Modification of the Response to Actinomycin D-induced Sublethal Damage by Simultaneous Recovery from Potentially Lethal Damage in Mammalian Cells

S. C. Barranco and D. R. Flournoy

Division of Cell Biology, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, Texas 77550

SUMMARY

Exponentially growing cells and cells that were allowed to enter a plateau or nondividing state of growth by depleting the medium of growth-essential nutrients were used in these studies. Nondividing Chinese hamster ovary cells in vitro are less sensitive to actinomycin D (AMD) than are exponentially growing cells. The cells were tested for their ability to recover from AMD-induced potentially lethal damage (PLD) or sublethal damage. The proliferating and the nonproliferating cells do not recover from PLD or sublethal damage, and they experience further reductions in survival when they are maintained under the medium conditions of their respective growth states.

However, when dividing cells are placed in conditions suboptimal for growth (incubated in depleted plateau medium after treatment with AMD), they did exhibit increased survival. The PLD recovery was so great under these conditions that it masked the true response of the cells to fractionated doses of AMD. When adjustments were made for the PLD recovery, the resulting data indicated a slight but measurable increase in survival. Since AMD inhibits cell progression in all stages of the cell cycle, this increase in survival observed with fractionated doses of AMD may be due to true recovery from sublethal damage, although the movement of cells into less sensitive stages of the cell cycle between treatments cannot be ruled out.

INTRODUCTION

Recovery from SLD is measured by the increase in survival, observed as the time interval is increased between 2 dose fractions of irradiation or drugs (3, 7, 13, 21). During the interval between the dose fractions, SLD from the 1st dose may be repaired and is thus unavailable to interact with SLD from the 2nd dose (12). Thus, the resulting survival may be higher after a fractionated dose schedule than when the total dose is administered as a single dose.

Recovery from PLD is measured by the increase in survival observed when cells are exposed to various growth conditions after single drug or irradiation treatments (3, 22). Conditions that are suboptimal for growth, such as low temperature, inhibition of protein synthesis, and maintenance of cells in nutritionally depleted medium, have been reported as favoring recovery from PLD (3, 5, 14, 19, 22, 29). Recovery from PLD occurs in vivo and in vitro (3, 16, 24) in dividing and nondividing mammalian cells (3, 15, 19), in oxic and hypoxic cells (14), and independently of recovery from SLD (3, 14, 19). Conversely, the expression of PLD is measured by a decrease in survival, usually under conditions that inhibit DNA synthesis (22).

The CHO cells used in our studies are able to recover from bleomycin-induced PLD (3). The incubation period between SLD-fractionated dose treatments exactly mimics that used to test for recovery from PLD, and the resulting data suggest that part of the recovery from SLD observed after fractionated dose treatments with bleomycin was due to recovery from PLD (3).

It has been reported (10) that the Chinese hamster cell line V79-753B exhibits an increase in survival after exposure to fractionated doses of AMD. It was suggested, however, that the rise in survival was possibly due to cells progressing into less sensitive stages of the cell cycle by the time the 2nd dose of AMD was administered and not to recovery from SLD.

The experiments that we report here suggest that the apparent "recovery" from SLD after fractionated dose treatments with AMD might be due entirely or in part to recovery from PLD.

MATERIALS AND METHODS

Cell and Culture Techniques. The CHO cell stocks were maintained in monolayer cultures in Hsu's modified McCoy's Medium 5A, supplemented with 20% fetal calf serum in a 5% CO2-95% air, humidified incubator at 37°. For experiments on exponentially growing populations, 10⁶ cells were placed into replicate 60- x 15-mm Petri dishes containing medium and 20% fetal calf serum, at least 18 hr before treatment.

Induction of Plateau Phase. Our plateau phase system has been described previously (2, 3). The following minor changes were made. To obtain plateau phase or nondividing cell populations, 5 x 10⁴ exponentially growing CHO
cells were placed into each 60- x 15-mm Petri dish containing 5 ml of medium with 20% fetal calf serum. The cells were not fed again until the experiments were performed, 115 to 120 hr later. Total cell counts were obtained at various intervals; the cell number increased exponentially ($T_p$, 15 hr) and then plateaued around 8 to $10 \times 10^6$ cells/dish after 72 hr. Survival was measured only on cells that had been in plateau phase for an additional 48 hr. At this time the labeling index was less than 2%, and essentially all of the cells were blocked in a G0-like compartment. The entry of plateau phase cells back into the cell cycle was followed on cells that had been resuspended ($10^9/$dish) into fresh medium. At 3-hr intervals, cells were pulse-labeled with $1 \mu$Ci $[^3H]$thymidine per ml (specific activity, 1.9 Ci/m mole) and total cell counts were performed. The cells reentered the cell cycle in a partially synchronized manner and progressed from G1 to S phase at 5 to 10 hr after subculturing. The cell number began increasing at 18 to 22 hr and doubled by 30 hr after the plateau cells were subcultured (2).

Survival Determinations. The effects of AMD on survival were determined on exponentially growing cells and on cells that had been maintained in plateau phase for at least 48 hr. Concentrations of from 1 to 5 $\mu$g AMD per ml were used. Cells were treated with AMD dilutions made in depleted plateau medium (plateau phase cells) or in fresh medium (exponentially growing cells). The depleted plateau medium was collected from plates of plateau phase cells, filtered to remove cells and debris, and then used for treatments or incubation after treatment for recovery tests. Replicate plates were used in each survival study and each experiment was performed at least 3 times. The averages of the survival fractions at each dose or time point were plotted in the subsequent charts. In all cases survival was determined by the ability of the treated cells to form colonies. After the treatments, the cell monolayers were washed twice with Puck’s Solution A and trypsinized (0.025% for 5 min), and known numbers of single cells were plated into Petri dishes and incubated for 6 to 8 days for colony formation (2, 6). Colonies were stained and counted. A cell was considered to have retained reproductive capacity (viability) if it gave rise to a colony of 50 or more cells (2, 6).

Recovery from PLD. PLD is damage that ordinarily causes cell death, but this may be prevented by appropriate posttreatment incubation such as exposure to cycloheximide (22), suboptimal temperatures (29), or incubation in depleted “conditioned” medium from plateau phase cultures (3, 20). In the experiments reported here, suboptimal growth conditions were achieved by holding plateau phase or exponentially growing cells in depleted plateau medium after treatment with AMD. After the treatment, one group of cells was plated immediately for colony formation, while other groups were washed and incubated at 37° in fresh or depleted medium for 1 to 4 hr before being plated for colony formation.

Response to SLD. To test for recovery from SLD, fractionated doses of AMD were used in treatments of dividing and nondividing cells. The treatments were fractionated using the integral dose concept of drug concentration and duration of treatment ($C \times T$); and the sum of the integral fractionated doses received by the cells was kept constant during any 1 experiment (3). For example, the sum of 2 fractionated doses of 5 $\mu$g/ml for 30 min $2(5 \times 30)$ equals the same integral dose of 300 as a single treatment with 5 $\mu$g/ml for 60 min. All cells received one-half of the integral dose at 0 hr. At the end of the 1st treatment, 1 group of cells was treated immediately with the same integral dose, thus allowing no time between treatments for recovery. The other groups of cells were washed and reincubated in the appropriate incubation medium, and they received their 2nd integral dose 1 to 4 hr later. All cells were plated for colony formation immediately after receiving the 2nd dose of AMD.

Chemical Solutions. The treatment solutions were always prepared immediately before use to ensure against loss of activity. AMD was dissolved in sterile water and diluted to the various treatment concentrations in fresh or depleted plateau medium. Because AMD is known to bind to cells and Petri dishes (11), all treated populations were extensively washed to remove extracellular AMD before further studies were performed on the cells. However, since radioactive AMD was not used (11), we have no way of knowing whether the washing was adequate.

RESULTS

Dose Response to AMD. The effects of AMD on survival of CHO cells treated in plateau or exponential phases of growth are presented in Chart 1. Dividing cells are more sensitive than are nondividing cells, and their survival curve is slightly biphasic, with the inflection point at the 2-$\mu$g/ml dose point. The survival curve for dividing cells is characterized as having no shoulder region with $D_0$'s of 0.9 and 1.7 $\mu$g/ml (1 hr treatment) for the sensitive and less sensitive slopes of the curve, respectively, and $n = 1$.

The survival curve for nondividing cells is also biphasic and contains a slight shoulder region with $n = 1.5$ and $D_0$'s for the sensitive and less sensitive slopes of 1.3 and 5.8 $\mu$g/ml (1 hr treatment), respectively.

Responses of Dividing and Plateau Phase Cells to PLD. In the experiments reported here, the survival responses of nondividing (plateau) cells to single doses of AMD were determined on cells treated and then either plated immediately for colony formation (no time allowed for recovery) or held for 1 to 4 hr in depleted plateau medium before subculturing. The plateau cells were held in depleted plateau medium (instead of fresh medium) to test for PLD recovery in the same environment that the cells were in before AMD treatment. It can be seen in Chart 2 that plateau cells treated with 2.5 $\mu$g AMD per ml for 30 min exhibited a survival fraction of 0.65 if they were plated for colony studies immediately after treatment. Cells treated and held for 1 to 4 hr in the nutritionally deficient medium experienced a reduction in the survival fraction to 0.50. Cells treated with 5 $\mu$g AMD per ml for 30 min also showed a decreased survival fraction upon incubation for longer than 2 hr in the depleted plateau medium. These data indicate that plateau phase cells held in the nutritionally de-
Responses of Dividing and Plateau Phase Cells to SLD.

Dividing cells were held in fresh medium between 2-dose fractions of AMD; plateau phase cells were held in depleted plateau medium. It can be seen in Chart 4 that plateau phase cells receiving 2 doses of 2.5 or 5 μg AMD per ml for 30 min (separated by the times indicated on the graph) did not exhibit recovery from SLD when held in depleted plateau medium. In fact, a reduction in survival was observed, suggesting either that the cells may become sensitized by the 1st dose of AMD or that the cells had progressed into more sensitive stages of the cell cycle by the time the 2nd dose fraction was given.

The response of dividing cells to fractionated doses of AMD are shown in Chart 5. Cells treated with 2-dose fractions of 0.8 μg AMD per ml for 30 min given 1 to 4 hr apart had a 2.5-fold-lower survival than cells that were given both doses within a 0 time interval. Cells receiving 2 doses of 2.5 μg/ml for 30 min (the approximate inflection point dose on Chart 1) showed only slight reduction in survival. A 10-fold reduction in survival was obtained in cell populations receiving 2-dose fractions of 5 μg/ml for 30 min when the fractions were given 1 to 4 hr apart.

Responses to PLD and SLD by Dividing Cells Held in Depleted Plateau Medium. Dividing cells treated with AMD and then held in fresh medium for 1 to 4 hr before being plated for colony formation do not recover from PLD or SLD (Charts 3 and 5). However, it can be seen in Chart 6 that incubation of AMD-treated dividing cells in depleted plateau medium resulted in higher survival values.

Parallel recovery experiments were performed to determine the contribution made by PLD recovery to the overall recovery observed in SLD-fractionated dose experiments.
Recovery from PLD can also occur between fractionated doses of irradiation or bleomycin (3, 19). As a test for recovery from SLD, plates of dividing cells received 5 μg AMD per ml for 30 min at 0 hr; then at 0- or 60-min intervals later, the cells received a 2nd 30-min treatment of 5 μg AMD per ml and were then plated for colony formation. Cells were incubated at 37° in depleted plateau medium between the 1st and 2nd treatments. As a test for recovery from PLD during this same interval, replicate plates of cells were treated for 30 min with a single dose of 5 μg AMD per ml at 0 hr. One group of cells was plated for colony formation immediately after treatment, thus allowing no time for recovery; the other cells were washed and incubated for 1 to 4 hr in depleted plateau medium at 37° to test for recovery under these conditions. Replicate plates of these cells were plated for colony formation only after the cells in the SLD experimental groups had received the 2nd dose of AMD. In this way, the cells in the PLD experiment were held in depleted medium for times equivalent to the various intervals between the fractionated doses given to the SLD groups and, therefore, could be used to determine the contribution made by recovery from PLD during this time.

In Chart 6 it can be seen that cells treated with 5 μg AMD per ml for 30 min and then plated immediately (PLD curve) had survival fractions of 0.086, whereas cells held 1 to 4 hr in depleted medium had up to 2.9-fold-higher survival values, indicating recovery from PLD.

Cells held in depleted plateau medium between the two 5-μg/ml-dose fractions of AMD (Chart 6, SLD curve) also exhibited increased survival values. The survival fraction increased from 0.02 (0 time interval between doses) to 0.07 when cells were held for 3 to 4 hr in the nutritionally deficient medium.

The fractional increases or decreases in survival were determined from the data in Charts 3, 5, and 6 and are expressed as recovery ratios in Chart 7. Increased survival under the conditions of the various experiments is indi-
S. C. Barranco and D. R. Flourny

depicting the increased survival observed when dividing cells were incubated in either fresh (FM) or depleted plateau phase medium (PM). The SLD-PLD curve represents the response to SLD (PM) once the contribution made by simultaneous recovery from PLD (PM) has been subtracted.


dicated by recovery ratios above 1 (on the chart); decreased survival is expressed as recovery ratios below 1. It can be seen in Chart 7 that AMD-treated cells cannot recover from PLD or SLD when they are incubated in fresh medium (PLD-FM, from Chart 3, and SLD-FM, from Chart 5). These curves are included in Chart 7 for comparison with curves depicting the increased survival observed when dividing cells were incubated in depleted plateau medium after treatment (SLD-PM and PLD-PM, from Chart 6). The SLD minus PLD curve (SLD – PLD) was obtained by reducing the SLD survival (and recovery ratio) points by the fraction due to recovery from PLD. These data suggest that part of the observed recovery from SLD was due to recovery from PLD.

DISCUSSION

Dose Responses of Dividing and Nondividing Cells to AMD. It is evident from these studies that dividing cells are more sensitive to AMD than are nondividing cells. The exponentially growing cells are 1.5 to 3.4 times more sensitive than are the nondividing cells (calculated from the $D_0$’s of the survival curves in Chart 1). These data are in agreement with recent reports by Wilkoff (30), Valeriote (28), and Djordjevic and Kim (8), but not with that of Piro et al. (23) who find V79 cells more sensitive to AMD in the plateau phase. The difference in this case may result from the fact they feed their plateau cultures, whereas ours is an unfed system with less than 2% labeling index. AMD inhibits DNA-directed RNA synthesis through its ability to complex with DNA (18, 25). It is this ability to complex DNA that probably accounts for the activity of AMD against both proliferating and nonproliferating mammalian cells. We are aware of the limitations inherent in comparing plateau phase cells in vitro with $G_0$ cells in vivo. However, these results (Chart 1) do suggest a possibility that killing of cells in the $G_0$ compartment in vivo may be obtained. A better understanding of the effects of anticancer drugs on these viable but nondividing cell populations has potential clinical significance. Since the plateau or $G_0$ cells generally are not killed by the cell cycle-specific drugs, these cells probably contribute to the repopulation or regrowth of the tumor. Therefore, it is extremely important to identify and characterize drugs that will influence the survival, repair, and repopulation kinetics of $G_0$ or plateau phase cells.

Response to PLD. Phillips and Tolmach (22) have suggested that the response of cells to irradiation may be modified by various posttreatment conditions that interfere with processes that normally result in either repair or expression of damage. An increase in survival may be observed after treated cells are held for several hr under conditions that prohibit protein synthesis and cell progression and, therefore, allow time for recovery (22). For example, irradiation of $G_1$ cells followed by incubation of the cells in the DNA synthesis inhibitors fluorodeoxyuridine or hydroxyurea resulted in lower survival (22). This suggested that DNA synthesis (unscheduled) may be required for repair of the PLD. This idea was further substantiated by postirradiation treatment with cycloheximide at doses that primarily inhibited protein synthesis, without altering the repair process, and resulted in higher survival than in cells receiving only irradiation. Therefore, an extremely important parameter to consider in the chemotherapy of tumors is the possibility that the tumor cells may recover from damage caused by the chemical treatment. The recovered cells, therefore, would be able to contribute to the regrowth of the tumor. Experiments were designed to measure recovery from drug-induced PLD, i.e., damage that would become lethal to the cell, if time and posttreatment conditions were not suitable for repair (22).

Dividing cells treated with AMD and then incubated 2 to 4 hr in nutritionally deficient medium showed an approximate 3-fold increase in survival (Charts 6 and 7). Plateau phase cells were unable to recover under the same incubation conditions (Chart 2). That the increased survival observed in dividing cells was related to incubation in depleted medium is supported by the observations that survival decreased when dividing cells were incubated in fresh medium after AMD treatment (Charts 3 and 7). These data suggest that dividing cells can recover from AMD-induced PLD, but only in conditions suboptimal for growth. We have presented other in vitro evidence that bleomycin-treated cells can also recover from PLD (3). The recovery ratios of nondividing cells were 2 times greater than those for dividing cells. These data are consistent with other in vitro and in vivo results (16, 24, 27).

Although the monolayers of cells were washed 2 times with medium immediately after treatment with AMD and then held in their respective incubation media, there is a slight possibility that some AMD remained bound to the cells and/or Petri dishes (11). This might account for the further reduction in survival observed (in Charts 2 and 3) after treatment with single doses of AMD, especially since...
the cell numbers in plateau and dividing populations were vastly different at the time of treatment, due to the methods of producing these populations (1.6 × 10^6 cells/ml in plateau and 3 × 10^6 cells/ml in dividing cells). In addition, the further reduction in survival may also be due to the direct damage of DNA and the inhibition of its repair by the binding of AMD to DNA (9).

Since incubation of the treated cells in nutritionally deficient plateau medium for several hr before plating them for colony formation favors recovery from PLD in dividing cells (Chart 6), but not in plateau phase cells (Chart 2), this would suggest that the kind of damage produced in plateau cells by a single AMD dose might be qualitatively or quantitatively different than the damage experienced by dividing cells. Why dividing but not plateau cells recover from PLD in depleted medium in our unfed plateau model system is unknown and is being investigated further in our laboratory.

Response to Fractionated Drug Doses (SLD). Since the size of the shoulder on X-ray survival curves is related to the capacity to accumulate sublethal radiation injury (12), experiments were performed to determine whether this phenomenon held true for chemically induced damage. In the experiments reported here, neither the plateau phase cells held in depleted plateau medium nor the dividing cells held in fresh growth medium between fractionated doses of AMD could recover from SLD. In fact, nondividing cells [having a small shoulder on the survival curve (Chart 1)] had from 1.5- to 2.7-fold-lower survivals to fractionated doses of AMD than they had when the same total dose was given as a single treatment (Chart 4). Dividing cells had 10-fold-lower survivals when 2 fractions of 5 μg AMD per ml were given (for 30 min each) than when the same integral dose was given as a single treatment (Chart 5). These data suggest either that the cells may have become sensitized by the 1st treatment or that the cells progressed into more sensitive stages of the cell cycle by the time of the 2nd dose fraction. Holding the treated plateau cells in depleted medium and the dividing cells in fresh medium closely approximates the nutritional and extracellular environments of these cells in the G0 and proliferating compartments in vivo, respectively, and thus may be predictive of the response of solid tumors and normal cell renewal systems.

The survival fraction (0.001) after 2 doses of 5 μg/ml for 30 min (Chart 5) was equivalent to the survival after a single treatment with 15 μg AMD per ml for 1 hr (unpublished results). Qualitatively similar effects were produced by fractionated doses of the nitrosoureas (3). Since the side effects of most cancer chemotherapy drugs appear to be dose-dependent, these results may be clinically relevant in that it may be possible to reduce the total drug doses and toxicity while achieving the same amount of cell kill through the use of fractionated dose treatments. However, these dose fractions are given 1 to 4 hr apart, instead of the usual 24- to 72-hr schedule used in most clinical protocols.

The Effects of Recovery from PLD on SLD Measurements. Little (19) has reported that, in addition to recovery from SLD, recovery from PLD can also occur between fractionated doses of irradiation. The CHO cells in our system express the same ability when exposed to bleomycin (3). However, when the recovery from PLD was accounted for, little if any recovery could be observed from bleomycin-induced SLD (3). A similar phenomenon exists with AMD. As long as dividing cells were held in fresh medium (to mimic the probable in vivo cellular environment) between fractionated dose treatments, no PLD or SLD recovery was observed (Chart 7). However, incubation of the dividing cell population in suboptimal growth conditions (depleted plateau medium) between dose fractionations resulted in an approximate 3-fold increase in survival (Charts 6 and 7, SLD curve). The cells also recovered from PLD (up to a 2.5-fold increase in the survival fraction) under these conditions (Charts 6 and 7). [Recall from “Materials and Methods” that the fractionated dose and PLD studies were performed as follows. One group of cells received both drug doses without any repair time allowed between fractions (0-hr survival points on SLD charts). The rest of the SLD groups received only one-half of the integral dose at 0 hr and were then incubated to allow for recovery, until they received the other half-integral dose 1 to 4 hr later. Other groups of cells (PLD) received a half-integral dose at 0 hr and were incubated as above to allow for recovery from damage, but unlike the SLD plates, were not given a 2nd half-integral dose. Instead, these cells were plated for colony formation only after the SLD plates received their 2nd half-integral dose. PLD groups handled in this manner (Charts 6 and 7) may be used to approximate the amount of recovery from PLD occurring from the 1st half-integral drug dose and before the 2nd half-dose fraction was given to the SLD groups.] The SLD(PM) survival curve (Chart 7) was adjusted for the contribution made by recovery from PLD(PM), and the resulting SLD-PLD curve suggested that at least part of the observed recovery from SLD might be due to the simultaneous recovery from PLD. The 1.5 recovery ratio observed in the SLD-PLD curve may be due to the progression of cells into more resistant phases of the cell cycle by the time the 2nd dose was given (10). However, it has been shown that AMD inhibits cell progression in all phases of the cell cycle (1, 4, 17, 26) at dose levels much lower than those used here. It is possible, therefore, that under certain culture conditions cells can recover from AMD-induced SLD.

From the data presented in this paper the following conclusions can be made. First, dividing cells are more sensitive than nondividing cells to AMD. Second, proliferating and nonproliferating cells cannot recover from PLD or SLD when the cells are incubated under the conditions of their respective growth states after treatment. In fact, sensitization and increased cell killing are observed with longer incubation times, possibly because of the effect of DNA-bound AMD on repair. Third, recovery from PLD and SLD does occur when treated dividing cells are held under conditions which are unfavorable for growth (i.e. depleted plateau medium). However, the recovery from PLD is so great under these conditions that it masks the true response of these cells to SLD. When adjustments are made for the PLD recovery, a small but measurable increase in survival is seen and may represent recovery from SLD.
ACKNOWLEDGMENTS

We wish to express our appreciation to Peggy Anderson for preparation of this manuscript.

REFERENCES


Modification of the Response to Actinomycin D-induced Sublethal Damage by Simultaneous Recovery from Potentially Lethal Damage in Mammalian Cells

S. C. Barranco and D. R. Flournoy


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/36/5/1634

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.