Jpn. J. Cancer Res. (Gann), 79, 236–243 ; February, 1988

The Dynamics of Tumor Cords in an Irradiated Mouse Mammary Carcinoma with a Large Hypoxic Cell Component

James V. Moore
Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, England

The tumor cord model represents a histologically based framework for interpretation of radiobiological phenomena, particularly the resistance to radiation conferred by absence of oxygen. For the mammary carcinoma T50/80 grown in B6D2F1 male mice, average oxygenation was poor, based on tumor growth delay after irradiation. There was no improvement in radiobiological oxygenation for several days after a high dose of radiation. This was consistent with events in the cords of the tumor, where although up to 20% of all cells became pyknotic by 8 hr, the cords did not shrink for at least 2 days. The cellular kinetics of populations of intact and dead cells, adjacent to and remote from the capillaries of the cords, were examined for up to 60 hr after irradiation and it was found that: (i) before treatment, average LI (adjacent) was 13% and LI (remote) was 2%, (ii) after irradiation, cells expressed pyknosis after passing through the S phase of the cell cycle, so that (iii) at early intervals there was a larger proportional rise in pyknotic cells in the adjacent than the remote zone. However, (iv) at later intervals there was always a higher proportion of dead cells in the remote zone.

Key words: Mouse — Mammary carcinoma — Tumor cords — X-rays — Cell kinetics

The tumor cord model[1, 2] provides a histological basis for the radiobiological inference that tumors contain fractions of 'well-oxygenated' cells (believed to exist near the tumor capillaries) and 'hypoxic' cells (thought to predominate in regions more remote from the vessels and adjacent to gross necrosis). Dynamic events in cords of irradiated tumors have been proposed to underlie the radiobiological phenomenon of reoxygenation after irradiation. Tannock and Howes[3] reported for a mouse mammary carcinoma in air-breathing animals, that extensive cell degeneration occurred 6 to 12 hr after irradiation, and was confined to zones of the cord near the blood vessel. Shrinkage of the cords occurred promptly, so that 12 hr after 20 Gy of gamma-rays the average cord width was only 50% that before treatment. Thus, even the most remotely-placed surviving cells would quickly come into closer proximity to the subtending vessel of the cord, which, providing conditions in the vessel remained unchanged, might result in an improvement in oxygenation of such cells. However, a different pattern of events was described in preliminary form by Moore,[4] for the mouse mammary carcinoma T50/80. In that tumor, no shrinkage of the cords occurred within 48 hr of irradiation and the distribution of histologically dead cells was a function of the time of assay. The present report describes the *in situ* radiobiology of T50/80 and examines the cellular events in the irradiated cords.

Materials and Methods

Mice Nine- to 10-week-old male mice of the B6D2F1 strain (the F1 hybrid of the cross of sib-mated lines C57B16×DBA2; Paterson Institute strains) were used. Mice were housed under a 12 hr dark (1800 to 0600 hr), 12 hr light regimen, and were provided with food and water *ad libitum*.
Tumor The poorly differentiated mammary carcinoma T50/80 arose in a 9-month-old female B6D2F1 mouse and has been passaged in male B6D2F1 mice before storage in liquid nitrogen. All experiments to be described were carried out with tumors of the 3rd or 5th passage generation only.
Irradiation Single or split doses of 290 kV X-rays (12 mA, HVL=2.3 mm Cu) were used at a dose rate of 4.9 Gy/min. Single doses were given at 1000 to 1100 hr, as was the first dose (22.5 Gy) in the split regimen, the second (12.5 Gy) being given 0.25 to 120 hr thereafter. During irradiation, the unanesthetized mice breathed room air, and the

tumor either lay freely in the beam path ('air') or had its blood supply occluded by a rubber press 15 min before and during irradiation ('clamped'). To determine further the radiobiological oxygenation status of the tumors, 1 mg/g body weight of the hypoxic cell sensitizer misonidazole (MISO; Roche, Welwyn Garden City) was injected intraperitoneally into mice either 30 min before or after 'air' irradiation.

Experimental The gross response of the tumor to irradiation under clamped, air, and misonidazole-sensitized conditions, was measured by the tumor growth delay assay.[5] Tumors were irradiated at a mean volume of 100 mm^3 and the time taken to grow to a size of 500 mm^3 was measured. In any single experiment six mice were used per experimental point, but most were repeated at least once and the data pooled, so that data points shown hereafter are usually for 12 to 18 mice. Growth delay was calculated as the time for individual treated tumors to reach 500 mm^3, minus the time for untreated tumors. From the individual delays, a mean delay ± 1 standard error was calculated for each radiation dose. For the split-dose experiments, mice were first treated at a size of 100 mm^3 and overall growth delay was calculated relative to this time of first treatment.

The histological response of the tumor to irradiation (a single dose of 35 Gy, in air) was measured in tumors of 500 mm^3 volume at the time of treatment. Two sets of experiments were carried out, with 3rd generation and with 5th generation tumors. In each experiment, two mice were sacrificed at intervals of 1 hr to 120 hr after irradiation. One hour prior to sacrifice, the mice were injected intraperitoneally with a single dose of 0.5 μCi/g of tritiated thymidine ([^{3}H]TdR, 21 Ci/mmol; Amersham). Tumors were fixed in three parts ethanol to 1 part glacial acetic acid, and 5 μm-thick sections were prepared and stained with hematoxylin and eosin. In each 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 gross necrosis were approximately parallel. Cord 'radius' was measured as the distance between the capillary endothelium and the first pyknotic cell at the viable/necrotic interface, for 100 cords in each tumor. From these 100 cords, the twenty whose radius fell most closely around the mean value were further analyzed. Within each cord, two zones of 100 μm parallel to the vessel, by 25 μm at right angles to the vessel, were scored. Zone '1' was directly adjacent to the capillary, and zone '2' adjoined the necrotic area. In tumor cords, values for the various cellular parameters to be described below have been shown to be a continuum of which these two zones represent the extremes.[6] Parameters measured for each zone were: (i) pyknotic index (PI), i.e. the number of cells with pyknotic or karyorrhectic nuclei, relative to all histologically intact plus dead cells; (ii) mitotic index (MI), the number of mitotic cells relative to all intact cells; (iii) [^{3}H]-TdR labeling index (LI), the number of (a) labeled histologically intact cells relative to all intact cells, or (b) labeled dead cells relative to all dead cells; (iv) the number of intact cells per zone, measured by counting the absolute number of cells with rounded, basophilic nuclei containing nucleoli. Measurements of cell number and sectional area were made using a semi-automatic image-analysis system (MOP-Videoplan, Zeiss, Welwyn Garden City). Data for the two tumors per generation were pooled. Thus individual data points to be shown later are for 40 cords and approximately 1500 to 2000 cells.

Results

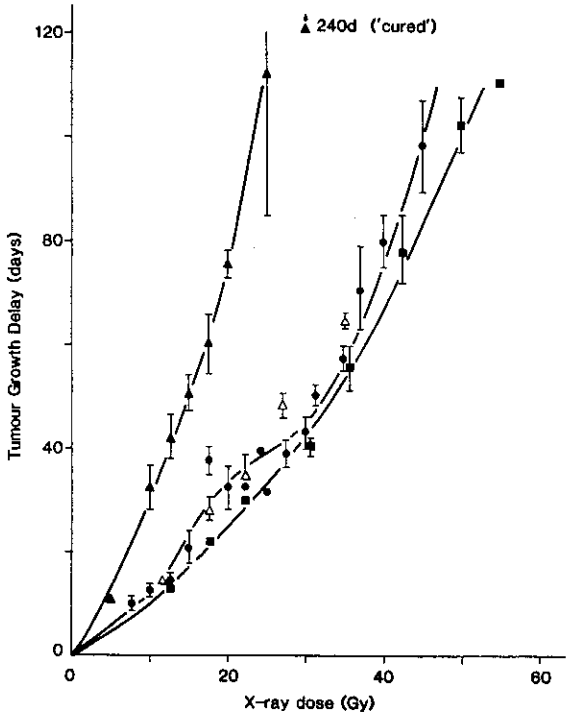
Tumor Growth Delay Curves of delay after single doses of X-rays are shown in Fig. 1. Smooth curves were fitted to the data by polynomial regression, with 4 degrees of regression. The mean ratio of doses for given growth delays can be summarized as:

(1) X-ray (clamped)/X-rays (air)
1.13 (P=0.73)

(2) X-rays (clamped)/MISO-X-rays (air)
2.22 (P=0.01)

(3) X-rays (clamped)/X-rays-MISO (air)
1.20 (P=0.42)

Noteworthy was the low average radiobiological oxygenation status of T50/80 tumors in air-breathing hosts (group 1; insignificantly different from the clamped values, by analysis of variance) and the large, highly significant sensitization by MISO given before (group 2), but not after (group 3), irradiation. There was some scatter of the data for X-rays alone 'in air' around 17 to 25 Gy, and the fitting program has drawn a kink in the curve at these points (note that at doses lower than these, the mean ratio of doses 'clamped/air' was still small; 1.23). It is commonly assumed that this kink in growth delay curves represents a transition to regrowth from a population of lower average radiobiological oxygenation. For the T50/80 tumor a dose of 22.5 Gy would be sufficient to reduce the tumor cell population to this level, hence the choice of this dose for the first irradiation in the split-dose experiment. Growth delays after split doses are shown in Table I. After a 22.5



Gy first dose, tumor shrinkage commenced only after 48 hr and reached 25% of the pre-treatment volume at 120 hr. Some slight sparing of the tumor (lesser growth delay) was evident between 3 and 48 hr interval between the first and second doses but, notably, there was no evidence for major changes in the relationship of the effects of air and clamped treatments.

Tumor Cords Major shrinkage of tumor cords in irradiated T50/80 did not occur until after 48 to 60 hr; only by 120 hr was average cord radius reduced to 50% of that of controls (Fig. 2a). Subsequent detailed histological and kinetic analysis was confined to the first 60 hr, i.e. when the overall cord structure and size remained relatively unchanged.

Fig. 1. Delay in growth of mammary carcinoma T50/80 as a function of radiation dose, given under two different conditions of oxygenation or with the radiation preceded or followed by injection of misonidazole. Inter-animal variation (bars on data points) is calculated as ±2 SE. (■) X-rays, tumor clamped; (●) X-rays, tumor 'in air'; (▲) Misonidazole, 30 min, X-rays in air; (△) X-rays in air, 30 min, misonidazole.

Table I. Delay in Growth of T50/80 as a Function of Radiation Dose

Dose (Gy)	Interval between doses (hr)	Relative tumor volume at time of 2nd dose	Growth delay (days)		
			air	clamped	*P* value of difference
0 (untreated or clamped only)	—	1.00±0.20	0.0±0.1	0.0±0.2	
12.5×1	—		14.8±1.5	14.1±1.0	0.36
22.5×1	—		33.1±0.9	30.1±0.3	0.12
35.0×1	—		62.3±1.2	56.1±3.7	0.080
22.5+12.5	0.25	1.00±0.21	64.5±3.6	65.1±0.9	0.28
〃	3.0	0.99±0.16	47.3±2.3	47.2±3.1	0.49
〃	6.0	1.00±0.19	50.6±1.5	49.2±1.0	0.23
〃	12.0	1.01±0.20	44.8±1.8	40.3±1.5	0.05
〃	24.0	1.02±0.22	42.6±2.6	49.4±1.5	0.02
〃	36.0	0.97±0.28	57.9±3.6	46.7±1.2	0.009
〃	48.0	0.83±0.23	51.4±2.9	47.1±1.8	0.12
〃	72.0	0.62±0.24	60.4±2.4	58.3±0.8	0.21
〃	120.0	0.25±0.14	68.2±3.3	63.8±2.5	0.16

Radiation was given either as a single dose, or as a first dose of 22.5 Gy 'in air,' followed at various intervals by a second dose of 12.5 Gy (given either in air or clamped). Errors are ±1 SE. Values for *P* indicate the probability that growth delays for air and clamped treatments are significantly ($P<0.05$) different.

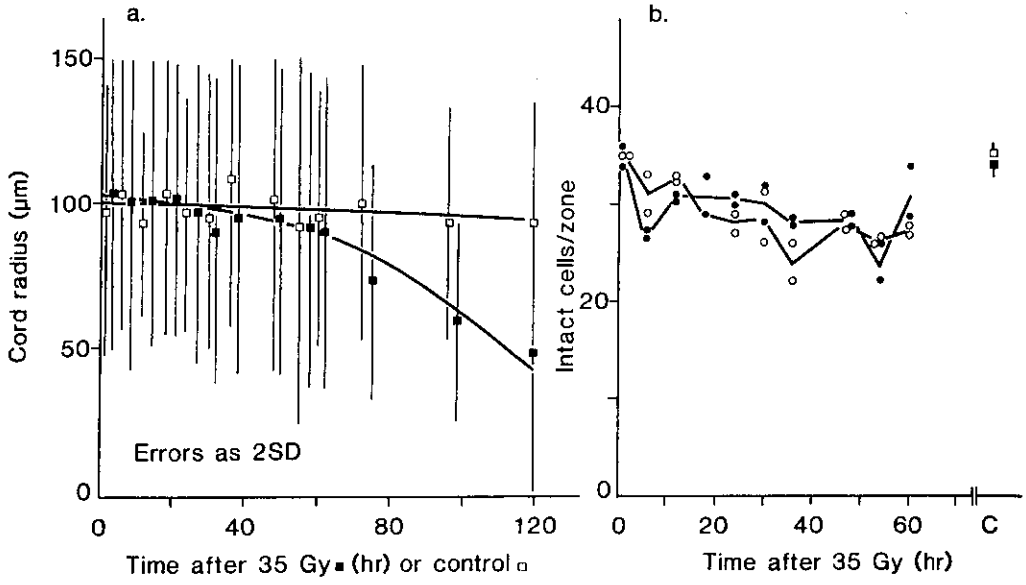


Fig. 2. (a) Average radius of tumor cords as a function of time after 35 Gy of X-rays (■), or for untreated controls growing over the same time period (□). Error bars have been drawn as 2 SD to show the range of variation to be expected for 100 measurements in a single tumor; inter-animal variation of the mean was much smaller (2SE≈10–20%). (b) Numbers of histologically intact tumor cells in equal-area zones of tumor cords: zone 1=cells 0–25 μm from the capillary (●), zone 2=cells 75–100 μm from the capillary (○). C indicates values for the respective untreated controls (■, □).

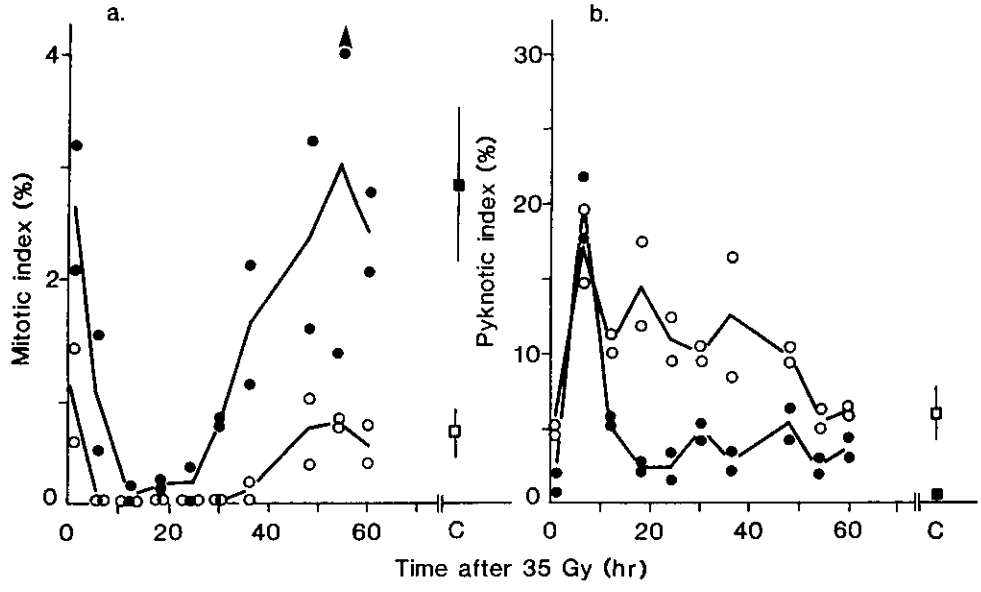


Fig. 3. (a) Mitotic index of histologically intact cells in two zones of cords, as a function of time after 35 Gy. Lines have been drawn at equal distances between the data points for experiments using two transplant generations of the tumor. Symbols as in Fig. 2(b). Errors as 2 SE. (b) Variation with time after 35 Gy, in pyknotic index for the two zones of the cords. Symbols as in Fig. 2(b).

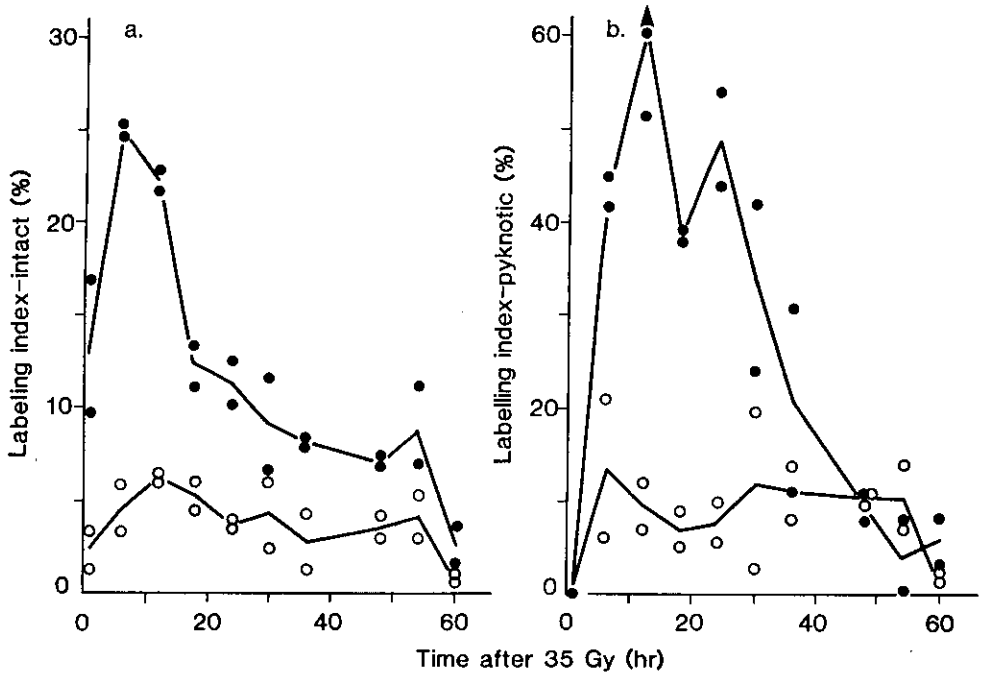


Fig. 4. (a) Variation with time after 35 Gy, in labeling index for histologically intact cells, for the two zones of the cords. (b) Variation in labeling index for histologically dead (pyknotic, karyorrhectic) cells, for the two zones. Symbols as in Fig. 2(b).

The number of intact cells per zone fell to an apparent nadir at 54 hr (Fig. 2b), when numbers were reduced to 70% of that before irradiation (average cell area of 41 μm^2, 1SD 10 μm^2, was not significantly changed). There was no evidence for an overall greater reduction in cell number among cell populations near the subtending vessel (zone 1).

The average mitotic index in control tumors was 2.8% in zone 1 and 0.7% in zone 2. MI in both cell zones fell abruptly after 35 Gy and remained suppressed for at least 24 hr (Fig. 3a). Thereafter, a wave of mitoses occurred until at 54 hr the MI was insignificantly different from that in controls. Mirroring the early fall in MI was a rise in the number of histologically dead cells, whose average area was 24 μm^2, 1SD 11 μm^2 (Fig. 3 b). At 8 hr, the absolute values for PI were the same for zones 1 and 2, i.e. a greater proportional rise in zone 1. Between 8 and 24 hr the PI fell sharply in zone 1 and more gradually in zone 2. At no point was PI in zone 1 significantly greater than in zone 2; for all except the 8 hr point the reverse was found.

The [^{3}H]TdR labeling index of intact cells of both zone 1 (value in controls=13%) and zone 2 (2%) initially rose approximately two-fold and then fell to very low values by 60 hr (Fig. 4a). Among histologically dead cells of zone 1, LI rose from zero to 60% in 12 hr, and in zone 2 from zero to an average of 12% (Fig. 4b). The subsequent decline in LI for the pyknotic cells qualitatively resembled that for the intact cells. For both zones 1 and 2 and for all time intervals, it was almost always the case that the proportion of labeled dead cells was higher than the proportion of viable cells that were labeled (on average, by a factor of 2).

Discussion

Comparison of growth delay in T50/80 after single doses of radiation given with the tumor clamped or 'in air', suggest that this

tumor may have a relatively high proportion of radiobiologically hypoxic cells (Fig. 1). An approximate value for the hypoxic fraction (f) can be obtained using the formula:

$$f = 0.5^{(Tc-Ta)/Td}$$

where Tc and Ta are the GD's after given doses of radiation, for clamped and air treatments, respectively, and Td is the volume doubling time for the tumors regrowing after irradiation (for a critical discussion of the method, see ref. 7). For T50/80, average (Tc − Ta) was 6.1 ± 1.5 days, and average Td for irradiated tumors was 8.5 ± 0.4 days. Therefore f = 61% (95% c.l. 44, 79). Of 11 rodent tumors similarly analyzed by Moulder and Rockwell,[7] only two had comparable or higher hypoxic fractions (the mouse mammary carcinoma EMT6 [69%] and sarcoma FA [95%]). The split-dose data (Table I) likewise indicate that there was little improvement in the radiobiologically defined oxygenation status of the tumor in the days following a first high dose. In this respect, T50/80 differs markedly in response from the C3H mammary carcinomas described by Tanaka *et al.*[8] and by Howes.[9] In both these cases, a reduction in the hypoxic fraction to below pretreatment level by 2 to 3 days after a first dose could be inferred. In T50/80, the 'sparing' that occurred between 0.25 and 3 hr intervals for both air and clamped tumors may reflect repair of sub-lethal injury. Only by 3 days, when tumor volume began to decline markedly, did growth delay after split doses return to the same level as for a single dose of 35 Gy (Table I). Again, the time-scale of changes in overall tumor volume differed between T50/80 and the mammary tumors studied by others,[8, 9] for which reduction in volume began within 1 day after high doses of radiation.

These differences between mammary tumors in overall response reflect differences in the behavior of the tumor cords. Although there was a prompt reduction in the number of intact cells in T50/80 (Fig. 2b) this did not result in immediate shrinkage of the cord (Fig. 2a), in contrast to the results of Tannock and Howes.[3] In T50/80 the cellular dynamics of the tumor cord after 35 Gy were complex. A qualitative model consistent with the temporal events occurring in zone 1 would be: a prompt suppression of mitosis (Fig. 3a) as cells were blocked in the G_2 phase of the cell cycle; and a rise in the proportion of intact labeled cells (Fig. 4a) occurring because (i) some cells would have moved from S to the blocked G_2 phase in the 1 hr period of availability of [^{3}H]TdR, and (ii) cells that died promptly (Fig. 3b) may have done so preferentially in phases of the cell cycle other than S. It is probable that some of these cells died in the G_2 phase, in that the proportion of dead cells that were labeled was disproportionately high (Fig. 4b) and these cells must have died within 1 hr of taking up [^{3}H]TdR. Disappearance of dead cells in zone 1 occurred relatively rapidly as evidenced by the marked fall between 8 and 24 hr (Fig. 3b), either because of 'export' of the dead cells into other zones, or because of dissolution *in situ* (the lifespan of pyknotic cells as recognizable entities appears to be 5 to 10 hr in the cords of rodent tumors[10]). It is noteworthy that as the proportion of labeled intact cells fell (Fig. 4a) so also did the fraction of dead cells (Fig. 3b), further supporting the argument that in this tumor cells were dying after passing through the S phase. Events in zone 2, whose undisturbed LI and MI were much lower than in zone 1, can be explained in the same general terms: a raised LI (Fig. 4a) and prompt increase in PI (Fig. 3b). The LI remained elevated until 60 hr and, assuming that the cells of this largely quiescent population also express death after S, this would account for the persistently high PI in this zone.

The observation by Tannock and Howes[3] of a greater number of dead cells in zone 1 than zone 2 at 6 hr after irradiation of a mammary carcinoma was then and subsequently interpreted in terms of radiobiology, i.e. that cells next to blood vessels are well-oxygenated and sensitive to killing by radiation, while cells remote from blood vessels are hypoxic and resistant to radiation. We suggest, however, that these observations may equally well have reflected cell kinetics than radiobiology: if only cycling cells die promptly and if the kinetics of the cells that are in cycle are the same,[11] then the population showing the greatest proportional rise in dead cells will be that with many or all cells in cycle — zone 1.[6] If one chooses to examine PI at any time other the first few hours, the conclu-

sion might be reached that the cells of zone 2 were more 'sensitive' to radiation. Interestingly, Scott[12] in 1958 pointed to a similar difficulty in the interpretation of some of the classical histological/radiobiological experiments of Mottram.[13]

The cells of ultimate relevance to radiobiology are those from which whole tumors regrow after irradiation and whose response may be quantitated indirectly by the growth delay assay. It is likely that after a dose of 35 Gy as used here, the reproductively surviving fraction (f) of even hypoxic cells will be very low, e.g. from the calculation above:

$$f[\text{hypoxic}] = 0.5^{(Tc/Td)} = 1.3\%$$

However, such cells exist in a milieu of all other cells. Yamaura and Matsuzawa described the total destruction of the corded central region of rat tumors as a result of the collapse of blood vessels.[14] In T50/80, even when the subtending capillaries appeared intact, a relatively small reduction in total cell number, the presence of space-occupying dead cells and a wave of mitoses in zones near the vessel make it less likely that surviving remote hypoxic cells would be brought or move rapidly into closer apposition with the vessel and its nutrient supply. Thus, the kinetics of cords in early transplant generations of T50/80 would militate against the large reductions in hypoxic fraction that have been described for other mammary carcinomas.[8, 9] It is of interest to speculate further, if cord dynamics of T50/80 are not conducive to survival of the remote cells, on the location of the poorly oxygenated tumor-populating cells which are sensitized by misonidazole in the growth delay assay. Within individual tumors, there was a very large range of tumor radii (Fig. 2b). This may be geometric in origin, but we have shown elsewhere that different tumors of the same class have systematically different cord radii, with thin cords occurring in poorly oxygenated tumors.[15] It is possible that in a proportion of cords within one T50/80 tumor, all cells, including those adjacent to the vessel, were hypoxic. It is known for T50/80 that misonidazole given before 40 Gy of X-rays not only ablates all cells of zone 2 but increases the average pyknotic index in zone 1, the putative 'well-oxygenated' cells.[16]

ACKNOWLEDGMENTS

I thank Mrs. Susan Yarnell for expert technical assistance. This work was supported by the Cancer Research Campaign (U.K.)

(Received Oct. 1, 1987/Accepted Dec. 4, 1987)

REFERENCES

1) Caspersson, T. and Santesson, L. Studies on protein metabolism in the cells of epithelial tumors. *Acta Radiol.*, Suppl. **46**, 76–80 (1942).
2) Thomlinson, R. H. and Gray, L. H. The histological structure of some human lung cancers and the possible implications for radiotherapy. *Br. J. Cancer*, **9**, 539–549 (1955).
3) Tannock, I. F. and Howes, A. The response of viable tumor cords to a single dose of radiation. *Radiat. Res.*, **55**, 477–486 (1973).
4) Moore, J. V. Cytotoxic injury to cell populations of solid tumors. *In* "Cytotoxic Insult to Tissue," ed. C. S. Potten and J. H. Hendry, pp. 368–404 (1983). Churchill Livingstone, Edinburgh.
5) Thomlinson, R. H. and Craddock, E. A. The gross response of an experimental tumor to single doses of X-rays. *Br. J. Cancer*, **21**, 108–123 (1967).
6) Moore, J. V., Hasleton, P. S. and Buckley, C. H. Tumor cords in 52 human bronchial and cervical squamous cell carcinomas: inferences for their cellular kinetics and radiobiology. *Br. J. Cancer*, **51**, 407–413 (1985).
7) Moulder, J. E. and Rockwell, S. Hypoxic fractions of solid tumors: experimental techniques, methods of analysis, and a survey of existing data. *Int. J. Radiat. Oncol. Biol. Phys.*, **10**, 695–712 (1984).
8) Tanaka, N., Tanabe, C., Okumura, Y. and Murakami, K. Post-irradiation kinetics of C3H/He mouse mammary carcinoma as regards tumor volume regrowth time and cell loss. *Strahlentherapie*, **155**, 58–62 (1979).
9) Howes, A. E. An estimation of changes in the proportions and absolute numbers of hypoxic cells after irradiation of transplanted C3H mouse mammary tumors. *Br. J. Radiol.*, **42**, 441–447 (1969).
10) Moore, J. V. Death of cells and necrosis of tumors. *In* "Perspectives on Mammalian Cell Death," ed. C. S. Potten, pp. 295–325 (1987). Oxford University Press, Oxford.

11) Tannock, I. F. The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumor. *Br. J. Cancer,* **22,** 258–273 (1968).
12) Scott, O. C. A. Some aspects of the effects of ionising radiation on tumors in experimental animals. *Adv. Biol. Med. Phys.,* **6,** 121–173 (1958).
13) Mottram, J. C. A factor of importance in the radiosensitivity of tumors. *Br. J. Radiol.,* **9,** 606–614 (1936).
14) Yamaura, H. and Matsuzawa, T. Tumor regrowth after irradiation. An experimental approach. *Int. J. Radiat. Biol.,* **35,** 201–219 (1979).
15) Moore, J. V., Hopkins, H. A. and Looney, W. B. Tumor-cord parameters in two rat hepatomas that differ in their radiobiological oxygenation status. *Radiat. Environ. Biophys.,* **23,** 213–222 (1984).
16) Moore, J. V. Tumor cords in irradiated carcinomas. *In* "Proceedings of 7th International Congress on Radiation Research" D1–23 (1983). Martinus Nijhoff, Amsterdam.