the therapeutic importance of the remote cells must rest on inferences from cellular kinetic and histological studies. The hypothesis that the remote cells are spared in

treatment with high doses of X-rays or CY prompts 2 questions: (A) Is there histological evidence for sparing of such cells, as had been suggested by earlier, qualitative studies [3, 4]?; and (B) Is the dynamic balance of the cord disturbed to such an extent that any spared cells could influence the therapeutic response of the tumor?

For both agents the initial large decreases in cell numbers and the increase in numbers of aberrant mitoses was complete by 2 or 3 days (Figs 1, 4). In neither case could we demonstrate a markedly smaller effect on the remote cells in terms of CD. For CY, both CD_{adj} and CD_{rem} were at 90% of their control values by 12 hr and 62% at 3 days. For X-rays the values were 72% at 12 hr, 75% at 3 days. The observation that mitosis was suppressed in $zone_{rem}$ 12 hr after CY makes it probable that the drug was reaching these cells (Fig. 3B). The rise in numbers of necrotic cells as a proportion of control levels was somewhat greater for $zone_{adj}$ of CY-treated cords than for $zone_{rem}$. This may be an indication of 'sparing', but numbers at the 2–3 day peak were not significantly different (Fig. 4A). For murine mammary carcinomas irradiated by X- or γ-rays, it was reported that 6–12 hr after treatment numerous pyknotic cells appeared that were confined to $zone_{adj}$ [3, 4, 13]. Our quantitative results for necrotic cells 12 or 24 hr after X-rays do not confirm these observations, nor do the numbers at peak suggest a reduced effect in $zone_{rem}$ (Fig. 4C).

With regard to progression across the cord, there are 6–7 layers of intact tumor cells in untreated cords of 3924A. A cohort of cells takes 52–56 hr to traverse the cord from vessel to necrosis (Moore, to be published), suggesting that the normal lifetime of the last row of intact cells is only 7–9 hr, before they enter the necrotic zone. In treated tumors this dynamic balance could be disturbed by a number of mechanisms, e.g. through failure to produce new cells so that remote cells do not move across the cord/necrosis boundary, and/or through autolysis of killed cells, resorption of products into the vasculature and subsequent inward collapse of the cord. In CY-treated cords mitosis was completely suppressed for only 12 hr. Thereafter the cells resumed division (Fig. 3B). Much of the subsequent proliferation was plainly abnormal in that, despite a residual LI_{adj} of 15–20% and a near-normal MI_{adj}, there was no recovery in the number of intact cells until 7 days. Nevertheless, necrotic cells resulting from aberrant mitoses remained space-occupying throughout the long period of measurement (Fig. 4A). The cords did not shrink (Fig. 1A), as might have occurred had there been rapid removal of dead cells together with immediate and prolonged suppression of proliferation of cells in $zone_{adj}$. It seems improbable that remote cells would have been 'saved' by being brought passively into closer apposition with the vessel, as has been postulated for other tumors [4], although the possibility of active inward migration cannot be discounted. After 7 days both zones repopulated rapidly and apparently 'simultaneously' (assuming normal kinetics were restored, the lag time between corresponding points in the 2 zones would be less than 28 hr). Slight shrinkage of cords did occur in irradiated tumors over the 2-week period of measurement, although the data are scattered (Fig. 1C). There was little mitotic delay (Fig. 2D) and the indices of proliferation in $zone_{adj}$ were never less than 25–50% of the control (Fig. 3C,D). Unlike CY, though, the LI and MI between 9 and 13 days remained lower than normal. This may be merely a measure of the longer growth delay induced by the radiation dose. However, Tannock and Howes [4] observed that cords of a murine mammary carcinoma failed to return to control thickness for at least 7 days after 20 Gy of X-rays, by which time the tumor had resumed gross regrowth. They speculated that a new equilibrium in cord thickness might have been established, perhaps as a consequence of damage to vasculature or alteration of blood flow in irradiated tumors.

The limitations of these descriptive assays of cytotoxic injury must be recognised. The argument for sparing of subpopulations in cords requires that a single administered dose result in different effective doses between zones. Can such differences be recognised in terms of morphological/kinetic parameters? Moore and Dixon [14] examined the effect of graded, administered

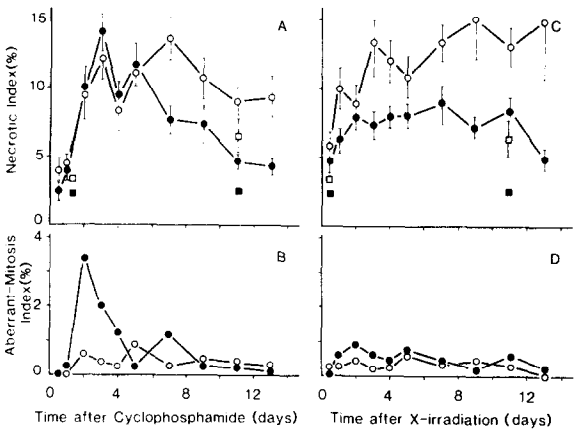

Fig. 4. (A,C) Necrotic index in $zone_{adj}$ (●) and $zone_{rem}$ (○) after CY(4A) or X-rays (4C). Untreated controls, $zone_{adj}$ (■), $zone_{rem}$ (□). (B,D) Mitotic index for aberrant mitotic figures in cords treated by CY (4B) or X-rays (4D). Symbols as for A,C. No aberrant mitoses were observed in controls.

doses of CY in a mammary carcinoma of the rat in terms of relative LI, MI and CD and of survival of clonogenic cells. Changes in MI most closely approximated the dose–response curve of clonogenic cells, and dose differences of 25 mg/kg were detectable. However, the MI curve still overestimated clonogenic survival, as did LI. Cellular density was poorly dose-responsive. From data collated by Steel [15], a dose of 150 mg/kg of CY would be expected to reduce the surviving fraction of clonogenic cells in solid tumors of rodents to somewhere between 5×10^{-2} and 5×10^{-4}. Similarly, 15 Gy of X-rays given 'in air' might yield a surviving fraction between 5×10^{-2} and 1×10^{-4} [15]. These values are at least 1 log lower than those obtained by calculating relative LI, MI and CD in 3924A cords (from Figs 1, 3). A closer relationship between morphological and clonogenic response may exist in the time at which, for example, LI and CD recover to control levels (between 7 and 11 days for both $zone_{adj}$ and $zone_{rem}$ after CY; Fig. 1), which coincides closely with the later stages of clonogenic recovery after treatment [16]. In turn, the time taken for clonogenic cell number to recover to the pretreatment level is markedly dose-dependent, both for drugs [17] and radiation [18]. Similar limitations apply when using NI (15% at peak; Fig. 4) to infer the behaviour of clonogenic cells. Large changes in the gross response of a tumor are certainly reflected by changes in NI. For example, growth delay after irradiation of a murine mammary carcinoma was enhanced 2-fold by the hypoxic cell sensitizer misonidazole and the NI of cords in the tumor was increased by the same factor [19]. However, the relationship between the two types of assay is unlikely to be simple and relative dose-responsiveness has yet to be examined.

The tumor cord model is important in that it attempts to relate radiobiological phenomena (sensitivity and resistance) to spatially distinguishable populations in histological specimens. The present study failed to detect major differences in the morphological/kinetic responses of cell populations within cords at doses of agent where growth delay curves would predict regrowth from 'resistant' populations. It seems reasonable to assume that many of the cells adjacent to vessels of cords will be clonogenic, but studies with *in vitro* tumor spheroids have shown that many (up to 85%) of the histologically intact remote cells are irreversibly non-clonogenic, even when transferred to an optimal growth environment [20]. There have been relatively few *in vivo* studies that test whether cords, or the areas in which cords are found, are relevant to the outcome of therapy. A careful study of human cervical tumors revealed that for a given clinical stage, tumors with larger average intercapillary distances and greater superficial necrosis had a higher probability of recurrence [21]. On the other hand, the results of 3 experimental series suggest that regrowth of heavily irradiated tumors with necrotic cores occurs from the well-vascularised tumor rim [22–24]. Thus the important question of the location(s) and nature of the treatment-resistant cells from which tumors must often regrow remains to be resolved.

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