LETHAL RESPONSE OF HeLa CELLS TO X-IRRADIATION IN THE LATTER PART OF THE GENERATION CYCLE

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ABSTRACT The age-response for the killing of HeLa S3 cells by X-rays during the latter part of the generation cycle has been examined in detail. As synchronous cells move from the G1/S boundary through S phase, the relatively high sensitivity of late G1 cells gradually decreases; minimum sensitivity is reached in mid-S and maintained during the remainder of that phase. The response of cells as they progress from S to the point in G2 at which they are temporarily arrested by radiation (or by inhibitors of protein synthesis) was measured in populations free of both S phase cells and late G2 cells that had passed the arrest point: cells retain their high resistance from early G2 up to the arrest point. The response of G2 cells that have passed the arrest point before being irradiated was examined by exposing randomly growing cultures to X-rays and collecting cells periodically thereafter, as they entered mitosis. Survival values very close to those of sensitive mitotic cells were found in the 2 h period after irradiation during which unarrested cells continued to reach mitosis. Values typical of late S/early G2 were found only after cells that had been arrested began arriving at mitosis. Thus, HeLa S3 cells undergo an abrupt increase in sensitivity at or near the arrest point. The sensitivity to a second irradiation of cells arrested in G2 by a conditioning X-ray dose increases rapidly in the early part of the arrest period.

INTRODUCTION

It is well established that the responses of eukaryotic cells to X-irradiation undergo temporal fluctuations during the generation cycle (Terasima and Tolmach, 1961; Elkind and Whitmore, 1967, pp. 85–103), and that these fluctuations reflect the cells' progression through the cycle. The age-response for cell killing (loss of colony-forming ability) in synchronously growing HeLa S3 cells follows a defined pattern with two peaks of sensitivity and two periods of resistance (Terasima and Tolmach, 1963*a*). Mitotic cells are relatively sensitive but pass rapidly into a more resistant state early in G1; increased sensitivity is then gradually reacquired until the cells reach the G1/S transition where sensitivity is again maximal. As the cell population enters and moves through S, sensitivity decreases once more, reaching the second resistant period in late S or early G2.

Age-responses are conveniently determined with synchronous cell populations, but because of the decay of synchrony which occurs as an initially synchronous culture progresses through the generation cycle, several features of the age-response for cell killing have been only imprecisely described. In particular, the duration of a sensitive or resistant period is not well defined, nor is it always clear whether a particular transition from sensitivity to resistance (or vice versa) occurs gradually or abruptly in an individual cell. Partly as a consequence of this lack of detailed information, the physiological basis for at least a major part of the age-response remains poorly understood. Nevertheless, certain features of age-responses have been delineated. For example, the decrease in sensitivity that is generally observed during S phase, which in HeLa S3 cells begins simultaneously with the inception of DNA synthesis (Terasima and Tolmach, 1963*b*) but in some types of cells is delayed (e.g., Sinclair and Morton, 1966; Rockwell and Kallman, 1974), is apparently gradual, and it is dependent on DNA synthesis (Terasima and Tolmach, 1963*b*).

The behavior of cells in G2, during which the resistance of late S phase cells must give way to the sensitivity of M cells, has been difficult to determine in mitotically synchronized populations because of the relatively brief duration of this phase (about 3 h) and the aforementioned loss of synchrony that develops during progression of cells through the earlier phases of the cycle. Synchronous G2 populations of HeLa S3 cells that had been purified by killing contaminating S phase cells with tritiated thymidine of high specific activity (HSA-[^{3}H]TdR) and by removing mitotic cells trapped by vinblastine showed a progressively increasing sensitivity with increasing average age of the population, but the detailed pattern of the change was not determined (Djordjevic and Tolmach, 1967).

When cells are X-irradiated they exhibit delayed progression through S and G2 (Okada, 1970). The G2 delay, which is dose dependent, is generally longer than the S delay and is characterized by a block in cell progression at a point in the cycle (arrest point) identical or close to the last point at which inhibition of protein synthesis prevents cells from reaching mitosis (Puck and Steffen, 1963; Okada, 1970). Cells irradiated late in G2, after they have passed the arrest point, enter mitosis without delay (Okada, 1970). HeLa S3 cells that are not blocked in G2 by X-rays can easily be separated from those that are: when randomly dividing cultures are irradiated, those cells that have passed the arrest point enter mitosis within at most 1.5–2 h, while cells that are blocked do not reach mitosis until they have recovered from the G2 arrest (e.g., 8–10 h after irradiation with a 500 rad dose). By collecting mitotic cells at various times after irradiation, the responses of arrested and nonarrested cells can be compared.

It has been suggested (Walters et al., 1969; Tolmach et al., 1971) that cell killing and the arrest of cells in G2 may be independently induced radiation effects. However, recent observations have shown a discontinuity at the arrest point in the expression of chromosome aberrations (Dewey, 1975) and of deficient DNA synthesis (Griffiths and Tolmach, 1975), both of which are strongly correlated with cell killing (Dewey et al., 1971; Griffiths and Tolmach, 1975). We have therefore reexamined the sensitivity of G2 cells in detail, to determine whether cell killing also displays a discontinuity at the

arrest point. The results show that irradiation with a given dose at any time between about the middle of S phase and the arrest point in G2 elicits a constant response, while cells irradiated after having passed the arrest point exhibit lower survival values typical of mitotic cells. In addition, the sensitivity of arrested cells has been measured and found to increase early in the arrest period.

MATERIALS AND METHODS

Culture Conditions

HeLa S3 cells, maintained as monolayer cultures in an atmosphere of 5% CO_2 in air saturated with water vapor, were grown in F10 medium (Ham, 1963) lacking thymidine and supplemented with 10% calf and 5% fetal calf sera (F10FC) and antibiotics. The source of the line and certain of its characteristics have been reported elsewhere (Griffiths and Tolmach, 1975). Synchronous populations were obtained by selective harvest of mitotic cells from cultures growing in plastic dishes (Terasima and Tolmach, 1963c) or bottles (Lindahl and Sörenby, 1966). In order to enhance synchrony in certain experiments, cultures were resynchronized by temporarily blocking cells at the G1/S transition by replacing the F10FC medium in early G1 with N16 medium (Ham and Puck, 1962) supplemented with 20% human and 10% horse sera (N16HHF), antibiotics, and 1 μM fluorodeoxyuridine (FUdR). Cells were released from the block 12–14.5 h later by replacing the N16HHF with F10FC containing 10 μM thymidine, except as noted. Experiments with randomly growing cultures were carried out after at least 48 h of growth following trypsinization (Phillips and Tolmach, 1966).

Irradiation

X-radiation was administered at 37°C at a dose rate of either 86 or 92 rads/min. The X-ray generator was operated at 220 kV constant potential, 15 mA, with 0.25 mm Cu + 1 mm Al added filtration, yielding a half-value-layer of 1.0 mm Cu.

Inactivation of S Phase Cells with HSA-[^{3}H]TdR

Tritiated thymidine of sufficiently high specific activity will rapidly kill a cell into whose DNA it has been incorporated (Whitmore and Gulyas, 1966). Hence S phase cells that might contaminate a G2 population can be selectively inactivated, thereby increasing the homogeneity of partially synchronous cultures. Populations containing a majority of G2 cells were treated with HSA-[^{3}H]TdR (18 or 27 Ci/mmol, 2 μCi/ml) in N16HHF medium for 2–7 h, as detailed in the Results. 1 μM FUdR was added to enhance the incorporation of the label. At the termination of the treatment, the dishes were rinsed and the medium was changed to F10FC containing 10 μM thymidine, except as noted.

Determination of Cell Survival

Survival was measured by scoring the number of colonies containing 50 or more normal looking cells 12–14 days after plating a known number of cells; plating efficiencies of unirradiated cells were in the range of 70–80%. Survival values were not corrected for single cell survival, but any increase in the average number of cells per colony, $\bar{N}$, at the time of irradiation was noted, and such changes are included in the respective figures. When no data are given, $\bar{N}$ did not increase above that normally seen 3 h after mitotic collection (1.65–1.8).

Arrest of Mitotic Cells with Vinblastine

Vinblastine sulfate (VLB) at a concentration of 0.03 μg/ml arrests HeLa S3 cells in metaphase (Palmer et al., 1960; Cutts, 1961), but has little if any toxic effect on interphase cells (Pfeiffer

and Tolmach, 1967), although cell progression might be slightly slowed (Madoc-Jones and Mauro, 1968). After incubation in the presence of this agent, the loosely attached mitotic cells were dislodged with growth medium, the cultures were rinsed, and fresh medium was again added.

Time-Lapse Cinemicrography

Selected microscope fields were photographed at approximately 5-min intervals, using procedures for exposure and analysis described previously (Hurwitz and Tolmach, 1969).

RESULTS

When randomly dividing HeLa S3 cultures are irradiated, cells continue to divide, in declining numbers, for about 1.5 h. Following this, there is a period, the length of which increases with dose, during which radiation-induced G2 arrest permits very few if any cells to enter mitosis. The arrested cells ultimately resume progression and reach mitosis. In the experiment whose results are shown in Fig. 1, cultures were irradiated with 200–600 rad doses, and mitotic cells were collected and plated for survival determination at various times thereafter. Little difference is seen in survival values for cells collected within the first 2 h after irradiation. Cells collected 1.5 h after irradiation

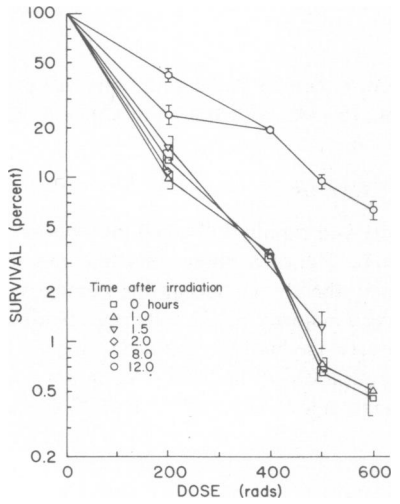

FIGURE 1

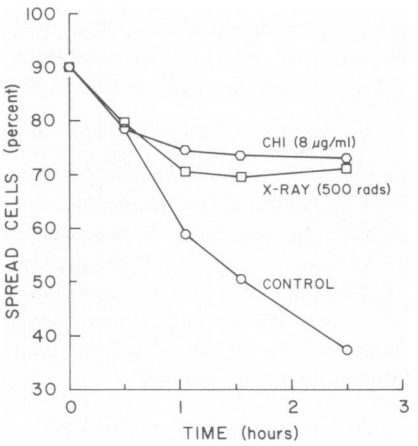

FIGURE 2

FIGURE 1 Dose-dependence of the survival of cells collected from randomly growing cultures at various times after irradiation. The means of two to four dishes are plotted, the bars indicating either the standard deviation, or if only two dishes, the range. Where no bar is shown, it lies within the symbol.

FIGURE 2 Progression of VLB-treated cells into mitosis in the presence of 8 μg/ml cycloheximide (CHI), after irradiation with 500 rads, and in controls receiving no further treatment. Treatments were carried out at zero time which was 6.75 h after release from an FUdR resynchronization block of mitotically collected cells. The fraction of spread cells was scored at each of the times indicated.

with 200 or 500 rads (few cells could be collected at this time) had slightly higher survival values than those collected earlier, but the increase is probably not significant inasmuch as cells collected still later (2.0 h after irradiation with 200 rads; higher doses could not be tested because of the very small number of cells that could be collected at this time) again showed the lower value. Thus, cells that are in late G2, past the point at which arrest occurs, have about the same radiation sensitivity as mitotic cells (0 h collection). In contrast, the survival values for cells collected 8 h after irradiation clearly indicate that cells that have been arrested, and which must therefore have been in S or early G2 at the time of irradiation, are much more resistant. By 12 h postirradiation, cells collected from the culture receiving only 200 rads were probably in early S at the time of irradiation; hence survival is significantly lower than in the cells of the 8 h collection. However, after a dose of 400 rads, G2 arrest is sufficiently long to eliminate any difference between the 8 and 12 h collections.

Arrested cell populations are probably quite heterogeneous with respect to cell age at the time of irradiation, some having been in G2 while others were in S. This heterogeneity derives from differences in the duration of G2 arrest in individual cells, arising from the increasing duration of arrest with age at the time of irradiation in this region of the cycle (Terasima and Tolmach, 1963*a*); some S phase cells may overtake G2 cells (at sufficiently high doses) and enter mitosis before the latter (Whitmore et al., 1967). Hence the shape of the age-response curve in early G2 cannot be determined by experiments of this type, even with more frequent measurements. In particular, it is not possible by this procedure to determine whether the response approaches that of late G2 (and M) cells gradually, or changes abruptly at some point between the beginning of G2 and the arrest point.

In order to measure the survival response of more homogeneous early G2 populations, experiments were carried out with synchronous cultures obtained by mitotic selection and purified by elimination of both S phase cells and G2 cells that had passed the arrest point. The method used by Djordjevic and Tolmach (1967) to increase the homogeneity of G2 populations was employed with slight alteration: the VLB (added 3.5 h after mitotic collection) together with the trapped mitotic cells were removed 2.5 h after irradiation instead of at the time of irradiation as in the earlier study. This permitted the removal not only of cells that had reached mitosis by the time of irradiation, but also of cells in late G2 that had passed the arrest point; study was thereby confined to early (prearrest point) G2 cells. Table I shows the results of treating cells with HSA-[^{3}H]TdR or cycloheximide (CHI) (see below) from 18.5 to 22 h after mitotic collection, with VLB from 3.5 to 22 h, and with certain combinations of these agents. It is evident that by 18.5 h post-collection about 60% of the cells had completed DNA synthesis (or were in the last stage of S), and that by 22 h (in the presence of VLB) almost 20% had entered mitosis. It is also seen that treatment with CHI for 3.5 h killed at most 10% of the cells.

Serial irradiation of the purified population permitted measurement of the response of G2 cells whose average age was progressively approaching the arrest point. However, the flow of cells into mitosis would not be halted in the unirradiated control cul-

TABLE I

EFFECT OF VARIOUS TREATMENTS ON SURVIVAL OF G2 CELLS*

Treatment	Survival
	% ± *SD*
HSA-[^{3}H]TdR	62 ± 10
VLB (0.03 μg/ml)‡	83 ± 9
CHI (8.0 μg/ml)	89 ± 1
VLB‡ + CHI	74
HSA-[^{3}H]TdR + CHI	60

*Treatment from 18.5 to 22 h after mitotic collection.

‡VLB present from 3.5 h postcollection; treatment terminated at 22 h, at the time of removal of accumulated mitotic cells.

tures on which fractional survival values must be based; hence it was necessary to mimic the radiation-induced G2 arrest. This was accomplished by adding CHI (Walters and Peterson, 1968) to parallel cultures at a concentration of 8 μg/ml. Treatment with either radiation or CHI results in a halt of cell progression into mitosis within about 1.5 h; Fig. 2 shows that in the presence of VLB, the fraction of spread cells decreases to a constant value within this time after treatment with either agent, while rounded mitotic cells continue to accumulate in the control. Fig. 3 shows the plating efficiency of cells in control and irradiated cultures as a function of time of treatment; the values have been corrected for the effect of HSA-[^{3}H]TdR and the small apparent toxicity of CHI (Table I). The decrease seen for the control cells reflects the normal movement of G2 cells into mitosis, and is consistent with previous results (Djordjevic and Tolmach, 1967). The number of colony-forming cells decreases at the same rate in the irradiated cultures as in the controls; the ratio, shown by the triangles, is essentially constant. It is concluded, therefore, that sensitivity to X-rays, as measured by loss of colony-forming ability, is constant during early G2.

The data presented above indicate that cells are relatively resistant from early G2 to the arrest point; survival is about 10% after a dose of 500 rads. Previous reports (Terasima and Tolmach, 1963*a*) have shown that HeLa S3 cells are relatively sensitive at the G1/S transition, and become more resistant as they progress through S, but it was not clearly indicated when maximum resistance is reached; in order to precisely locate the age-response curve during S phase, it is necessary to monitor cell progression. This matter was therefore reexamined with resynchronized cultures (see Materials and Methods), movement through S being followed by treating parallel dishes with HSA-[^{3}H]TdR for 1.5 h at various times after bypass of the FUdR block; the decrease in lethality from this treatment served as an indicator of progression into G2. Before adding the HSA-[^{3}H]TdR, the F10FC medium containing unlabeled thymidine (1 μM), which had been added to bypass the FUdR block, was removed, and the cultures were rinsed with N16HHF to remove as much thymidine as possible. The action of HSA-[^{3}H]TdR was stopped in the unusual fashion (Materials and Methods) except that the concentration of thymidine added to the F10FC medium was 1 μM.

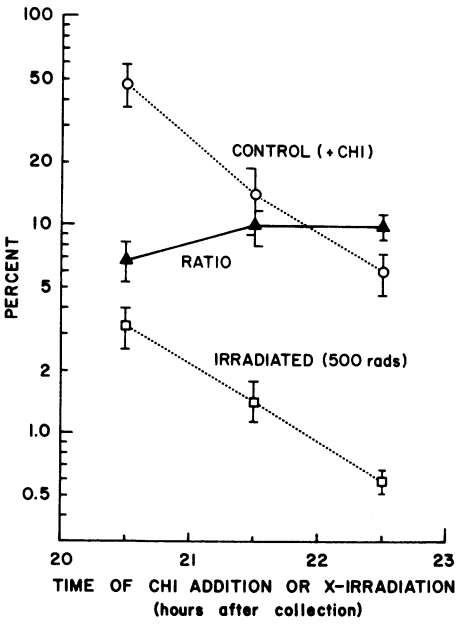

FIGURE 3

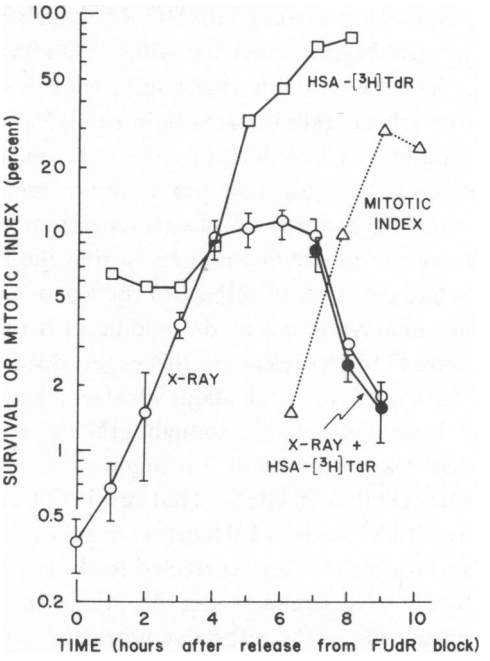

FIGURE 4

FIGURE 3 Colony formation as a function of time of treatment of mitotically collected cells with VLB (added 3.5 h after collection) and either CHI (○) or 500 rad doses of X-rays. (□). The values give the plating efficiencies corrected for killing of S phase cells by HSA-[^{3}H]TdR and the slight toxicity of CHI. The ratio (▲) represents the percent survival after irradiation.

FIGURE 4 Survival of cells irradiated with 500 rads and/or treated with HSA-[^{3}H]TdR at various times following release from a 14.5 h FUdr resynchronization block of mitotically collected cells. □, HSA-[^{3}H]TdR added at the times indicated and removed 1.5 h later; ○, irradiated with 500 rads at times indicated; ●, treated with HSA-[^{3}H]TdR for 1.5 h and irradiated with 500 rads at the times indicated (1.0 h after addition of the HSA-[^{3}H]TdR); △, mitotic index.

Survival in cultures to which HSA-[^{3}H]TdR was added at progressively later times after release from the FUdR block is shown in Fig. 4 (squares). For the first 3 h, the treatment resulted in the loss of about 94% of the microcolonies; presumably, 3–6% non-S phase cells were present at the time of treatment, though their origin is unclear. When the HSA-[^{3}H]TdR was added 4 h after bypassing the block, the percentage of cells surviving the treatment increased to 8.7%, indicating that by 4 h postrelease some cells were already in G2 or in the last stages of S, and survival continued to rise as the initiation of treatment was further postponed. The last two points on this curve probably underestimate survival because 20–30% of the cells were in mitosis at those times, and some of these were undoubtedly lost when the medium was removed and the dishes rinsed.

The open circles in Fig. 4 show colony survival in cultures (not treated with HSA-

[^{3}H]TdR) irradiated with 500 rad doses at various times after release. It is clear that survival began to increase within 1 h after bypass of the FUdR block and continued to increase until 4 h after release when it reached a level comparable to that observed for early G2 cells in the experiment of Fig. 3. By this time the majority of cells has presumably reached the latter half of S. Survival did not change significantly for an additional 3 h, indicating that sensitivity remains relatively constant during the period in which the majority of cells progress from late S into early G2. While it is possible that there are minor fluctuations during the latter part of S that might be obscured by heterogeneity with respect to the age of individual cells, we conclude that cells reach maximal resistance at the middle of S phase and retain it until they pass the arrest point (7 h after release in this experiment; the triangles in Fig. 4 show that the mitotic index reached a peak about 9 h after release.)

It seemed possible, though unlikely in view of the results presented above, that the decrease beginning at 7 h might also partially reflect a higher sensitivity of cells in early G2 than in late S. That this is not the case was shown by adding HSA-[^{3}H]TdR to parallel cultures 1.0 h before irradiation, and removing it 0.5 h after irradiation. The survival values were corrected for killing by the HSA-[^{3}H]TdR alone, and are shown by the solid circles in Fig. 4. It is clear that although survival seems to be slightly lower than in the respective untreated cultures (probably reflecting an increased proportion of sensitive postarrest G2 cells resulting from removal of the resistant S phase cell fraction) the differences are very small, and certainly the response of the treated cells changes in the same fashion as does that of untreated cells; that is, the 7 and 9 h values are reduced proportionally.

The results described up to this point clearly show that sensitivity to X-rays remains approximately constant from early G2 until the arrest point, where it abruptly increases to that found for mitotic cells. These experiments give no indication, however, of the radiation sensitivity of cells that have been arrested in G2 by an earlier exposure to X-rays. In order to determine the sensitivity of arrested cells, two-dose irradiations were carried out, the first dose serving to arrest the cells. Mitotically collected cells were resynchronized with 1 μM FUdR and irradiated with 400 or 650 rads in late S/early G2, 5.5 h after release from the FUdR block. At various times after the conditioning dose, the cultures were again irradiated with 400 or 650 rads, respectively. The results (Fig. 5) indicate that as the interval between irradiations is increased from 0 to 2 h, survival increases, probably as a result of the repair of sublethal damage (Elkind and Sutton, 1959). As the interval is extended further, survival begins to decrease. In this experiment, survival began decreasing by 3 h in the cultures receiving a 400 rad initial dose, while in cultures receiving 650 rads, the decrease was delayed 2 h longer.

Fig. 6 shows the changes in mitotic index and in the fraction of cells divided in these populations as a function of time. The increase in response (Fig. 5) seems definitely to occur before the escape of cells from G2 arrest. That is, if cells progress to mitosis after escape from arrest at the same rate as unarrested cells traverse this portion of the cycle (Fig. 2), then escape occurred no earlier than 1.5 h before mitosis, while survival began to fall before this time.

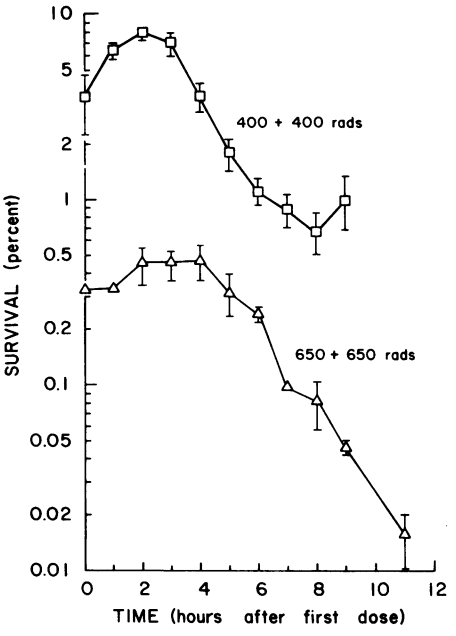

FIGURE 5

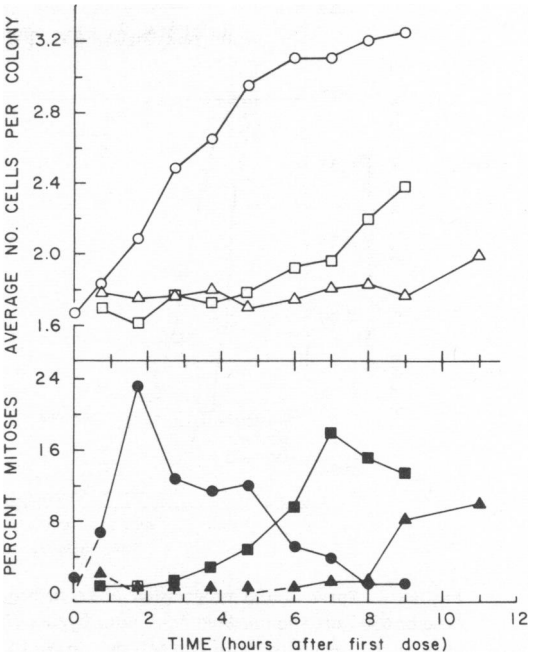

FIGURE 6

FIGURE 5 Survival of cells exposed to two doses of 400 rads each, or two doses of 650 rads each, separated by various intervals of time, the first dose being administered 5.5 h after release from a 12.5 h FUdR resynchronization block of mitotically collected cells. Survival after the first 400 rad dose was 25%, and after the first 650 rad dose, 6.5%.

FIGURE 6 Cell progression, as indicated by the increase in cell number (upper plots) and changes in mitotic index (lower plots), in unirradiated and irradiated cultures. 5.5 h after bypass of a 12.5 FUdR resynchronization block of mitotically collected cells, cultures were irradiated with 400 rads (squares) or 650 rads (triangles). The circles refer to unirradiated controls. The lines projecting downward from several of the symbols in the lower part indicate that the mitotic index lies between zero and the value plotted.

This finding was confirmed and extended by an additional experiment in which the rate at which arrested cells become more sensitive was compared with the rate at which irradiated cells recover from arrest, i.e., enter mitosis. Cultures were treated as in the experiment of Fig. 5, except that only one dose regimen (650 + 650 rads) was used. Two additional cultures were followed for cell progression, entry into mitosis being monitored by time-lapse cinemicrography. One of these (23 cells) received a 650 rad dose at the same time the conditioning dose was administered, while the other (30 cells) served as an unirradiated control. The step functions in Fig. 7 show the entry of cells into mitosis in these two cultures. It is apparent that a median delay of about 11 h was induced by a 650 rad X-ray dose, and that the variance in the delay is large (as has been observed previously). Thus, while 29 of the control cells divided over a period of 3.5 h, a period of almost 15 h was required for division of 22 irradiated cells (one cell in each culture failing to divide within 43 h after irradiation).

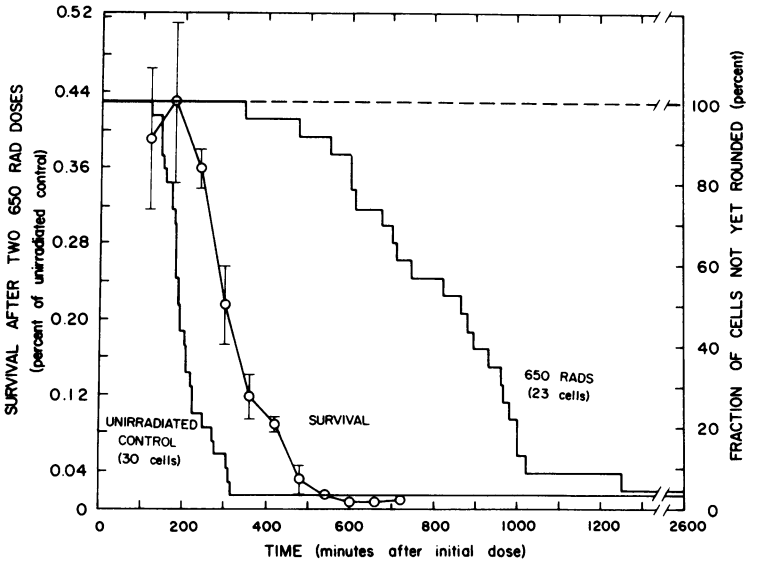

FIGURE 7 Survival and progression of resynchronized cells arrested in G2 by a conditioning dose of 650 rads administered 5.5 h after bypass of the FUdR block. The circles show the survival of cells irradiated with two 650 rad doses, the second dose being administered at various times after the conditioning dose. The step functions represent progression of control and irradiated (conditioning dose only) cells into mitosis as determinnd by time-lapse cinemicrographic observations of cell rounding.

The circles in Fig. 7 show the survival data. The ordinate has been adjusted so that peak resistance (180 min point) falls at 100% on the progression curves. The change in response to the second dose can thereby be compared directly with the rate of progression of cells into mitosis. As in the previous experiment, it is clear that arrested cells acquire increased sensitivity to a second dose several hours (9 h for the median cell) before they reach mitosis. Furthermore, the slopes of the curves indicate that the rate at which increased sensitivity is acquired is much greater than the rate at which the cells of the irradiated population enter mitosis, and only slightly slower than the rate for unirradiated cells.

Rigorous comparison of these rates would require construction of an age-survival curve from the rate of progression, making the assumption that sensitivity increases abruptly at mitosis. (Since it is impossible to detect the escape of cells from arrest, the transition must be assumed to occur as cells enter mitosis; the actual transition, of course, occurs several hours earlier.) The assumption of an abrupt change implies that the overall sensitivity of the population can be determined from the relative proportions of cells having either of only two sensitivities, one characteristic of premitotic cells and the other of mitotic cells. The equation $S = S_r f + S_s(1 - f)$ would obtain, where S is the predicted fractional survival, S_r the fractional survival of resistant (premitotic) cells, S_s the fractional survival of sensitive (mitotic) cells, and f the fraction of premitotic cells (not yet rounded). The measured fractional survival values at maximal and minimal resistance, 4.30×10^{-3} and 8.0×10^{-5}, respectively, would be taken as the respective values of S_r and S_s. However, because of the 54-fold difference in

these values, the second term becomes significant only when $1 - f$ approaches unity, and since the present progression data apply to only 23 cells, one of which does not round for division, $1 - f$ never becomes large enough to allow a contribution from this term. Hence, direct comparison of the time course for change in survival with that for cell rounding is justified.

Rigor would also demand that calculation of the survival values in Figs. 5 and 7 be made on the basis of single cells instead of microcolonies, as the number of cells per microcolony, $\bar{N}$, did not remain strictly constant during the interval over which the second dose was administered. When values for $\bar{N}$ (obtained from the cinemicrographic observations) are taken into account (Elkind and Whitmore, 1967, Chap. 2) for the experiment of Fig. 7, maximal survival ($\bar{N} = 1.8$) decreases from 4.30×10^{-3} to 2.46×10^{-3}, and survival at the most sensitive point (660 min; $\bar{N} = 2.0$) decreases from 8.0×10^{-5} to 4.2×10^{-5}. Minimum survival is therefore actually 58.6-fold smaller than maximum, and if the latter is set to equal 100%, the former is corrected from 100/53.8 = 1.86% to 100/58.6 = 1.71%. However, as this difference is negligible, the shape of the survival curve is not altered.

DISCUSSION

These results clearly indicate that HeLa S3 cells undergo an abrupt transition with respect to X-ray-induced cell killing at or near the G2 arrest point. Cells irradiated in G2 before they have reached the arrest point exhibit survival values typical of (resistant) late S cells, while cells irradiated after having passed the arrest point exhibit survival values that are closely similar to those of (sensitive) mitotic cells. The findings are summarized in the upper curve of Fig. 8 where the survival probability of a microcolony of multiplicity 1.8, irradiated with 500 rads, is plotted as a function of time after release from temporary arrest at the G1/S transition. That the resynchronization treatment with FUdR does not perturb the radiation response is indicated by the fact that survival in late S/early G2 is the same in resynchronized cultures (Figs. 3, 4) as in cultures not so treated (Fig. 1), about 10% after a dose of 500 rads.

It is not known whether an abrupt transition at the arrest point occurs universally, but as most cells exhibit maximal sensitivity in mitosis (Sinclair, 1968), as well as X-ray-induced arrest in progression during G2 (Okada, 1970), it seems likely that such behavior will be widely found. In this connection, we note that it has been reported (Sinclair and Morton, 1966) that a marked decrease in survival occurs when S phase cells are eliminated from a mitotically synchronized Chinese hamster V79 cell population (not resynchronized at the G1/S transition) containing mostly late S and early G2 cells. It was concluded that the entire G2 phase is probably equally sensitive in V79 cells. A similar, though smaller, decrease in survival has been reported (Djordjevic and Tolmach, 1967) for mitotically collected HeLa S3 cells (again not resynchronized at G1/S) when cultures containing S (mostly) and G2 cells were freed of the S phase cells. It seems likely, on the basis of the results found in the present work, that the age-heterogeneity present by the time the unresynchronized cultures reached G2 can account for the earlier results: the cultures probably contained a greater proportion of sensitive postarrest cells than do resynchronized cultures.

The results presented in Figs. 5–7 indicate that cells arrested in G2 by a conditioning

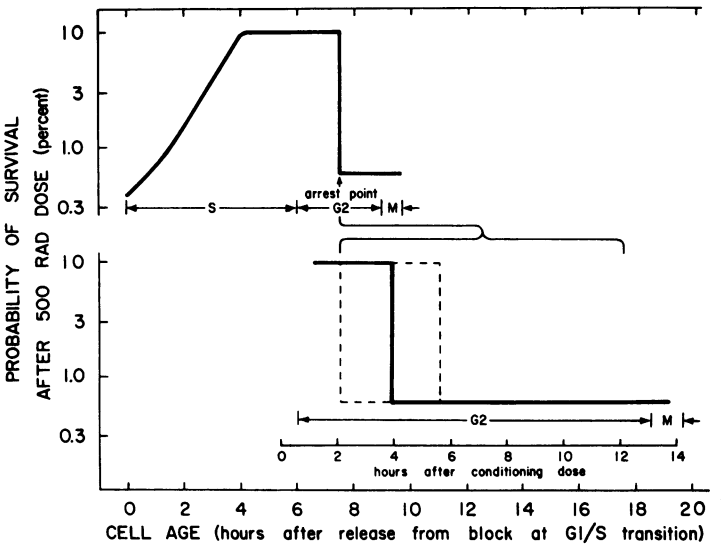

FIGURE 8 The upper curve shows the probability of a microcolony (multiplicity approximately 1.8) surviving irradiation with 500 rads at various times during the latter part of the generation cycle, after resynchronization of the G1/S boundary. The line is drawn from the experimental results presented in Fig. 4 for S and G2 cells, Fig. 3 for prearrest G2 cells, and Fig. 1 for pre- and postarrest G2 as well as mitotic cells (differences in cell multiplicity being taken into account in collating the data). The sharp drop represents the transition at the arrest point. The lower curve represents the probability of survival of a 1.8-cell microcolony to a second dose of 500 rads (survival after the first dose being taken as 100%), administered at various times after treatment with a conditioning dose of 500 rads delivered 5.5 h after resynchronization at the G1/S boundary. The bracket represents the duration of the G2 arrest induced by the conditioning dose (determined from interpolation of the data in Fig. 6 and from other experiments not shown). The dashed lines indicate estimates of the earliest point (inception of arrest) and the latest point (3.5 h later; see Fig. 7) at which arrested cells might undergo the transition from the sensitive to the resistant state, while the heavy line indicates the most probable transition point. This curve does not include changes in survival that would result from repair of sublethal damage.

dose of X-rays acquire the sensitivy of postarrest (and mitotic) cells soon after their arrest, although exactly how soon is not clear. If progression of the irradiated cells in these experiments was the same as that of controls, most of them would have reached the arrest point 1.0–1.5 h after receiving the conditioning dose, since half of the control cells divided within 3 h (Figs. 6 and 7) and the postarrest portion of G2 occupies 1.5–2.0 h (Fig. 2). Sensitivity appears to have begun increasing shortly after the cell reached the arrest point, i.e. between 3 and 4 h after the initial 650 rad dose. The steep slope of the survival curve in Fig. 7 provides an additional indication that the transition occurs early during arrest, i.e. before development of the dispersion in cell age seen at entrance into mitosis. According to this interpretation of the data, the temporal displacement of the two curves in Fig. 5 would indicate that the interval between arrest and change in radiation sensitivity is dose dependent. However, the change in response could occur sooner after arrest than the data suggest, as it has been reported that irradiation may retard the progression of cells toward the arrest point (Highfield and

Dewey, 1975). (If dose dependent, such retardation could give rise to the temporal discrepancy of the curves in Fig. 5.) Moreover, a very early increase in sensitivity might be obscured by an increase in survival deriving from further repair of sublethal damage. Hence the change in response cannot be precisely located. Our estimate of its range is indicated by the dashed lines in the lower section of Fig. 8, which depicts the presumed change in response to a dose of 500 rads shown by a microcolony of multiplicity 1.8 that has been arrested by a conditioning dose of 500 rads.

The slope of the age-response curve in Fig. 7 gives an indication of the rapidity with which the change in sensitivity occurs in individual cells, and of the population age-dispersion of the change. If it is assumed, on the basis of the preceding results (Figs. 1 and 3), that cells in G2, including those arrested by irradiation, possess one sensitivity up to the arrest point and a greater sensitivity thereafter, then survival of arrested cultures will be determined by the proportion of resistant and sensitive cells present at any time. If the sensitivity of arrested cells changes slowly, or if individual cells change from the resistant to the sensitive state at widely different times during the arrest period, the rate of change in the response of cultures containing such cells would be expected to be markedly slower than the rate of progression of unirradiated cells into mitosis. It is clear from Fig. 7 that the former is almost as great as the latter (while it is much greater than the rate at which the irradiated cells reach mitosis). Thus, not only do arrested cells apparently become more sensitive immediately or soon after they reach the arrest point, but the rate of transition from the resistant to the sensitive state in individual cells is presumably rapid, and cells seem to undergo this transition at about the same point in the arrest period.

At least two types of explanations can account for the abrupt increase in sensitivity observed at the arrest point. The first depends on the concept of cellular repair of radiation damage; the second involves the idea that a discrete cellular event occurs at or near the arrest point. Explanations of the first type assume that the time available for repair of damage before its "registration" or "fixation," i.e., the initiation of a process that results in the expression of that damage (in the present case, ultimate cell death), influences the probability that the damage will be expressed. This type of model was invoked previously (Terasima and Tolmach, 1963*a*) in explanation of the greater X-ray response of late than of early G1 cells; DNA synthesis was suggested as the critical process (Kimball, 1961). In the present case, mitosis would be the critical process; such an assignment seems reasonable not only on the basis of the well studied production of chromosome aberrations (Lea, 1955), but also because kinetic evidence has been adduced that mitosis is a preferred period in the generation cycle for the expression of radiation damage. For example, cells irradiated with 500 rads spend about twice as long in mitosis as do unirradiated cells, defective mitoses occur with high frequency after irradiation, and (at least in certain systems) cells suffer permanent mitotic arrest as a consequence of a lethal exposure to X-rays (Hurwitz and Tolmach, 1969). Arrest in G2 might permit the cell to eliminate damage that would be lethal if mitosis occurred immediately or within a short time after irradiation. Because an increase in dose produces both a longer delay and more cell killing (and from the very

fact that cells are killed by X-rays), the potential elimination of damage would have to be highly incomplete, or the irradiated cell would have to contain additional, nonrepairable kinds of damage. Moreover, if it is assumed, in conformance with current ideas, that lethal damage resides in the DNA or chromosomes, and if the damage is repaired in a time-dependent fashion beginning immediately after irradiation (Sax, 1939; Painter, 1970), survival should increase as the time between irradiation and mitosis is increased from 0 to 2 h. That this is not the case is shown by the data in Fig. 1: cells collected 2 hr after receiving a 200 rad dose exhibit the same survival level as mitotic cells. The data in Fig. 7 also indicate that repair processes operating on this time scale do not affect the survival level: response to the second dose begins to increase at least 3 h before cells begin to enter mitosis (and the median cell enters mitosis 9 h after the response is half-maximal). Hence, if repair is relevant to the transition in response at the arrest point, its rate must be slower than known DNA and chromosome repair rates. Accordingly, while explanations of the first type are not ruled out (e.g., much of the lethal damage might reside in structures other than DNA or the chromosomes), they are not very attractive.

Many possible explanations of the second type can be imagined. This type of model has been offered in explanation of the large decrease in radiation response that occurs in early G1: on the basis of ultrastructural studies, Dewey et al. (1972) have proposed that a decondensation of chromatin fibers occurs, resulting in a decreased probability of interaction between lesions. In the present case, it is possible, for example, that a change occurs in cellular metabolism or in the conformation of chromatin at the arrest point, resulting in an increased sensitivity to radiation. Alternatively, the change could take place in the repair capabilities of the cell, rather than in its intrinsic sensitivity; i.e. a cell may be unable to repair radiation damage after passing the arrest point, for reasons other than insufficient time before reaching mitosis. In either situation, response to radiation administered just past the arrest point or just preceding mitosis would be the same, as is observed, since at neither time would the cell be able to repair the damage. That there is indeed a specific point in G2 where the cell undergoes a discrete metabolic change is suggested by the fact that a number of distinct transitions occur nearly simultaneously, about 1 h before mitosis. These involve not only radiation-induced phenomena—arrest itself, the expression of chromosome aberrations at the next mitosis (Dewey, 1975), the expression of deficient DNA synthesis in the next generation (Griffiths and Tolmach, 1975), and the abrupt change in survival described here—but also phenomena that are not associated with radiation, including loss (with respect to progression into mitosis) of sensitivity to inhibitors of protein synthesis and to high concentrations of actinomycin D (Highfield and Dewey, 1975).

Choice between the two types of explanations might be afforded by the imposition of a delay in the progression of postarrest cells to mitosis, by some means that neither interferes with repair nor introduces other lesions. Unfortunately those inocuous agents that would be expected to delay postarrest cells, such as low or high temperatures, or hypoxia, seem also to inhibit repair of sublethal damage (e.g., Berry and Oliver, 1964; Ben-Hur et al., 1974; Howard, 1968) and might similarly inhibit repair

that could occur as a result of the imposed delay. Despite the absence of the desired test, the multiplicity of phenomenologically distinct changes in cellular behavior at the arrest point suggest that a fundamental cellular transition occurs there.

The results presented here have potential applicability to the problem of rationalization of radiotherapeutic regimens. If the response of a tumor cell population to a course of (fractionated) X-ray therapy is to be calculated, using measured cellular responses and a suitable mathematical model (e.g., Steward and Hahn, 1971), the detailed age-response function must be known or assumed. Data concerning the responses of cells in G2, and in G2 arrest, are directly pertinent to such an undertaking.

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