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Evidence that Repair and Expression of Potentially Lethal Damage Cause the Variations in Cell Survival after X Irradiation Observed through the Cell Cycle in Ehrlich Ascites Tumor Cells

GEORGE ILIAKIS AND MICHAEL NÜSSE

Institut für Biophysik and Gesellschaft für Strahlen- und Umweltforschung, Paul-Ehrlich-Str. 20., D-6000 Frankfurt/M, West Germany

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The survival of synchronously growing Ehrlich ascites tumor cells (EAT cells) was measured after X irradiation in various stages of the cell cycle. Cells at the beginning of S or in G2 + M phase showed a high level of killing, whereas cells irradiated in G1 or in the middle of S phase were more resistant. These changes resulted from a change in the survival curve shoulder width (D_q) as cells passed through the cell cycle, and the mean lethal dose (D_0) remained practically unchanged (0.8 ± 0.05 Gy). When synchronization of the cell population was further sharpened using nocodazole, exponential survival curves were obtained at the beginning of S phase and at mitosis with a D_0 = 0.8 Gy. When cells (in all stages) were incubated in balanced salt solution for 6 hr after irradiation, repair of potentially lethal damage (PLD) was observed, resulting in an increase in D_q, while D_0 remained constant. Treatment of the cells after irradiation with either caffeine (2–6 mM) or β-arabinofuranosyladenine (β-araA) (60–100 μM) or hypertonic medium resulted in an expression of PLD and reduced the D_q of the survival curve, which approached or reached an exponential line with D_0 = 0.8 ± 0.1 Gy. We measured the rate of the loss of sensitivity of these treatments that we assume reflects the rate of repair of PLD. For caffeine (6 mM) treatment (S cells, 5 hr) we found a repair time constant (t_{50}) or about 1 hr, similar to that observed for repair of PLD in growth medium containing 0.5 μg/ml aphidicolin. With hypertonic treatment we detected two repair components, a fast one that restored the slope of the survival curve, and a slow one with a t_{50} of about 1 hr that restored the shoulder of the survival curve. PLD induced by irradiating in G1 phase was repaired when cells were arrested for some hours either in G1 phase or in the subsequent mitosis but was not repaired if the cells were arrested in S phase. PLD induced in S or G2 + M phase was repaired only when the cells were arrested in the cell cycle before division. Results indicate that the shoulder width D_q of the survival curve in cells irradiated at various stages of the cell cycle results from repair of PLD. This repair of PLD probably takes place in the interval between irradiation and the next S phase or mitosis and is therefore minimal for cells irradiated at the G1/S border or in mitosis (D_q = 0). PLD still unrepaired when the cells reach these phases is assumed to be expressed, as was found for PLD repaired in cells incubated in balanced salt solution, for some hours after irradiation. We therefore suggest that the variations observed in cell survival through the cell cycle might reflect variations in the final amount of PLD either repaired or expressed as the cells progress through the various stages of the cell cycle.

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INTRODUCTION

Survival of mammalian cells after irradiation can be modified by a variety of postirradiation treatments. This modification has been attributed to the production by X rays of potentially lethal damage (PLD) whose repair or expression was thus modified (*1*). Treatments like incubation of cells after irradiation in balanced salt solution (*2*), with certain metabolic inhibitors (*1*), at low temperatures (*3*) or in the plateau phase of growth (*4–8*) resulted in increased cell survival that was interpreted as repair of PLD. Treatment in anisotonic medium (*9, 10*) or incubation with caffeine (*11–15*) caused a decrease in cell survival interpreted as an increase in the expression of potentially lethal damage.

Ehrlich ascites tumor cells grown in plateau phase in suspension repaired potentially lethal damage if they were not transferred to fresh medium until some hours after irradiation (delayed plating; DP) (*16, 17*). When these cells were treated immediately after irradiation either in hypertonic medium (*18*) or with β-arabinofuranosyladenine (β-araA) (*19, 20*) radiation-induced PLD was expressed. This expression of PLD modified the shoulder of the survival curve but left the slope almost unchanged. Exponential survival curves were obtained when the cells were treated either with 120 μM β-araA for 4 hr or in hypertonic medium (Pos/Piso = 2.5) for 40 min. Similar expression of PLD was also observed in "aged" plateau-phase EAT cells grown as a monolayer (*21*) when these were subcultured in fresh medium immediately after irradiation.

These results implied that the shoulder of the survival curve resulted from repair of X-ray-induced PLD. Further, since β-araA is an inhibitor of the DNA-dependent DNA polymerases α and β [e.g., (*22–24*)], it was suggested that the repair of PLD might involve DNA polymerization.

A variation in cell survival after X irradiation occurs through the division cycle (*25*). Although the response of the various cell lines examined has not been found to be invariant, some common features have been found (*26, 27*). Among the differences observed are the alterations that the changes in survival cause in the parameters of the dose–effect curve. In HeLa cells (*28*), for example, mainly the slope of the survival curve changed when cells were irradiated in various phases of the cell cycle, whereas in other cell lines (*26*) more change in the shoulder width of the survival curve was seen.

In preliminary experiments with synchronized Ehrlich ascites tumor cells we observed survival changes almost exclusively attributable to variations in the shoulder width of the survival curve during the cell cycle. Since we have already shown that the shoulder of the survival curve results from repair of X-ray-induced PLD as discussed above, we asked the question: could variations in repair and/or expression of PLD through the cell cycle be the cause of the variation in cell survival. In this paper we investigate this possibility.

MATERIALS AND METHODS

A line of mouse Ehrlich ascites tumor cells (EAT cells) first isolated by Löwenthal and Jahn (*29*) and subsequently subcultured by Karzel (*30*) was used. The cells were grown in suspension in A2-medium (*16*) supplemented with 20% horse serum.

Cultures of exponentially growing cells were prepared (cells were passaged daily by dilution to 2×10^5 cells/ml) at an initial concentration of 10^5 cells/ml. The cells were incubated at 37°C. CO_2 gassing was not necessary for daily subcultured cells due to the presence of HEPES in the medium. The cells were allowed to grow to a concentration of $5–8 \times 10^5$ cells/ml, and were then used as a source of synchronously growing cell populations.

Synchronized cells were prepared by a method combining cell selection and chemical block (*31, 32*). Briefly, G1 cells were selected from an exponentially growing culture using velocity sedimentation in sucrose gradients (80 to 250 g/liter) in a zonal rotor (TZ 28, Sorvall) with a reorienting gradient. In this way $4–6 \times 10^7$ cells in G1 phase were selected. These G1 cells were either used immediately (or sometimes after an incubation of 6–8 hr at 10°C) or incubated for 6–8 hr in fresh medium containing either 2 m*M* thymidine or 1.5 μg/ml aphidicolin (*33*). Aphidicolin and to a lesser extent thymidine greatly reduced DNA synthesis and caused an accumulation of the selected G1 cells at the beginning of S phase. No significant variations in the radiation response of the cells were observed when aphidicolin was used instead of thymidine to resynchronize cells. The distribution of DNA content in this resynchronized population was practically indistinguishable from that of the original G1 cells. This indicated that the cells must have accumulated at the very beginning of S phase; they will therefore be called G1/S cells in this work. After incubation, thymidine or aphidicolin was washed out and cells were resuspended in fresh medium (F-medium). At different times thereafter samples were taken for the experiments. Three hours after block release the cells were in mid-S phase, and after 6 hr they were mostly in G2 + M phase. The durations of the cell cycle phases were: $t_{G1} = 2.4$ hr, $t_S = 5.0$ hr, and $t_{G2+M} = 2.6$ hr.

The survival of cells was measured by plating cells on soft nutrient agar after appropriate dilution as previously described (*29*). 2×10^4 cells/ml preirradiated with 50 Gy X rays (feeder cells) were mixed with nutrient agar to improve the plating efficiency of the cells (40–80%). Twenty to five hundred colonies were counted per dish; duplicate dishes were prepared for each point. All survival curves shown are single-cell survival curves. The standard errors in the estimation of survival were less than 15% unless otherwise stated. The curves were fitted to the data by eye. The experimental results were confirmed in several independent experiments.

For some experiments on repair of PLD, cells were incubated in a balanced salt solution (BSS) devised for EAT cells. Composition and properties of this salt solution have been described (*34*). In some experiments conditioned medium obtained from plateau-phase cultures (C-medium) was used. It was obtained from 4-day-old cultures prepared at an initial concentration of 10^5 cells/ml.

The following drugs were used: aphidicolin (a gift from Dr. Todd, ICI) dissolved in DMSO (10 mg/ml) and stored frozen; nocodazole (EGA-Chemie) dissolved in DMSO (4 mg/ml) and stored frozen; chromatographically pure β-araA (H. Mack, Illertissen, West Germany, a gift from Prof. Dr. W. E. G. Müller, Mainz) dissolved in balanced salt solution (10 m*M*); caffeine (Merck) dissolved in balanced salt solution (100 m*M*); thymidine (Serva) dissolved in water (200 m*M*).

Cell cycle distributions of the cell populations were checked by measuring DNA distributions of cells in a flow cytometer (ICP 21, Phywe, West Germany). Cells were

stained with the fluorochrome Hoechst 33258 (Riedel-de Haen, West Germany) according to Böhmer (*35*). For further details concerning measurement and evaluation of the histograms see Ref. (*34*). Figure 1 shows DNA histograms of G1 cells immediately after selection, cells in S-phase (at various times after block), and cells in G2 + M phase (6 hr after block release). The DNA histogram obtained with an asynchronously growing population is also shown for comparison.

Cells were irradiated in air in suspension (in 3-cm petri dishes, 2–3 ml per dish, stirred during irradiation) with 140-kV X rays (0.7 mm Al filtration) having an effective photon energy of about 20 keV with respect to the photon attenuation coefficient in water. Absorbed dose was measured with an energy-independent ferrous sulfate dosemeter (36). The absorbed dose rate was about $\dot{D}$ = 10 Gy/min. Cells were plated either immediately after irradiation (IP) or after a few hours treatment under various postirradiation conditions (DP).

RESULTS

Cell Survival Variations and Ability to Repair PLD in Cell Cycle

The survival of synchronized EAT cells exposed to 6 Gy of X rays at various stages of the cell cycle and plated immediately after irradiation is shown in Fig. 2 (solid triangles). G1 cells were obtained by velocity sedimentation and were used for 0, 1, and 2 hr. All subsequent measurements were performed with cells that had been resynchronized by a 6-hr thymidine block (2 m*M*). Cell survival decreased as the cells approached the G1/S border, increased during S phase (maximum at 6 hr), and

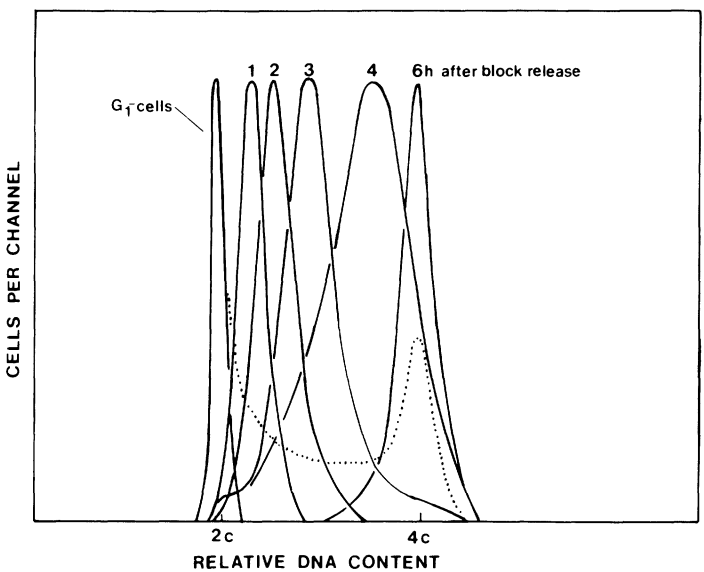

FIG. 1. DNA histograms of G1 cells obtained immediately after selection by centrifugation in sucrose gradients (see Materials and Methods) as well as of G1 cells resynchronized with a 6-hr thymidine block at various intervals after release of the block. The dotted line shows for comparison the DNA histogram of asynchronously growing cells used for the selection.

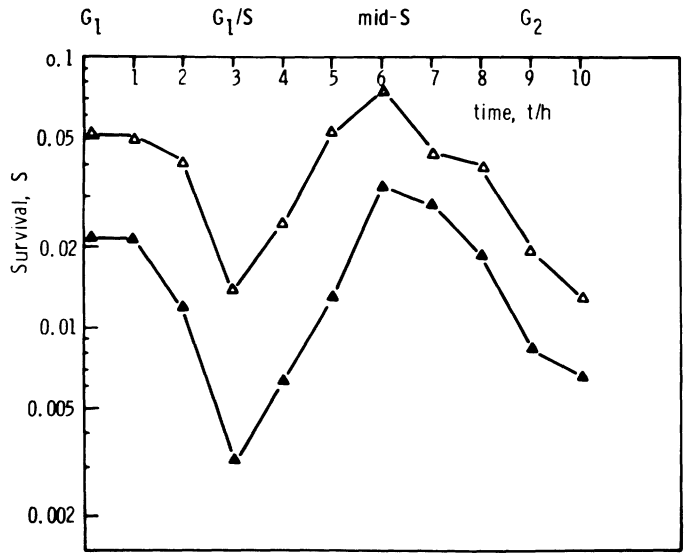

FIG. 2. Survival of X-irradiated (6 Gy) synchronized cells plated either immediately after irradiation (solid triangles) or after a 6-hr incubation in BSS (open triangles). The results up to t = 2 hr were obtained with selected G1 cells; the subsequent measurements were performed with cells resynchronized by a 6-hr thymidine block (2 mM) (also see text).

decreased again as the cells progressed toward G2 phase. This pattern was reproducible in all experiments, but some variation in the survival values was observed, especially for cells exposed in G2 + M phase or (to a lesser extent) at the G1/S border.

Incubation of cells after irradiation in BSS for 6 hr before plating resulted in a significant increase in cell survival in all stages of the cell cycle (open triangles in Fig. 2), indicating repair of X-ray-induced PLD under conditions delaying or inhibiting progression of the cells through the cell cycle [this was checked by flow cytometry; see also (*37*)]. Potentially lethal lesions were thus expressed when the cells progressed, after irradiation, without delay through the cell cycle.

The variations in survival through the cell cycle reflected mainly variations in the D_q of the survival curve; the slope remained unchanged. This is shown in Figs. 3a and b, where survival curves obtained with cells irradiated in various stages of the cell cycle are shown. The curves in the figures are labeled with the time at which the cells were irradiated after release of the thymidine block (t = 0 for G1/S cells; the survival of G1 cells is shown in Fig. 3a by the open circles). The D_q of the survival curve was at its minimum at the G1/S border and in the G2 + M phase (1.0–1.5 Gy), where the lowest survival levels were observed (Fig. 2), whereas the D_0 remained constant (0.8 ± 0.5 Gy) for all stages of the cell cycle.

When cells were resynchronized in M phase by incubation for various intervals with nocodazole (0.4 μg/ml, 6 hr after release of thymidine block), the results shown in Fig. 4a were obtained. Nocodazole is a reversible inhibitor of microtubuli polymerization (*38, 39*) that inhibits cell division and accumulates cells in metaphase. This was checked in EAT cells microscopically and by flow cytometry. The D_q of the survival curve was found to decrease as the cells accumulated in M phase, and

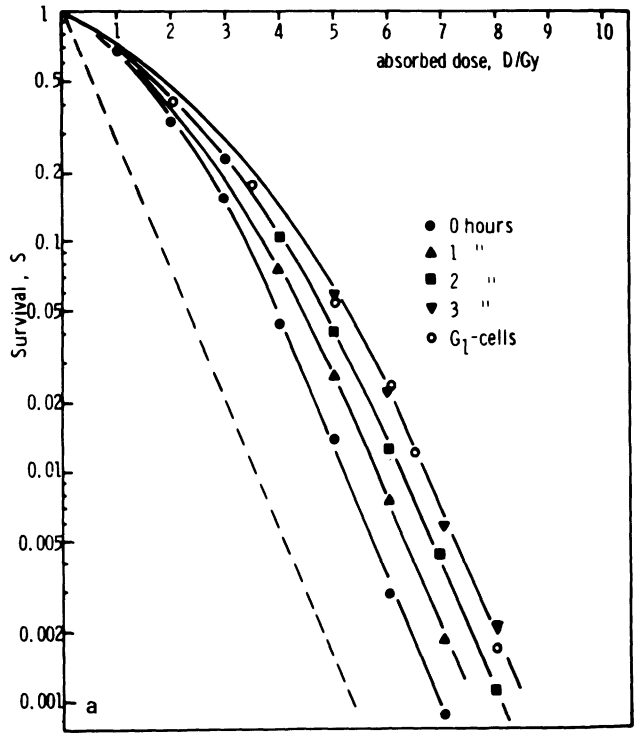

FIG. 3a. Survival curve of synchronized S-phase cells irradiated at various times (0–3 hr) after release of the thymidine block and plated immediately. The survival of cells irradiated in G1 phase is shown by the open circles. The broken line is an exponential survival curve with a slope equal to the final slope of the survival curves measured at the various phases of the cell cycle.

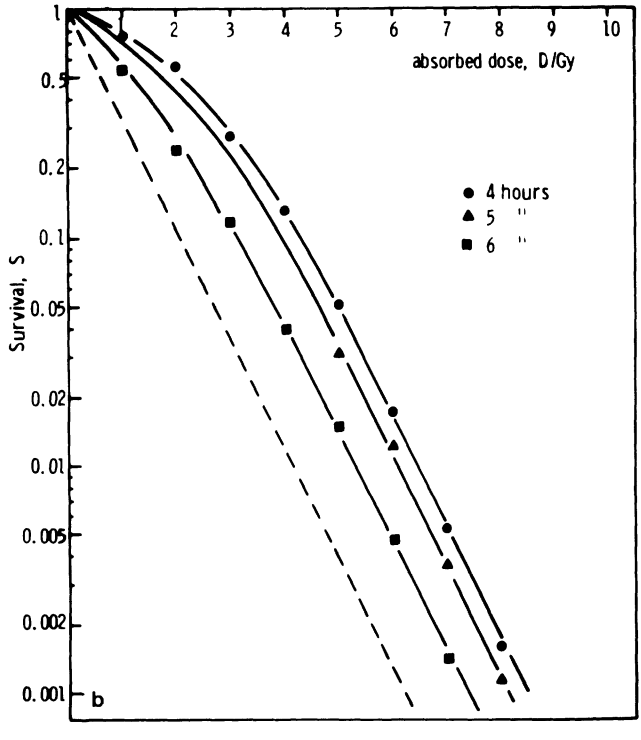

FIG. 3b. Details as in Fig. 3a except that cells were irradiated 4–6 hr after release of the thymidine block.

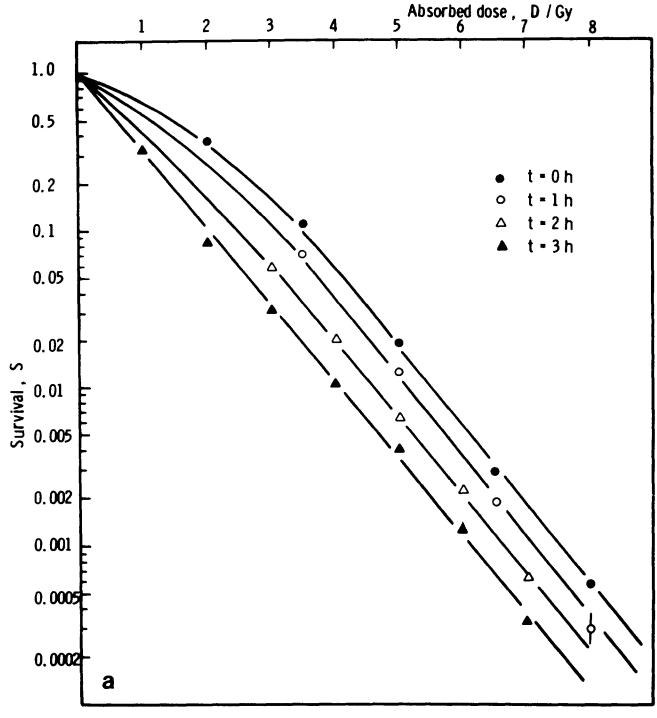

FIG. 4a. Survival curves of G2 + M cells (6 hr after resynchronization with thymidine) obtained at various intervals after addition of nocodazole (0.4 μg/ml) to accumulate cells in metaphase.

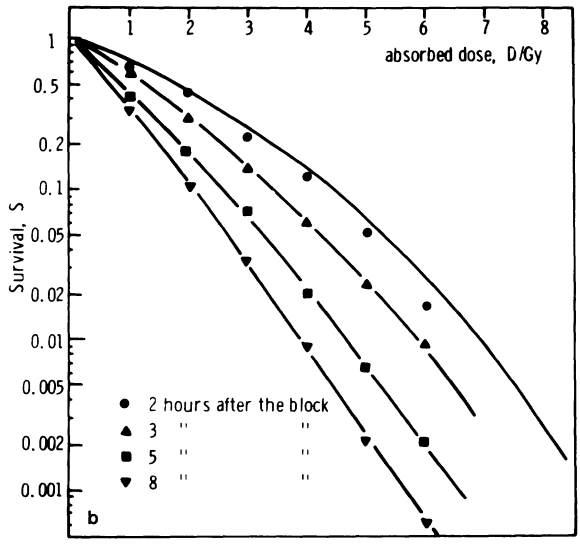

FIG. 4b. Survival curves of cells irradiated in G1 phase that were harvested 2 hr after washing out nocodazole (0.4 μg/ml) added to G2 + M cells for 4 hr (solid circles without line) compared with the survival curve obtained for G1 cells immediately after selection by centrifugation (upper survival curves without points). The solid triangles show survival when cells were incubated after nocodazole treatment for 3 hr with aphidicolin (1.5 μg/ml) to accumulate cells at the beginning of S phase; the solid squares and inverse triangles show survival after 5 and 8 hr incubation, respectively, in aphidicolin-containing medium.

it was zero for cells treated with nocodazole (mitotic index 90%) for 3 hr. The D_0 was 0.8 ± 0.05 Gy.

Cells that had been accumulated in M phase by nocodazole divided with a small delay (~1 hr) when resuspended in fresh medium without nocodazole and were in G1-phase after 2 hr. The survival obtained for these cells after irradiation with various doses is shown by the solid circles in Fig. 4b. No significant differences from the survival curve obtained with G1 cells irradiated immediately after selection by centrifugation were observed (upper line in Fig. 4b).

When nocodazole-treated cells were allowed to progress through G1 phase in fresh medium containing 1.5 μg/ml aphidicolin, a specific inhibitor of α-polymerase (*40, 41*), the cells traversed G1 phase unimpeded and accumulated at the G1/S border. This accumulation was due to a complete inhibition of replicative DNA synthesis by aphidicolin (*42*). The survival curves obtained at different times in the presence of aphidicolin after release of the nocodazole block are shown in Fig. 4b. As the cells progressed through G1 phase the shoulder decreased and was essentially zero after 8 hr. At this time the cells had accumulated at the G1/S border. This was also checked by flow cytometry.

Since the resynchronization treatments with nocodazole or aphidicolin did not in themselves affect cell survival or diminish the repair capability of the cells [(*42, 52*); this will be also discussed later in this section], we assume that the elimination of the shoulder occurs merely as the result of the accumulation of cells at the G1/S border or in M phase with a high degree of synchrony and that in those stages of the cell cycle the survival curves are exponential.

In Figs. 5a and b survival curves are shown for G1 and mid-S cells, respectively, which were irradiated and plated either immediately after irradiation (solid circles) or after 6 hr incubation in balanced salt solution with (open triangles) or without (open circles) aphidicolin (0.5 μg/ml). The survival increase observed in cells held in BSS was due to repair of PLD, and affected merely the D_q of the survival curve and was unaffected by the presence of aphidicolin, which was not toxic at the concentration used. Thus the variation in cell survival due either to a progression of the cells through the cell cycle or to repair of PLD reflected variations in the D_q of the survival curve; the D_0 remained constant (0.8 ± 0.05 Gy).

Expression of PLD Caused by Incubation with β-Arabinofuranosyladenine (β-araA)

When synchronized G1 cells were irradiated and incubated for 4 hr in BSS containing various concentrations of β-araA, the results shown in Fig. 6 were obtained. The repair of PLD usually observed in cells incubated in BSS after irradiation and before plating was inhibited in the presence of 60 μ*M* β-araA (open circles for DP, open triangles for 60 μ*M* β-araA-treated cells); in addition, PLD normally repaired in cells plated immediately after irradiation (solid circles for IP) was expressed. One hundred micromolar β-araA was still more effective in expressing PLD (solid triangles).

β-araA affected only the D_q of the survival curve. The D_0 remained unchanged; this was also found for plateau-phase cells (19, 20). Due to the toxicity of β-araA to synchronized G1 cells (PE = 60% for 0 *M* β-araA, 49% for 60 μ*M* β-araA, and 39%

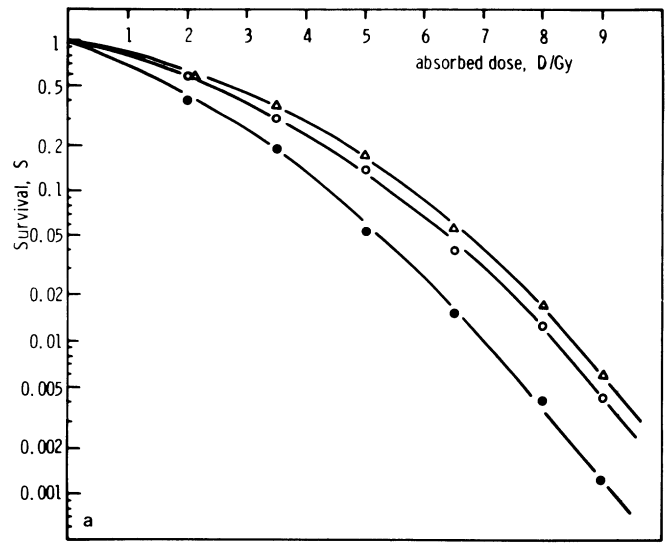

FIG. 5a. Survival curves of G1 cells plated either immediately after irradiation (solid circles) or after 6 hr incubation in either BSS (open circles) or BSS containing 0.5 μg/ml aphidicolin (open triangles).

for 100 μM β-araA), it was not possible to completely eliminate the shoulder of the survival curve by increasing the drug concentration as was previously found (*19, 20*). Nevertheless, the results indicate that the shoulder of the survival curve relies, at least in part, on PLD repair. β-araA was toxic to S cells and less effective on G2 cells.

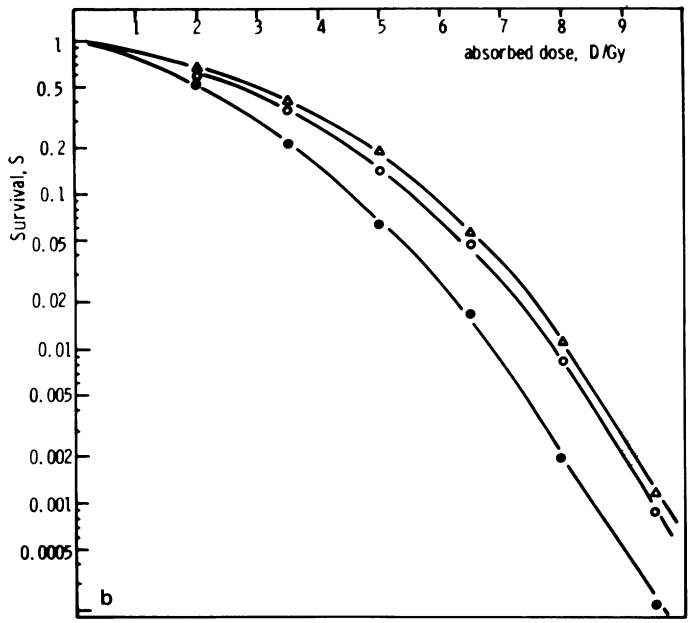

FIG. 5b. Details as in Fig. 5a, except that cells were irradiated in mid-S phase.

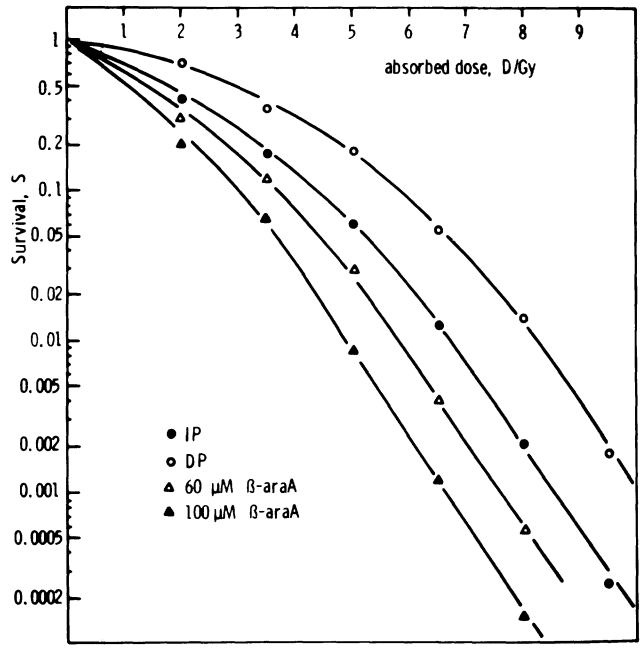

FIG. 6. Survival curves of cells irradiated in G1 phase and plated either immediately (solid circles), after a 4-hr incubation in BSS (open circles; delayed plating), or in BSS containing two concentrations of β-araA (triangles).

Expression of PLD Caused by Incubation with Caffeine

Caffeine expressed X-ray-induced PLD when given to the cells after irradiation in the various phases of the cell cycle. The results are shown in Figs. 7a and b. Eight hours treatment (time necessary for maximal effect; more details on the effects of caffeine on EAT cells will be presented elsewhere) of S cells with caffeine in F-medium at various concentrations after X-ray exposure resulted in expression of PLD that caused a drastic reduction of the D_q of the survival curve (0.5 Gy for 4–6 m*M* caffeine). When the cells were preincubated with caffeine for 30 min (shorter times gave variable results) before irradiation and subsequently held in the presence of caffeine for 8 hr, the survival curve was an exponential line with a $D_0 = 0.8 \pm 0.05$ Gy. Similarly, treatment of irradiated G2 (Fig. 7a, right panel) and G1/S cells (Fig. 7b, right panel) with 6 m*M* caffeine for 8 hr in F-medium (caffeine was also given 30 min before exposure to X rays) resulted in exponential survival curves. G1 cells were rather refractory to caffeine treatment and exponential survival curves were obtained only after 33 hr treatment (Fig. 7b, left panel). The exponential lines obtained in all phases of the cell cycle had a similar D_0 (0.8 ± 0.05 Gy).

Caffeine at the concentrations used did not influence the plating efficiency of the controls by more than 20%, except for G1 cells after long incubation periods, where the PE after 33 hr treatment was 15% compared to 45% of the controls or those treated for 12 hr with caffeine. In cases where cell division took place during incu-

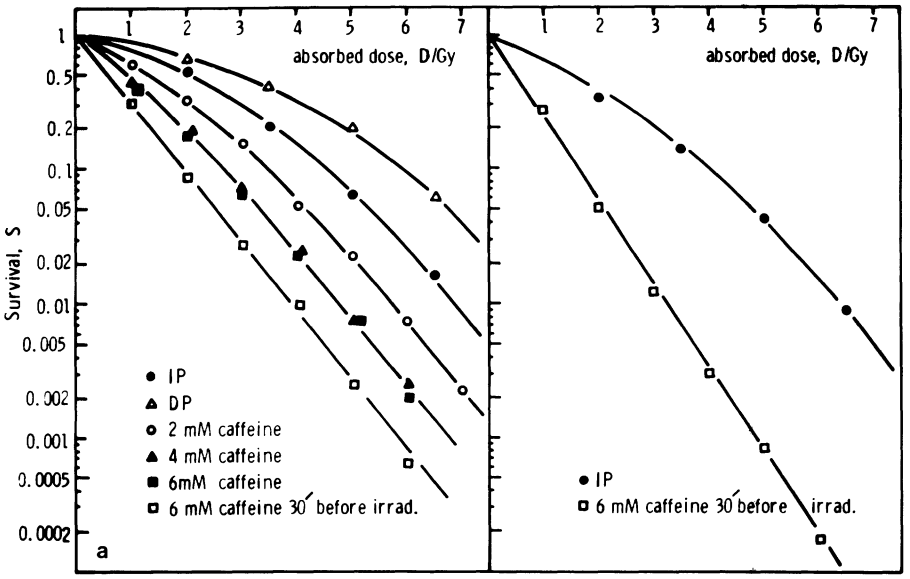

FIG. 7a. Survival curves of G1 cells (left panel) and G1/S cells (right panel) treated with caffeine as indicated.

bation with caffeine, the number of cells were counted and used to calculate cell survival.

To measure the rate with which irradiated cells lost sensitivity to caffeine and thus to obtain information about the rate of repair of potentially lethal lesions that were

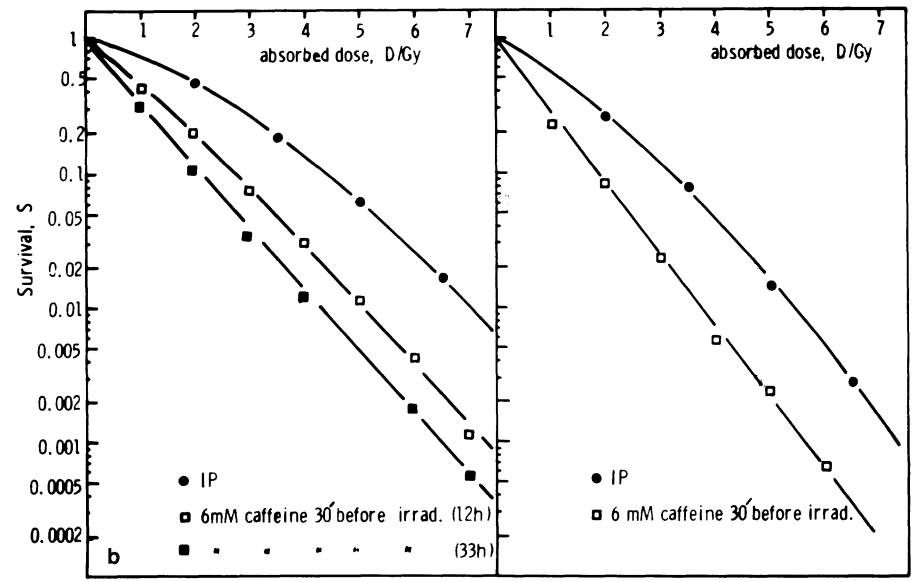

FIG. 7b. Survival curves of S cells (left panel) and G2 + M cells (right panel) treated with caffeine as indicated.

expressed in the presence of 6 m*M* caffeine, S cells were treated with caffeine for 5 hr beginning at various times trep after irradiation. The results are shown in Fig. 8. In this experiment 5 hr treatment with caffeine were enough to achieve expression of unrepaired PLD in S cells to an extent comparable to that observed in the results of Fig. 7b. An increase in cell survival was observed as trep increased, which for a given dose started from a survival level corresponding to that obtained when 6 m*M* caffeine was given immediately after irradiation (Fig. 7b), and after about 3 hr reached survival levels similar to those observed with untreated cells plated immediately after irradiation. This indicated that at 3 hr the caffeine-sensitive steps of the repair were completed. The t_{50} (time necessary to reach 50% of the observed survival increase) was about 1 hr.

The dotted line in Fig. 8 shows the increase in survival as a function of time when S cells irradiated with 6 Gy were incubated in F-medium containing 0.5 μg/ml aphidicolin (delayed plating DP). The t_{50} observed in this case was similar to that measured for caffeine (~1 hr).

Expression of PLD Caused by Incubation in Hypertonic Medium

To study the effect of hypertonic treatment in synchronized cells, we irradiated G1- and S-phase cells with various doses and incubated them immediately in F-medium that was made hypertonic (osmotic pressure Pos) by the addition of NaCl (Pos/Piso = 2.5). The cells were returned to isotonic (osmotic pressure Piso) F-medium and plated at various times thereafter. The results obtained with G1 cells are shown in Fig. 9a and those for S cells in Fig. 9b. Twenty minutes of incubation

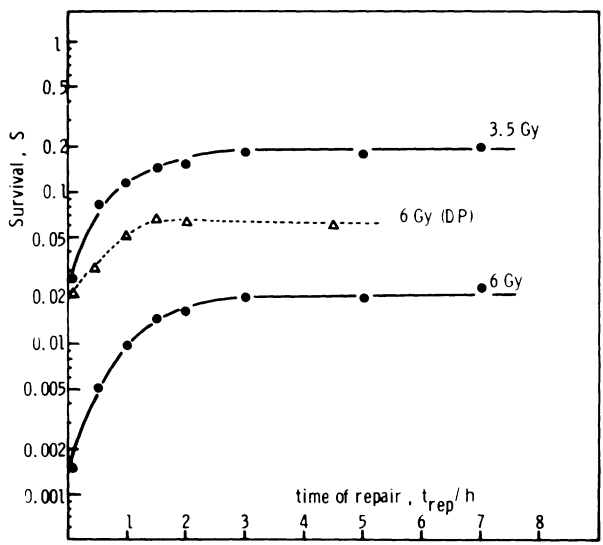

FIG. 8. Loss of sensitivity of S cells to caffeine as a function of time trep after irradiation (after trep cells were treated with 6 m*M* caffeine for 5 hr). The dotted line shows the survival increase as a function of time due to repair of PLD in S cells incubated in F-medium containing 0.5 μg/ml aphidicolin.

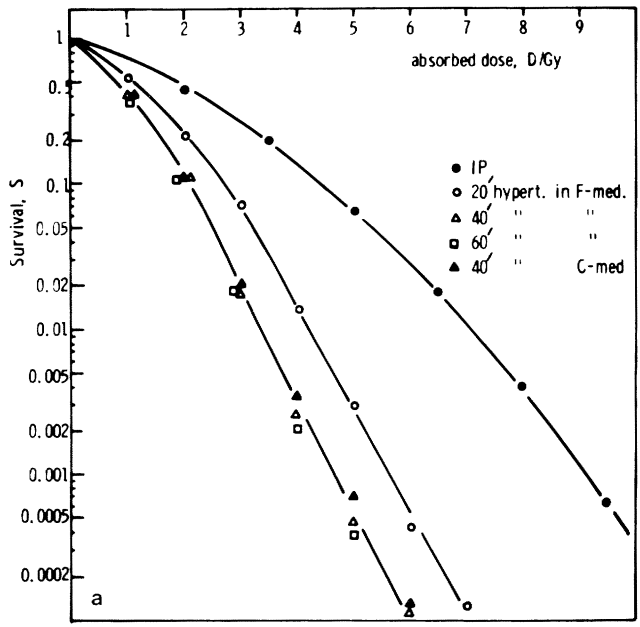

FIG. 9a. Survival curves of G1 cells obtained after incubation for various intervals in hypertonic (Pos/Piso = 2.5) F-medium or C-medium.

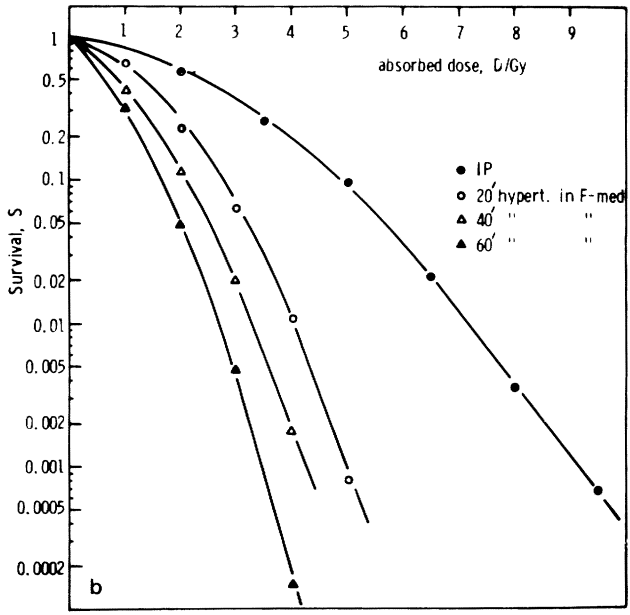

FIG. 9b. Survival curves of S cells obtained after incubation for various intervals in hypertonic (Pos/Piso = 2.5) F-medium or C-medium.

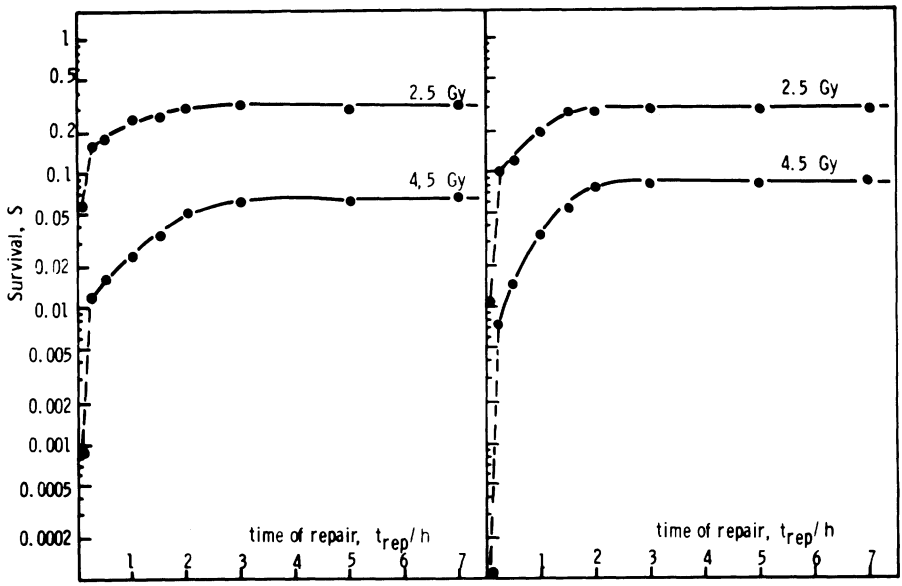

FIG. 10. Loss of sensitivity to 40 min hypertonic treatment (Pos/Piso = 2.5) for G1 cells (left panel) and S cells (right panel) as a function of the repair time trep after irradiation (in F-medium).

in hypertonic medium caused a considerable expression of PLD, and the survival curve (open circles) fell below that for cells plated immediately after irradiation (solid circles). A larger effect was observed when the cells were treated for 40 min, but only a small additional expression was observed after 60 min treatment (especially in G1 cells). No differences in the amount of PLD expressed were observed whether the cells were incubated in hypertonic F-medium or in hypertonic conditioned medium (C-medium, see Materials and Methods) (solid triangles in Fig. 9a). Expression of PLD caused by treatment of the cells in hypertonic medium affected both the D_q and the D_0 of the survival curve; the change in D_0 was more pronounced in S cells (0.4 Gy for S cells and 0.6 Gy for G1 cells treated for 60 min; D_0 of controls 0.8 Gy). A 40-min treatment was adopted for further experiments as was also used previously (*18*), which did not measurably affect the plating efficiency of the controls.

When cells in G1 or S phase were treated in hypertonic F-medium (40 min) for various times after irradiation, kinetic curves were obtained showing the loss of sensitivity to the treatment and indicating the rate at which the sensitive steps to hypertonic treatment of the repair of PLD were completed. The results are shown in Fig. 10 (left panel for G1 cells, right panel for S cells). A rapid increase in cell survival was observed in the first 15 min (shown by the broken line in Fig. 10) and was followed by a slower increase for the next 3 hr. At this time cell survival had reached values corresponding to those observed in untreated cells plated immediately after irradiation. The rate at which cell survival increased after the first rapid increase corresponded to t_{50} values of about 1 hr, increasing with decreasing dose. Larger t_{50} values were found for G1 than for S cells. Qualitatively similar results were found for G2 cells.

The survival curves obtained when S cells were treated in hypertonic medium at various times after irradiation are shown in Fig. 11. In the first 15 min, where the rapid increase in cell survival was observed (Fig. 10), we mainly found a change in the D_0 of the survival curve, which reached 0.7 Gy, similar to the value of 0.8 Gy observed for untreated cells. At longer intervals the D_0 remained unchanged, and repair of PLD increased mainly the D_q of the survival curve, which at 2 hr reached the value observed in untreated cells plated immediately after irradiation (solid triangles).

Fate of Potentially Lethal Lesions as the Cells Progress in the Cell Cycle

The reparability of PLD induced by X rays in cells irradiated in a particular phase of the cell cycle was checked either in the phase where the irradiation was given or in one of the subsequent phases of the cell cycle by arresting cells either by incubation in BSS or with aphidicolin or nocodazole.

Aphidicolin selectively arrested cells in S phase by reversibly inhibiting DNA replication via inhibition of DNA polymerase α (*40–42*); it was used at a concentration of 0.5 μg/ml. At this concentration it inhibited DNA synthesis to about 10% of the controls without affecting the repair capability or the plating efficiency of the cells (*52*). Nocodazole selectively arrested the cells in M phase (metaphase) by reversibly inhibiting microtubule polymerization [(*38, 39*), M. Nüsse and H. J. Egner, in preparation]. Cells were treated at a concentration of 0.4 μg/ml. At this concentration and for treatment times (in mitosis) not exceeding 4 hr nocodazole did not affect the

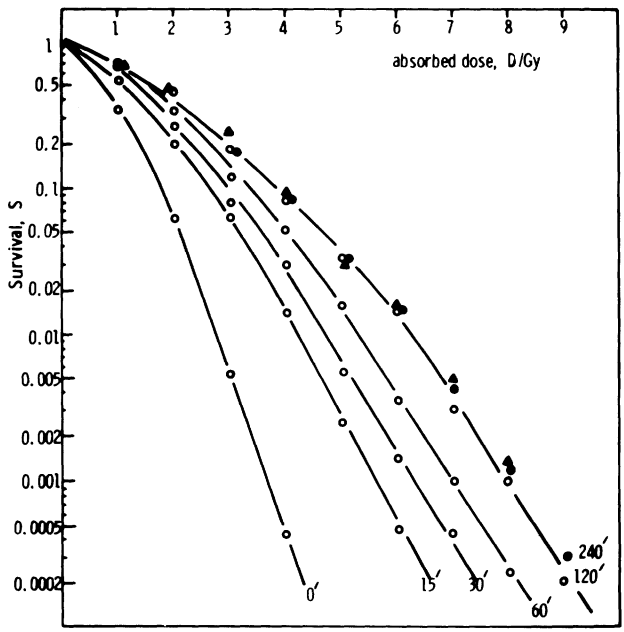

FIG. 11. Survival curves of S cells irradiated and treated in hypertonic F-medium (Pos/Piso = 2.5, 40 min) at various times thereafter as indicated. The solid triangles show the survival of untreated cells plated immediately after irradiation.

repair capability or the plating efficiency of the cells (less than 20% decrease in the plating efficiency).

The results are summarized in Table I. A (+) was inserted in the table when the repair of PLD in cells arrested in a phase of the cell cycle subsequent to the phase where radiation was given (either by incubation in BSS or with the above-mentioned inhibitors) was comparable to that observed in cells arrested for repair of PLD in the phase of the cycle where cells were irradiated; (−) signifies that no repair was observed in cells arrested in a phase of the cell cycle subsequent to the phase in which the cells were irradiated.

These results indicate that PLD remains reparable until the cells traverse either the G1/S border or mitosis as they progress through the cell cycle after irradiation. The fact that cells irradiated in G1 phase repaired PLD when arrested by nocodazole in mitosis, although of interest by itself (these results will be discussed in more detail elsewhere), is not relevant for cells growing either *in vivo* or *in vitro,* since under such conditions no mitotic block occurs. Stages in the cell cycle are thus indicated that, when reached by the irradiated cells, result in a cessation of PLD repair; PLD is then expressed either immediately or in a subsequent division cycle.

DISCUSSION

The results presented in the previous sections show (a) that repair of PLD can be observed in all phases of the cell cycle when the cells are delayed in their progression by incubation either in BSS or (for S cells) in medium containing aphidicolin and that this repair of PLD affected only the D_q of the survival curve; and (b) that treatments such as incubation with β-araA or caffeine or in hypertonic medium permit expression of PLD normally repaired in cells plated immediately after irradiation and reduce or eliminate the shoulder of the survival curve of cells in all phases of the cell cycle leading to the same "basic" exponential line (D_0 = 0.8 Gy). These results also indicate that repair of PLD led to the appearance in every phase of the cell cycle of the shoulder on the survival curve, starting from this exponential line. Since all variations in survival through the cell cycle were found to reflect variations

TABLE I

Repair of PLD in the Stage of the Cell Cycle Where Cells Were Irradiated as well as in Subsequent Stages[a]

Phase of cell cycle where cells were irradiated	*Phase of cell cycle where cells were arrested for PLD repair using treatment 1, 2, or 3*						
	G_1	G_1/S	*mid-S*	$G_2 + M$	*M*	G_1	G_1/S
G_1	$^{+}1$	$^{-}2$	$^{-}1, 2$		$^{+}3$		$^{-}$
mid-S			$^{+}1, 2$	$^{+}1$	$^{+}3$	$^{-}1$	$^{-}2$
G_2 + M				$^{+}1$	$^{+}3$	$^{-}1$	$^{-}2$

[a] See text.

Note. 1: Treatment in BSS for 4–6 hr. 2: Treatment in F-medium containing 0.5 μg/ml aphidicolin. 3: Treatment in F-medium containing 0.4 μg/ml nocodazole.

in the D_q of the survival curve, we assume that cells irradiated in various phases of the cell cycle had different D_q values because they were able to repair PLD to a different extent. These differences in the amount of PLD repaired could be due to repair time restrictions produced by the progression of the cells to cycle stages where PLD repair ceases.

Studying the fate of potentially lethal lesions whose repair is promoted by incubation in BSS or other treatments delaying progression through the cell cycle, we observed that PLD repair ceased when the cells traversed the G1/S border or entered mitosis. Since the survival curves obtained with cells irradiated either at the G1/S border or in mitosis were exponential, we assume that when the cells reach those stages of the cell cycle repair of PLD ceases, and the lesions remaining are finally fixed in subsequent cell divisions. Thus the amount of PLD repaired by cells in a given phase of the cell cycle, and therefore the D_q value of the survival curve, is expected to depend on the time available between irradiation and next S phase (G1/S border) or mitosis. This assumption allows an explanation of the decrease in cell survival observed as the cells move either from G1 to S phase [see also (*28*)] or from S phase to mitosis, as well as the higher sensitivity of G1 cells in cell lines with short G1 phase (*26, 27, 44*). We also predict on this basis that any treatment of cells that delays progression through the cell cycle without affecting their repair capability would be expected to result in a survival increase provided that (a) the delay is induced before the cells progress to the next S phase or mitosis and (b) the time available up to the next S phase or mitosis in cells normally progressing through cell cycle is not enough for the cells to repair all PLD. Support for these ideas comes from experiments with EAT cells, where incubation in BSS or F-medium containing aphidicolin or nocodazole considerably increased cell survival, as well as in experiments with other cell systems where incubation in BSS also resulted in a considerable increase in cell survival [e.g., (*2*)].

It is also possible that division delays induced by irradiation such as G2 arrest are exploited by the cells to repair PLD before mitosis, thus increasing the cell survival, and that the reduction in the G2 arrest is the reason for the higher sensitivity observed in AT cells (*50, 51*). The similar sensitivity observed in HeLa cells (*49*) for the interval in the cell cycle between mid S phase and G2 arrest can also be explained by considering the increase in the duration of the G2 block as the cells approach G2 phase [see (*31*)]. High sensitivity to radiation in mitosis is beyond the G2 block, and irradiated mitotic cells (nocodazole accumulation) divide without delay (M. Nüsse and H. J. Egner, in preparation). These findings might support the suggestion that the target for G2 block might not be the DNA (whereas it is most probably the target for cell killing) but might possibly be the nuclear membrane (*48*).

The results indicate further that in addition to PLD, the repair of which results in the appearance of the shoulder of the survival curve and which is expressed if still unrepaired when the cells reach G1/S border or M phase (to be termed α-PLD), PLD that also affects the slope of the survival curve can be expressed (to be termed β-PLD) using various postirradiation treatments, such as incubation in hypertonic medium (caffeine also produced a change in the slope under certain conditions). The main difference between the two suggested forms of PLD is that α-PLD is expressed, in addition to specific postirradiation treatments, by the cell itself at certain stages

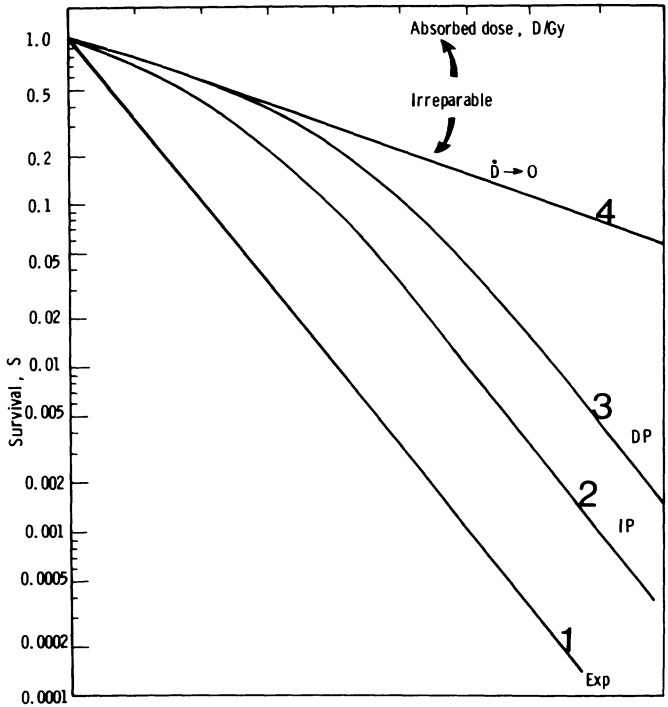

FIG. 12. Diagrammatic survival curves drawn from survival curves measured in EAT cells (see text).

of its life presumably by interfering with biological processes taking place in the cell at those stages. This expression is expected to occur in any cell (although to various extent depending on its age), is independent of any external treatment of the cell, and is coupled with biological processes inevitable for the cell, such as DNA synthesis and mitotic division, whereas β-PLD is expressed only after the application of treatments to which it is sensitive. Differences might also be found in the repair time constant as indicated in Fig. 10. The α-PLD is repaired with a t_{50} of 1 hr; β-PLD in contrast may show much faster repair kinetics.

These assumptions are summarized in Fig. 12. Irreparable lesions presumably lead to cell death in cells irradiated at low dose rate as indicated by curve 4 in the figure.[1] Reparable lesions can be expressed by various means resulting in a survival level below that defined by curve 4. The expression of damage responsible for lethality up to curve 3 (DP) might be the result of either an accumulation of PLD in the DNA (thus giving rise to irreparable damage) or a dose-dependent expression process competing with repair. The expression of PLD responsible for lethality up to the exponential survival curve 1 (obtained at the G1/S border, in mitosis and after the treatments used) is presumably mediated by the progression of the cells through cell cycle taking place in S phase or mitosis. This kind of PLD expression is suggested to be a

[1] A. Häuser and W. Pohlit, Production of irreparable lesions in mammalian cells irradiated at extremely low absorbed dose rate. Manuscript submitted for publication.

process taking place when cells reach specific stages of the cycle and not a continuous process competing with repair. Postirradiation treatments such as incubation with β-araA or caffeine or in hypertonic medium also permit expression of α-PLD. Expression of damage leading to survival level below those of curve 1 (β-PLD) can be achieved by specific treatments such as incubation in hypertonic medium, which might modify the lesions in such a way that subsequent repair becomes impossible.

The nature of PLD is unknown, although its sensitivity to treatments like β-araA indicates that it may be localized in the DNA. Therefore it is not possible to draw an unequivocal picture of the possible expression processes active at the G1/S border and in mitosis. It has been proposed that double-strand breaks (dsb) might be the potentially lethal lesions (*45–47*); in this case only about 1 dsb in 40 is expected to be dangerous to the cell and to interact with the biological processes leading to expression in G1/S phase or mitosis. That is because 40 dsb are produced per Gy (*47*) in EAT cells. The D_0 of the basic exponential survival curve, implying one lethal event per cell on average, was 0.8 Gy. The expression of those dsb might then be mediated by structural distortions occurring in the DNA in their presence at the beginning of S phase and in mitosis and resulting in either an inhibition of the repair, a fixation of lesions, or both (either simultaneously or in a subsequent phase of the cell cycle). The location of the dsb in the DNA could then be important to their sensitivity to fixation processes.

These results should be regarded as a first crude approximation that does not exclude the action of additional processes leading to the final effect. This is a possible interpretation and holds at first only for EAT cells or lines where mainly the D_q varies through the cell cycle. An extrapolation to cell systems showing mainly variations in the D_0 through the cell cycle, although possible, is at the moment premature.

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