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REPAIR IN IRRADIATED ASCITES TUMOUR CELLS GROWING IN VIVO

An investigation of split doses related to cell cycle

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Since the early days of radiation therapy it has been found that when a given dose is divided into several fractions the biologic effect decreases, compared with the same dose given as a single dose. In clinical practice, the time interval between the fractions may vary from a few hours to 2 to 3 days. However, whether a better tumour control is obtained with any one of those intervals is still a matter of dispute (TUBIANA 1977). In experiments, after the introduction of the single cell tissue culture technique by PUCK & MARCUS (1956), split dose irradiation has been extensively used to investigate the repair of sublethal injury. ELKIND & SUTTON (1960) described increased sensitivity to radiation after a period of some hours of better survival—a phenomenon subsequently attributed to changes in the distribution of cells in the cell cycle (ELKIND et coll. 1965). This type of reaction has also been found in tissues growing in vivo including tumours (TILL & MCCULLOCH 1963, WITHERS 1967, BELLI et coll. 1967, WITHERS & ELKIND 1968, EMERY et coll. 1970, MALONE et coll. 1972).

In previous experiments using single doses of irradiation the kinetics of cell proliferation in the Bp8 mouse ascites sarcoma were investigated (CAO et coll. 1982a, b, c). Characteristic early changes were found in the flow of cells through the cell cycle with a linear increase with dose for the mitotic delay, the G_1 depletion and the G_2 blockage. The blockage of G_2 cells causing increased duration of G_2 was the main factor responsible for the increase of the total cell cycle time. After release of the G_2 blockage about 24 hours after irradiation a persistent partial G_2 blockage was demonstrated. Furthermore, a dose dependent decrease in the number of tumour cells after reaching plateau values was found which made it feasible to perform a quantitative evaluation of the radiation effects.

The effect of 2 equal doses of irradiation (2×2.5 Gy) given at intervals of 3, 12, 24 and 48 h has been compared with the effect of a single dose of 5 Gy. These intervals were chosen on the basis of the knowledge of the changes in the distribution of cells in the cell cycle after the first conditioning dose of irradiation: 3 h after irradiation with 2.5 Gy, mitotic cells and G_1 cells had decreased, while S-phase and G_2 cells had somewhat increased; 12 h after irradiation, G_1 cells and 24 h after irradiation S-phase cells had decreased to minimum values, but G_2 cells were accumulated to a maximum value; 48 h after irradiation, G_1 and S-phase cells were near the control values, G_2 cells had decreased but were still higher than the control value.

Material and Methods

The experimental procedures used have been described in detail by CAO et coll. (1982a, d). Briefly,

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male NMRI mice aged about 3 months with a body weight of 25 g were used. Bp8 mouse ascites sarcoma cells were transplanted intraperitoneally with a fixed number of 18×16^6 cells into each of 4 to 8 mice. On the fourth day after inoculation the mice were exposed to whole body irradiation with a dose of 2.5 Gy (250 kV, 15 mA, 0.5 mm Cu added filtration, SSD 50 cm, 1.33 Gy/min). At intervals of 3, 12, 24 or 48 h the second dose of 2.5 Gy was given; during the intervals the mice were returned to their cages.

The ascites volume was measured by an isotope dilution technique. Based on the known ascites volume and the cell concentration the total number of cells was estimated. The mitotic index was determined from 1 000 cells. The cellular DNA content was measured using a rapid-flow cytofluorometric method as described previously (TRIBUKAIT et coll. 1975). After correction for the background the proportion of cells in the various compartments of the cell cycle was determined from the areas of the histogram assuming a Gaussian function for the G_1 and G_2+M maxima and attributing the remaining area of the histogram to the S-phase cells. From the proportion of cells in the various parts of the cell cycle and the total number of the cells, the total numbers of cells in various parts of the cell cycle were determined.

The calculation of the cell flow through the cell cycle is based, as described previously (CAO et coll. 1982 b), on the knowledge of the change in the total number of cells in the various parts of the cell cycle with time. In the present report, the flow rate of the total number of cells from one compartment to the following one will be described as well as the flow rate related to the pool size of the compartments and the average of the inflow and outflow per unit time divided by the number of cells in the various compartments, and the inverse of these values giving the mean duration of the part of the cell cycle.

For determining the mouse survival after irradiation, 12 animals in each group were observed daily.

Results

The total numbers of tumour cells after two equal doses of 2.5 Gy with the intervals of 3, 12, 24 or 48 h appear in Fig. 1. The results are compared with control values and with values after 2.5 and 5.0 Gy single dose irradiation. After 2.5 and 5 Gy single dose irradiation the plateau phase of growth is clearly reached at a lower level compared with controls in a dose dependent way. After split dose irradiation the number of cells is significantly increased already at 3 h compared with 5 Gy single dose irradiation; a repair value of about 70 per cent can be calculated on the basis of the total number of cells at the plateau phase. Split times of 12 and 24 h did not result in a further increase in the total number of cells; a 24 h split time, however, subsequently resulted in the same value as a 1×2.5 Gy single dose irradiation. When the split dose interval increased to 48 h the effect of the first dose seems to be almost completely repaired.

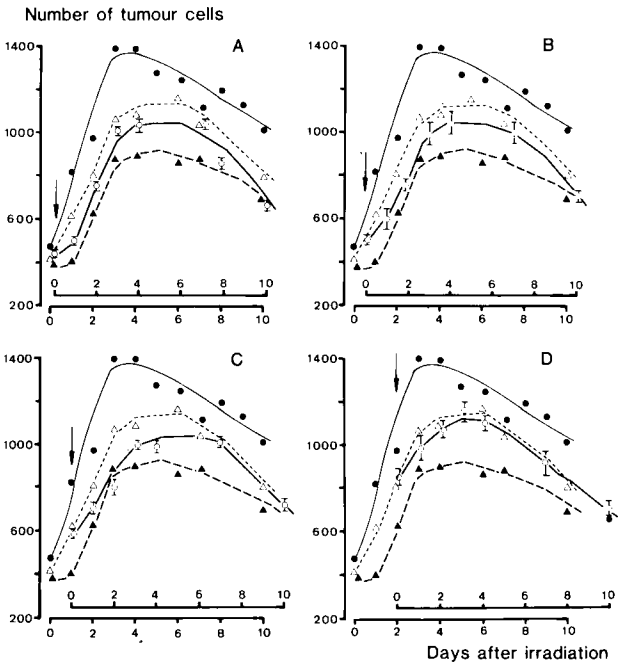

Fig. 1. Total number of Bp8 mouse ascites sarcoma cells (10^6) after a split dose irradiation of 2×2.5 Gy with an interval of 3 h (A, ○), 12 h (B, ○), 24 h (C, ○) and 48 h (D, ○) compared with that of single dose irradiation of 2.5 (△) or 5.0 (▲) Gy, and non-irradiated control values (●). The arrows indicate the time when the second dose of 2.5 Gy was given. The upper part of the time scale indicates the time after irradiation of the second dose, the lower part indicates the time scale of the first and single doses of irradiation. SEM of 4 to 8 animals are representative for all points. The animals were irradiated on day 4 after the inoculation.

The changes in the distribution of cells in the cell cycle are shown in Fig. 2. The marked shift during the first 24 h after a single dose irradiation from G_1 towards G_2 is obvious. Split dose irradiations with 3 and 12 h intervals resulted in an increased G_2 blockage compared with 2.5 Gy only but clearly to a lesser extent than with 5 Gy. With a 24 h split dose interval no further increase occurred, while after a 48 h interval about the same degree of G_2 cell

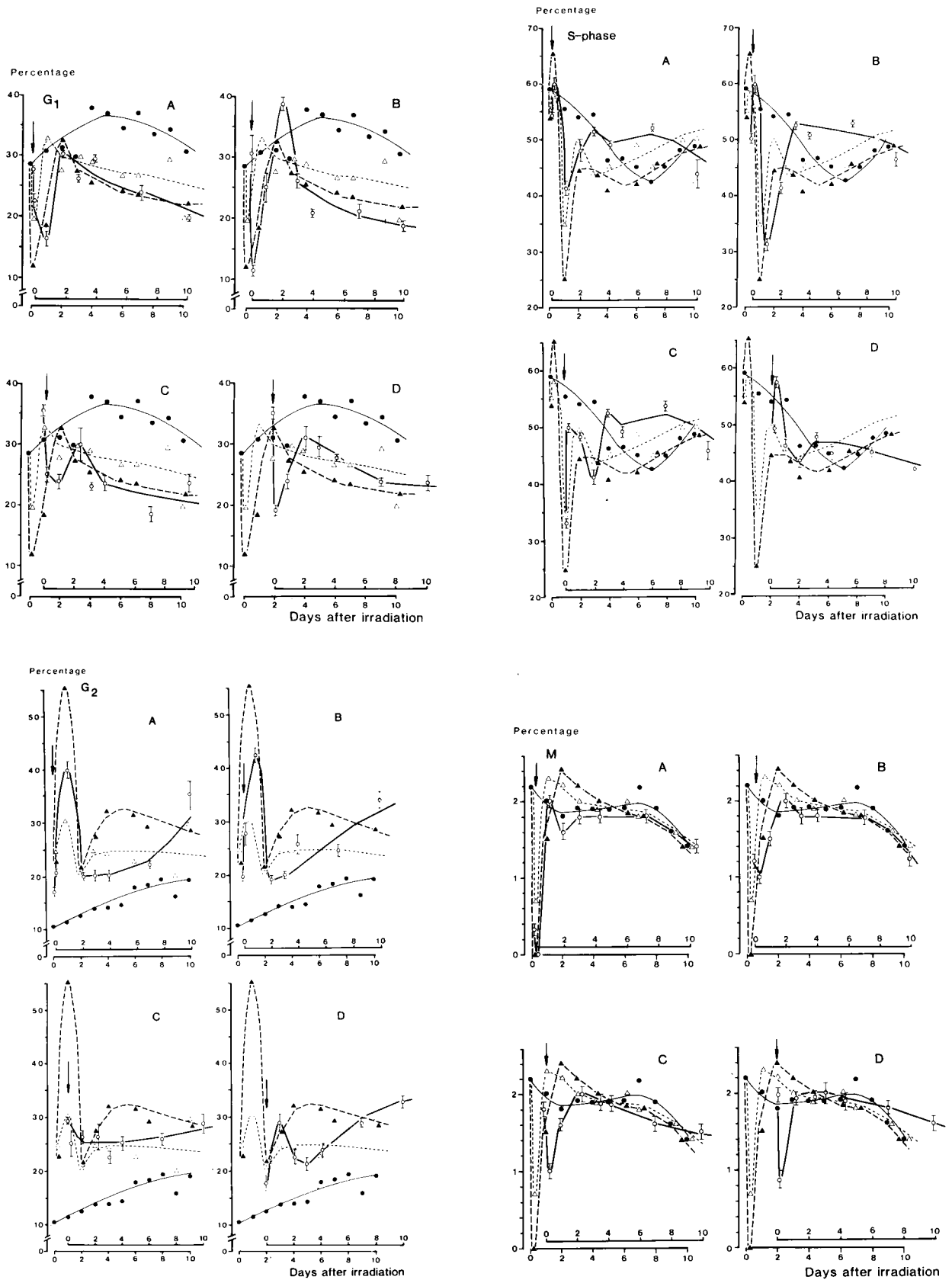

Fig. 2. Proportions of cells in G_1, S, G_2 and M after a split dose irradiation compared with those after a single dose irradiation of 2.5 or 5.0 Gy and with non-irradiated control values. For symbols and explanations see Fig. 1.

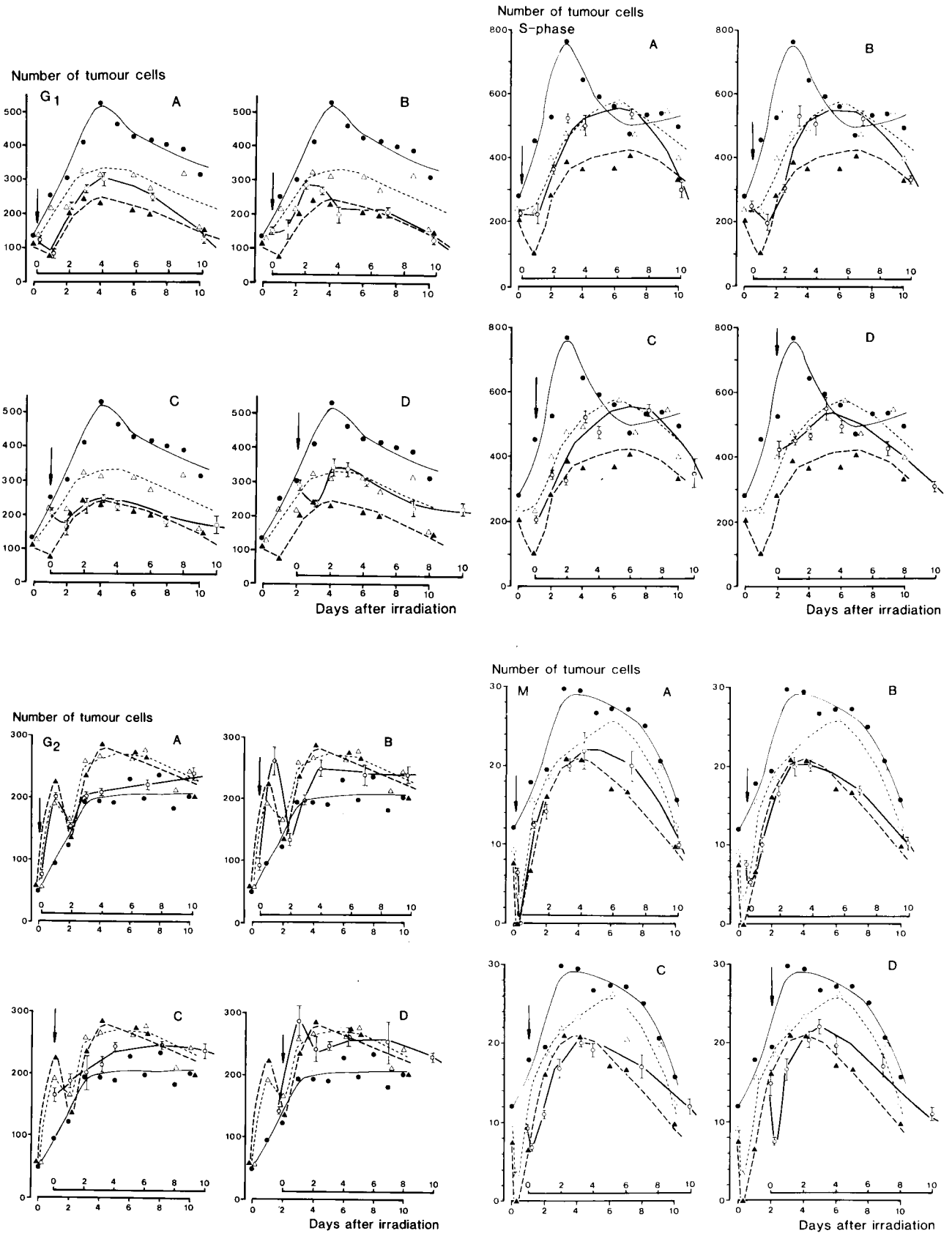

Fig. 3. Total number of cells in G_1, S, G_2 and M (10^6) after a split dose irradiation compared with that after a single dose irradiation of 2.5 or 5.0 Gy and with non-irradiated control values. For symbols and explanations see Fig. 1.

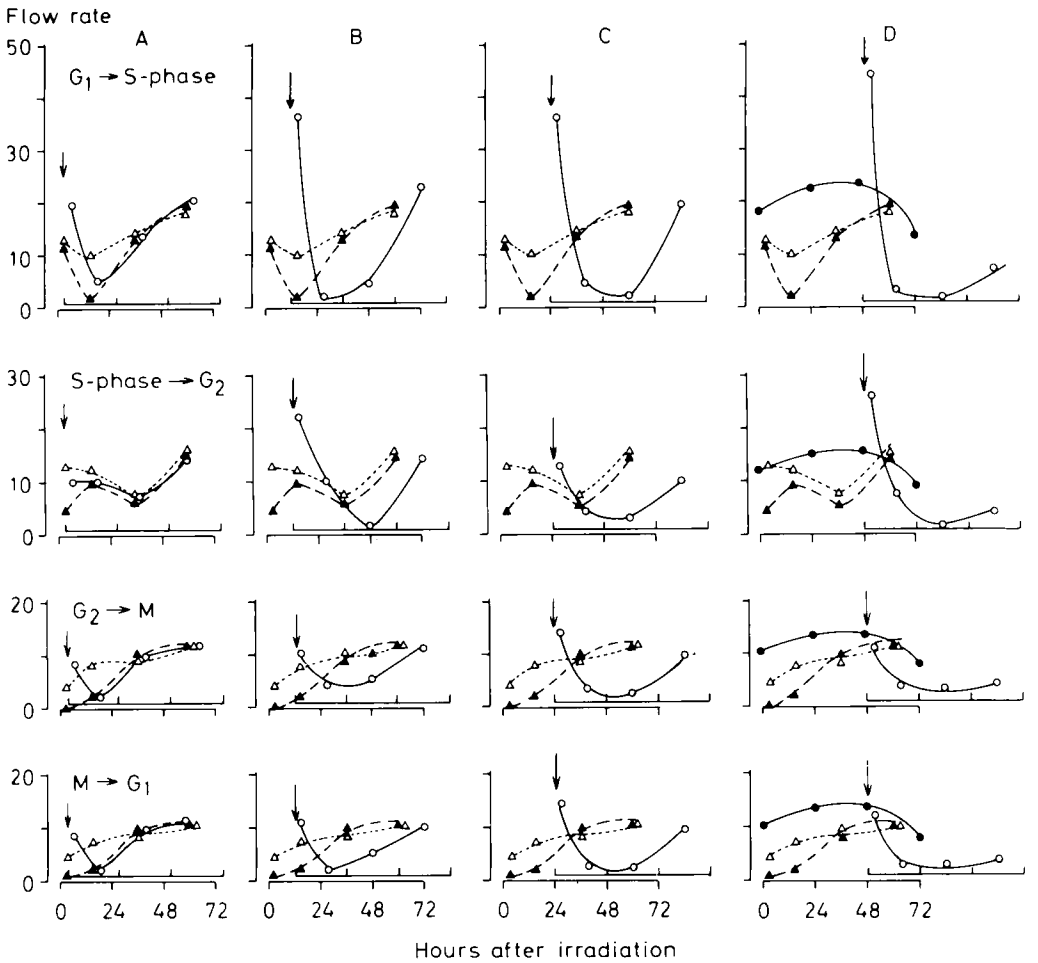

Fig. 4. Flow rates of cells (10^6/h) from G_1, S, G_2 and M to the following compartments after a split dose irradiation (○) compared with those after a single dose irradiation of 2.5 (△) or 5.0 Gy (▲). The arrows indicate the time when the second dose was given. Non-irradiated control values are given in D (●). For further explanations see Fig. 1.

accumulation was seen as after a single dose 2.5 Gy irradiation. The remaining partial blockage in G_2 after the release of the early G_2 blockage corresponded initially to the 2.5 Gy single dose irradiation but subsequently increased. The proportion of S-phase cells after a single dose irradiation shows an early dose dependent increase, followed by a decrease due to G_2 blockage and mitotic delay. Split dose irradiation generally resulted in an early increase in S-phase cells for all intervals. The proportion of mitotic cells in split dose irradiation decreases further for the 3 h split interval, the interval for which low values were found already after the single conditioning dose. At later times, when the mitotic cells approached or reached the control level, reactions similar to those for 2.5 Gy single dose irradiation were seen.

The absolute numbers of cells in the various parts of the cell cycle given in Fig. 3 show a more profound and longlasting disturbance in the growth of the tumour cell population. After irradiation with single doses of 2.5 or 5 Gy the numbers of G_1, S-phase and M cells decreased after having reached the plateau phase of growth in a dose dependent way; the G_2 cells, on the other hand, generally showed no dose dependence and after the release from the G_2 blockage they maintained unchanged values at a higher level than the controls. After split dose irradiation at all intervals the S-phase cells mostly corresponded to the values following a 2.5 Gy single dose irradiation. The G_1 cells may either show the patterns following single doses of 5 Gy with 12 or 24 h split times or 2.5 Gy after the 48 h split interval; only the values of the 3 h split interval are intermediate between those for 5 and 2.5 Gy. The G_2 values reached the plateau values for the 2.5

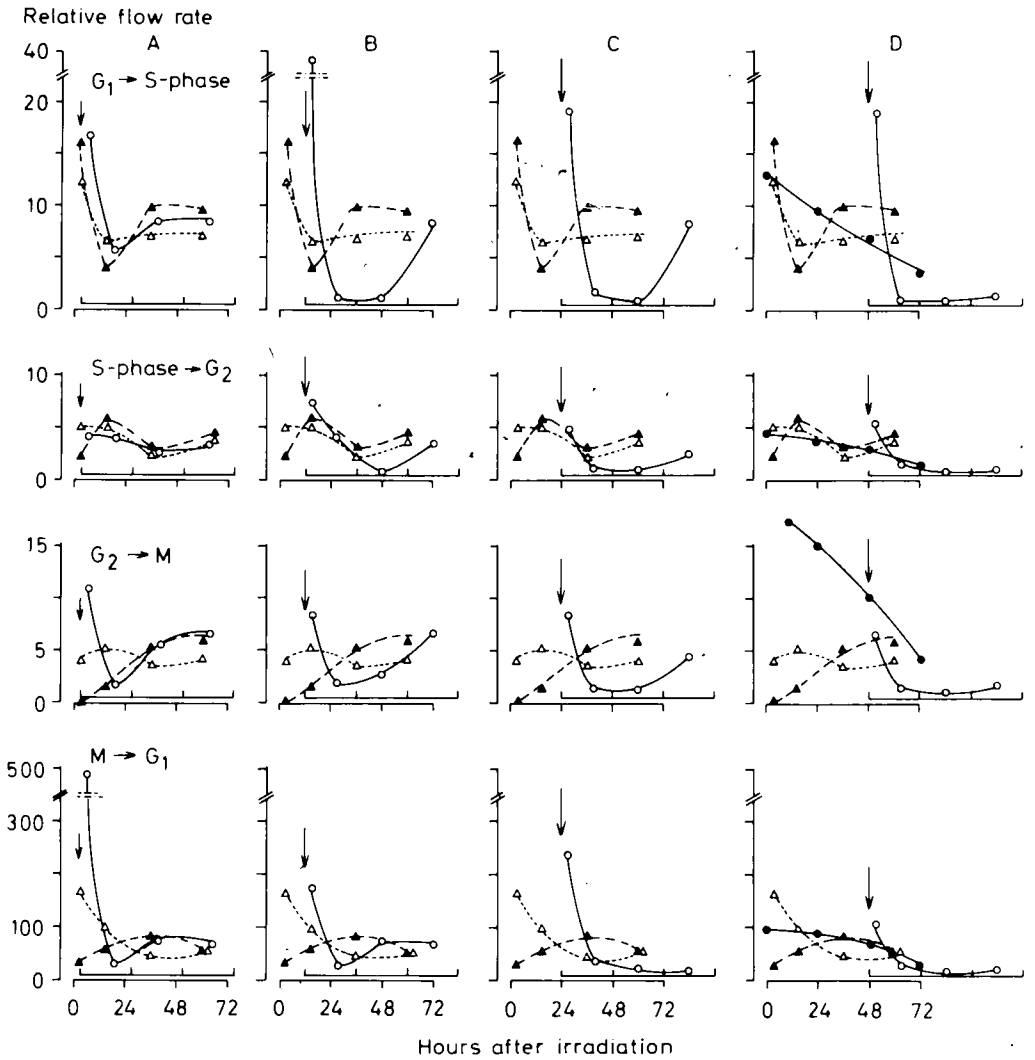

Fig. 5. Flow rates of cells related to cell pool size (per cent/h) from G_1, S, G_2 and M to the following compartment after split dose irradiation (○) compared with those after a single dose irradiation of 2.5 (△) or 5.0 (▲) Gy. Non-irradiated control values are given in D (●). For further explanations see Fig. 1.

and 5 Gy single dose experiments soon after 12 and 48 h split intervals but increased after a delay subsequent to 3 and 24 h intervals. The M cell values were mostly similar to those for the 5 Gy single dose experiments.

Calculations of the flow of the cells through the cell cycle, illustrated in Figs 4 to 6, show the immediate changes during 72 h following irradiation. The flow rate of the total number of cells from one compartment to the following one (Fig. 4) reveals, after the second split dose, an early increase, mostly from G_1 to S-phase but also partly from S-phase to G_2, and taking only the reactions of the single doses into consideration, also G_2 to M and M to G_1. This increase in the cell flow was followed by a general decrease 6 to 24 and 24 to 48 h and an increase 48 to 72 h. The same behaviour was also found when the values of the absolute cell flow rate were related to the pool size of the relevant compartment, except in the case of the flow from S-phase to G_2 for which the early increase seems to be only a consequence of the somewhat higher pool size (Fig. 5).

Thus, the early increase in the proportion of S-phase cells 6 h after the second split dose irradiation (Fig. 2) is due to an increased inflow of cells from G_1 while the outflow from S-phase to G_2 relative to the inflow is decreased.

In calculating the total cell cycle times (Fig. 6) after a transient decrease, a marked increase with maximum prolonged values was found in between 24 and 48 h for all split intervals except the 3 h interval, this latter interval mostly behaving as the

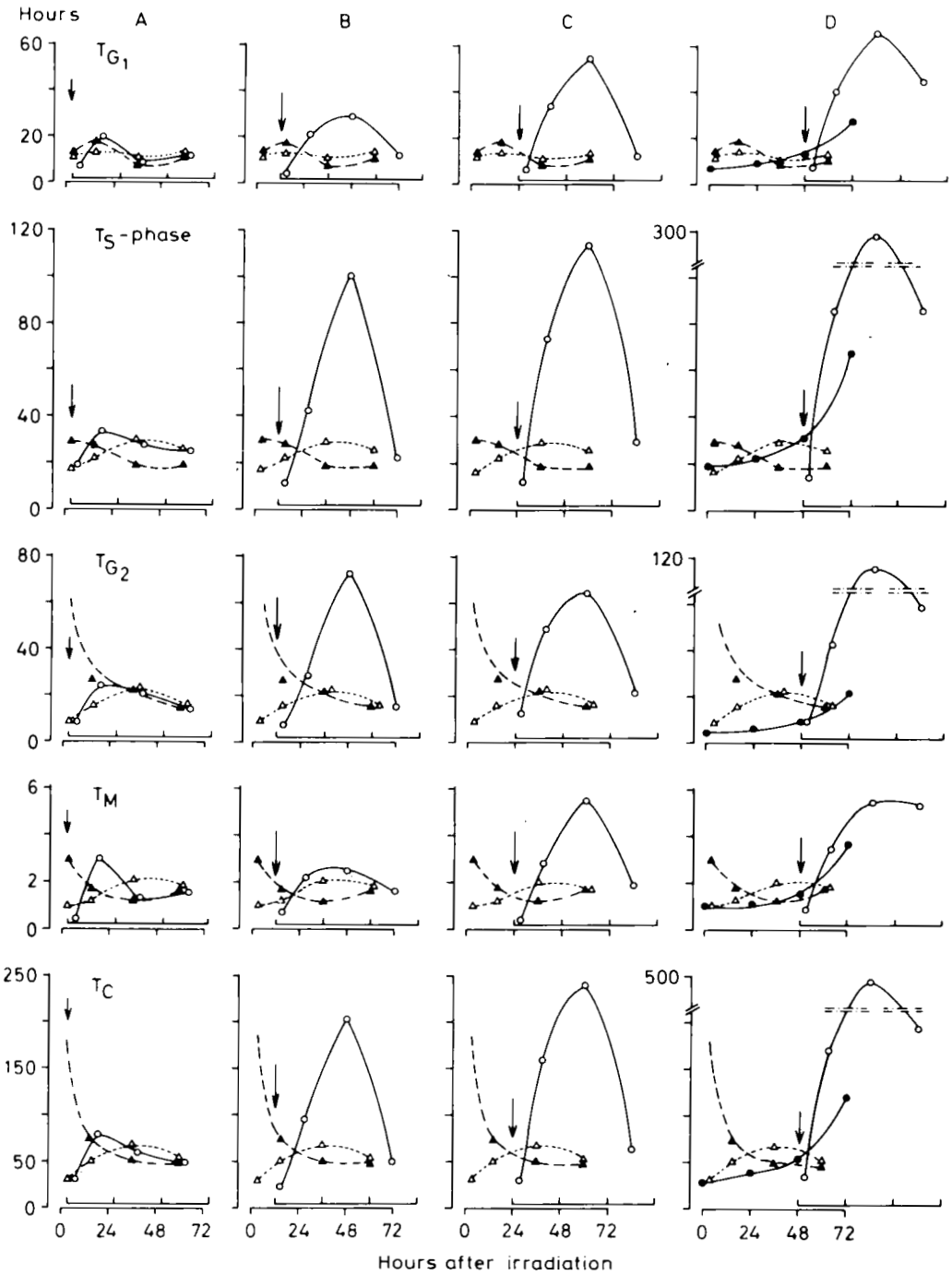

Fig. 6. Durations of G_1, S, G_2 and M and of the total cell cycle time (Tc) after a split dose irradiation (○) compared with those after a single dose irradiation of 2.5 (△) or 5.0 (▲) Gy. Non-irradiated control values are given in D (●). For further explanations see Fig. 1.

2.5 Gy single dose irradiation. In this prolongation of the cell cycle time, the S-phase contributed with about 50 per cent while G_1 and G_2 contributed with about 25 per cent each.

The ascites volume, the cell concentration, the relative cell volume, the percentage of normal and dead cells, and the ratio mitotic/G_2 cells did not differ significantly for the various split dose intervals. (Data are not given.) The survival of the animals following a single dose of 2.5 Gy was prolonged and had an LD_{50} of about 37 days. With increasing doses the survival decreased, and the

LD_{50} after a single dose of 5 Gy was slightly reduced to about 30 days. In the split dose experiments this value after an interval of 3 h was between the 5 and 2.5 Gy single dose values, while at increased split dose time intervals the survival times corresponded to those for 2.5 Gy single doses (Fig. 7).

Discussion

In the reactions of tumours to radiation given as a series of fractionated doses, repopulation, reoxygenation, recovery from sublethal injury and redistribution in the cell cycle have been considered as significant factors. Since the various phases of the cell cycle differ in their capacity to accumulate repairable sublethal injury (ELKIND 1967, SINCLAIR 1970) recovery and redistribution, i.e. the ability of a proliferating cell population to re-establish the initial distribution in the cell cycle between two fractions of irradiation, are closely linked.

In the present experiments, using rapid-flow cytofluorometry for measuring the cellular DNA content attention was paid to changes in the distribution of cells in the cell cycle both after the first conditioning dose of 2.5 Gy and following the second dose given at time intervals between 3 and 48 h. Since in ascites tumours both reoxygenation and repopulation are of little or no significance and the cell material is composed almost entirely of tumour cells, these experimental conditions are suitable for investigations on recovery in relation to cell distribution in the cell cycle.

For split dose irradiation growth curves for the total number of cells were found somewhere in between the 2.5 and 5 Gy single doses. In order to quantify the repair between the split doses the area between the two single doses was measured and taken as 100 per cent; the areas given by the 5 Gy single dose curve and the 2×2.5 Gy split doses curves express the degree of repair. It is possible to distinguish two rates at which repair proceeds (Fig. 8), the first with a mean rate of at least 20 per cent per hour and the second with a rate of about one per cent per hour. It is debatable whether these different repair rates reflect different types of repair. This repair pattern with time has been described in a large number of different experimental systems (ELKIND & SUTTON 1959, 1960, HORNSEY & VATISTAS 1963, BERRY & OLIVER 1964, WHITMORE et coll. 1965, WITHERS, BELLI et coll., BERRY 1967, SCHAER & RAMSEIER 1973, HERTZEL et coll. 1976, TUBIANA). In addition, an increase in the radiation sensitivity between 3 to 12 h after the conditioning dose has been described (ELKIND & SUTTON 1959, 1960, HORNSEY & VATISTAS, BERRY & OLIVER, WHITMORE et coll., BELLI et coll.). In the present experiments this time interval was not analysed and it cannot be excluded that the same time of reaction was also present.

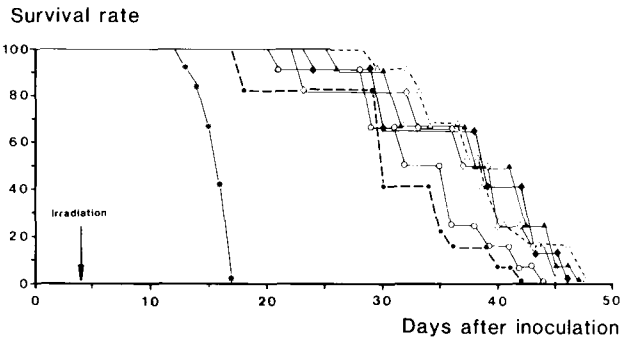

Fig. 7. The percentage mouse survival rates after split dose irradiation of 2×2.5 Gy with intervals of 3 (○), 12 (◇), 24 (◆) and 48 (▲) hours compared with those after single dose irradiation of 2.5 (△) and 5.0 (●) Gy, and with non-irradiated control values (*). Each group consisted of 12 mice. Inoculation of 18×10^6 Bp8 mouse ascites sarcoma cells on day 0, irradiation of the first dose on day 5 is indicated by an arrow.

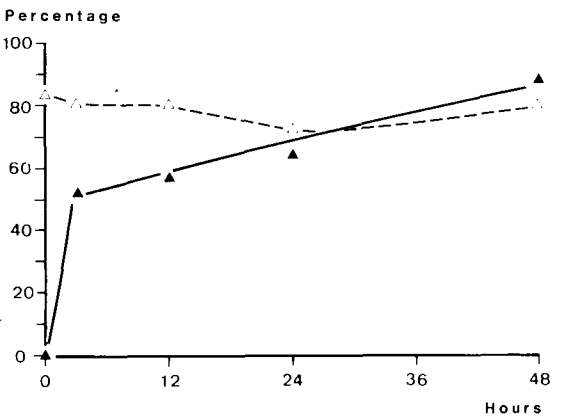

Fig. 8. Recovery (▲) and calculated mean cellular radiation sensitivity (△) of Bp8 ascites sarcoma cells. At zero time a single dose of 5 Gy was given or a split dose of 2.5 Gy followed by another split dose of 2.5 Gy at 3 to 48 hours. The mean cellular radiation sensitivity was calculated from the distribution of cells in the cell cycle at various times of irradiation.

In order to discuss the repair in relation to the distribution of cells in the cell cycle, it was assumed that the sensitivity to radiation of G_2 and M cells has a relative value of 1.0, while G_1 cells have a relative value of 1.3 and S-phase cells a value of 2—roughly the values found for Chinese hamster cells (SINCLAIR). The proportion of cells in the cell cycle for the non-irradiated cell population in the

present series was 34 per cent for G_1, 55 per cent for S-phase, 9 per cent for G_2 and 2.1 per cent for M. The calculated total cell cycle time was 32 h with a duration for G_1=7 h, S-phase=19.5 h. G_2=4.5 h, and M=1 h (CAO et coll. 1982 d). Three hours, 12 h, 24 h and 48 h after the conditioning dose the proportions of G_1 cells were 28, 31, 37 and 34 per cent, respectively. The corresponding values for S-phase were 54, 50, 33, and 49 per cent, for G_2 17, 18, 29 and 15 per cent and for M 0.3, 1.2, 1.4, and 1.6 per cent, respectively. The mean cellular relative resistance calculated from these distributions of cells in the cell cycle and from the values given are 1.65 for controls, 1.61, 1.60, 1.44 and 1.59 for the irradiated population at the various times. These values expressed as a percentage of the maximum possible radiation sensitivity (=2.0) also appear in Fig. 8. Obviously, the mean cellular sensitivity differs only slightly and deviates only significantly at 24 h, at which time the proportion of G_2 cells is increased to the maximum extent while the proportion of S-phase cells is decreased to the maximum extent.

Similar results are obtained if it is assumed that G_2 and M cells have no capacity for repair of sublethal injury (the cell survival curves have no shoulder) while S-phase and G_1 cells have this capacity for repair but various assumptions are made for the Dq values of their shoulders. Taking the total number of cells at the plateau phase of growth as an end-point, a shoulder type curve has been found for the dose response with a Dq of about 2 Gy for a cell population composed of different fractions of cells in the cell cycle corresponding to 4 days of growth (CAO et coll. 1982 d). Comparing the recovery with the relative resistance to radiation (Fig. 8), no deviations from the expected course of recovery can be found and on the basis of a changed cell distribution, no increase in the sensitivity between 3 and 12 h can be predicted. When the conditioning dose is increased, more marked early changes in the composition of the cell population have been observed (CAO et coll. 1982 a). At the same time, the fraction of lethally damaged 'doomed' cells increase which makes the calculations of the relative radiation sensitivity of a cell population more doubtful.

In addition to the cell cycle phases with differences in their capacity for sublethal injury other, non-cell cycle, specific changes may be present after a conditioning dose and may influence cell survival in a series of fractionated doses. Comparing the flow of cells through the cell cycle after split doses with that following single doses (Figs 4–6) some differences are obvious. In the split dose experiments after a transient early increase in the flow rate, the flow rate decreases with a maximum 24 to 48 h after the second dose. This prolongation is most marked for the split dose intervals of 48 h and it may be suggested that it is associated with the change in growth pattern reaching the plateau phase. This explanation is, however, less likely for the split dose intervals of 12 and 24 h. The implication of this prolongation of the cell cycle, in which the S-phase contributes most, may be that repair of potentially lethal injury is promoted. It has been found that suboptimum conditions for growth, imposed after a single dose, facilitate the repair of potentially lethal injury (PHILLIPS & TOLMACH 1966, BELLI & SHELTON 1969, LITTLE 1969, HAHN & LITTLE 1972, DRITSCHILO et coll. 1976).

Calculating the total number of cells in the various parts of the cell cycle, it has been found both in non-irradiated animals and animals exposed to single doses of whole body irradiation, partial body irradiation and protracted whole body irradiation that the total number of G_2 cells generally demonstrated dose independent constant values after reaching the plateau phase of growth. In contrast, the other cell types showed dose dependence and a decrease after reaching a maximum value (CAO et coll. 1982 a, c, d, 1983). A similar behaviour has now also been found following split dose irradiation supporting the suggestion that the G_2 cells might be specifically involved in auto-regulatory processes of cell growth in this ascites tumour.

The maximum prolongation of the survival of animals after whole body irradiation has been found for doses of 1.75 and 2.5 Gy but the survival began to decrease after 5 Gy and was less than in non-irradiated animals after 8 Gy (CAO et coll. 1982 a). This behaviour can be explained by normal tissue bone marrow injury superposed on the injury of the tumour cells. The slight increase in survival after a 3 h split dose interval and the maximum increase in survival after 12 h split dose intervals indicate that the recovery of the bone marrow is completed at the latter time.

SUMMARY

Split dose irradiation with 2×2.5 Gy at intervals of 3 to 48 h was compared with the effect of a single irradiation with a 5 Gy dose in Bp8 mouse ascites sarcoma growing in vivo. The total number of cells and, using rapid-flow

cytofluorometry, the proportions of cells in the various parts of the cell cycle were determined up to 10 days after irradiation. In addition, from sequential analyses of the total number of cells in the various compartments of the cell cycle, the flow of cells through the cell cycle was calculated. As judged by the growth in the total number of cells, a rapid repair of at least 20 per cent per h up to 3 h is followed by a slow repair of about one per cent per h during the following 45 h. The proportion of cells in the cell cycle following the conditioning dose did not differ enough to expect a significant influence on the mean radiation sensitivity of the cell population. At split time intervals of 12 to 48 h, after a transient early increase of the flow of cells through the cell cycle, the flow of cells was markedly reduced in all parts of the cell cycle with a maximum 24 to 48 h after the second dose of irradiation. This increase in the cell cycle time was not found following single dose irradiation and may influence the repair of potentially lethal cell damage.

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