Actinomycin D: Suppression of Recovery in X-Irradiated Mammalian Cells

Abstract. *The lethal effect of two x-ray doses can be considerably enhanced if cultured cells are exposed to actinomycin D between doses. Net survival can be modified by temperature as well as actinomycin treatment. These effects are related to repair of sublethal damage and to cell kinetics between doses.*

For the past several years, we have been studying the survival of cultured Chinese hamster cells and *L* cells exposed to fractionated, acute, x-ray doses (*1*). When a given x-ray dose was delivered in two parts, we found that, as a function of time between the two exposures, the net survival of the cell follows a curious kinetic pattern which is qualitatively the same for both cell lines. Generally similar results have been described for other mammalian cells assayed both in vitro (*2*) and in vivo (*3*), as well as for plant cells (*4*). We now report the dependence of net survival of cells on active metabolism (*5*) and actinomycin D treatment.

A typical curve for the net survival for the Chinese hamster cell subline *V79-379-A* after exposure to two doses is shown in Fig. 1, curve *A*. Cells were grown overnight attached to glass in a "CO_2 incubator" in the presence of about 1.3×10^4 x-ray sterilized cells per milliliter of medium. From earlier studies (*6*), we knew that after about 17 hours at 37°C these cells would be in asynchronous, exponential growth. Therefore, at the time of the first or conditioning dose (433 rad) each potential colony-forming unit consisted of about three cells (doubling time, 8 to 9 hours), and immediately after the conditioning dose the average surviving multiplicity was about 1.4 cells. This multiplicity is not important in the interpretation of curves like curve *A* since the net survivals shown in Fig. 1, as well as in the figures to follow, are < 0.1. This means that each potential colony-forming unit immediately after the second exposure consisted of about one surviving cell (*1*). Hence, all the results obtained with cells attached to glass show the net survival of essentially single cells which were in the logarithmic phase of growth, as a function of time between two exposures.

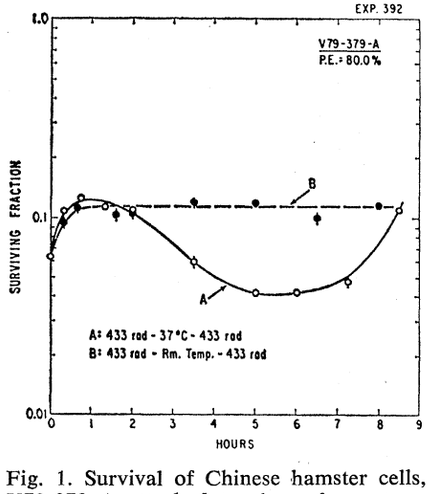

Fig. 1. Survival of Chinese hamster cells, *V79-379-A*, attached to glass after a two-dose exposure to x-irradiation. Single cells were grown overnight at 37°C in a "CO_2 incubator" and in the presence of about 1.3×10^4 x-ray sterilized cells per ml. Between two doses of 433 rad each, given at room temperature, cells were incubated at 37°C, curve *A*, or room temperature (about 25°C), curve *B*. Temperature shifts were effected by changes of media. After the second doses, cells were incubated at 37°C for colony formation. *P.E.*, plating efficiency. Uncertainties are standard errors. X-ray source: 55 kv, 722 rad/min.

In Fig. 2, curve *A* indicates the net survival of single *L* cells as a function of time between the doses 700 rad and 500 rad. For this experiment, cells were grown, and irradiated in suspension, about 2×10^5 cells per milliliter, and after the second exposure cells were diluted and plated for colony formation.

We have called the curves showing survival after two doses of x-rays "recovery curves" since in our earlier work with Chinese hamster cells (*1*, *7*), we showed that the structure of such curves results mainly from oscillations in $\tilde{n}$, the extrapolation number (*8*), of the survival curves of those cells which survive the conditioning dose. Immediately after a conditioning dose of, for example, 433 rad, $\tilde{n}$ is reduced to about 1.0 because the survival of single Chinese hamster cells after 433 rad is close to the exponential, terminal portion of the survival curve of the starting population, the threshold being largely eliminated or the "quasi-threshold dose" (*9*) being reduced to practically zero. At the maximum in the recovery curve, $\tilde{n}$ returns to about half of its initial value (part of the threshold has returned); at the minimum $\tilde{n} \cong 1.0$ (no threshold again); and by about half a doubling time after the minimum, $\tilde{n}$ returns to its initial value (full threshold). After a conditioning dose of 433 rad, division of surviving Chinese hamster cells is suppressed for 4 to 5 hours (*6*), and after 700 rad, the division of *L* cells is delayed for 15 to 20 hours.

In Figs. 1 and 2, curves *A* are for cells actively metabolizing between exposures, that is, cells kept at 37°C. Consistent with these changes in threshold already described, we interpret these recovery curves as follows (*7*, p. 129). The prompt increases in survival result mainly from the repair of sublethal damage in cells that survive the conditioning dose. Concomitantly, surviving cells "progress" toward division, taking on, as a result, survival responses leading to a narrowing of the threshold for Chinese hamster cells at the minimum in curve *A*. Whether or not the minimum for *L* cells also corresponds to $\tilde{n} \cong 1$, for neither cell

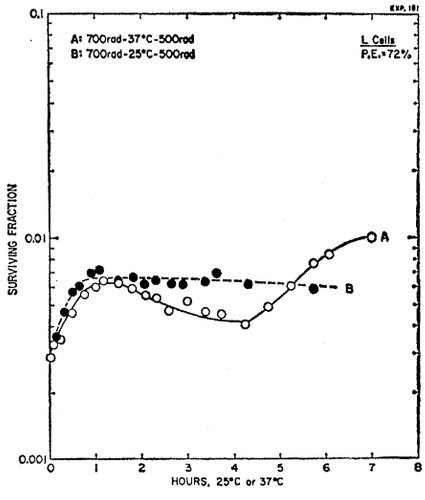

Fig. 2. Survival of *L* cells to two-dose exposures of x-rays. Cells were grown in suspension at 37°C. At a concentration of about 2×10^5 cells per milliliter, they were irradiated at 37°C first with 700 rad and then with 500 rad at the times shown. Between exposures, the incubation temperature was 37°C, curve *A*, or 25°C, curve *B*; the temperature of 25°C was reached within 6 minutes by transferring the spinner flask to a water bath of that temperature. After the second doses, cells were diluted and plated for colony formation. X-ray source: 280 kv (peak), 466 rad/min.

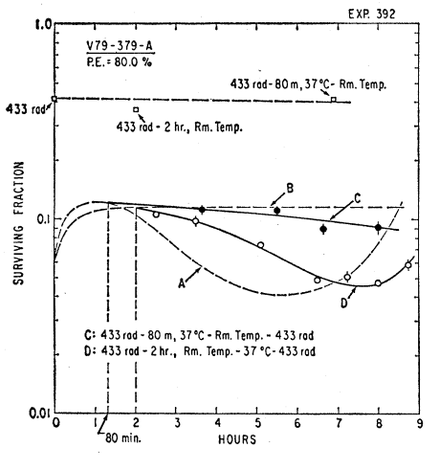

Fig. 3. The effects of temperature shifts on the net survival of Chinese hamster cells to two doses of 433 rad each. These results were obtained as part of the experiment in Fig. 1; curves *A* and *B* refer to Fig. 1. The data along curve *C* were obtained by incubating cells at 37°C for 80 min after exposure to 433 rad before cooling them to room temperature. The data along curve *D* were obtained by incubating cells at room temperature for 2 hours after a dose of 433 rad before raising the cell temperature to 37°C. Temperature changes were effected by the addition of appropriate amounts of media at 4° or 60°C. First doses were given immediately and second doses at the times shown. The open squares show that there was little effect on survival after a single 433 rad dose due to the temperature shifts noted. Other details as for Fig. 1.

line do we associate the minimum with a reversal of the recovery processes which give rise to the maximum. We base this on the fact that an asynchronous population consists of cells having a mixture of survival responses (*10*). Because of this, the distribution of cells (with respect to position in the division cycle) which survive a conditioning dose is distorted compared to that of the starting population. Those cells which survive the first dose progress toward division; the composite survival response can undergo oscillations as various moieties recover and take on different survival responses.

To test the foregoing, we have examined the dependence of recovery on temperature (*5*). Curves *B*, Figs. 1 and 2, show the dependence of net survival on time when cells are kept at room temperature (about 25°C) between doses. In both instances, survival increases promptly without delay and remains constant in the regions of the minimum observed at 37°C. In a separate experiment, neither control nor irradiated cells (both surviving and nonsurviving) divided at room temperature during periods considerably longer than those shown. These results show that the prompt increase in survival is only weakly dependent on active metabolism; moreover, they suggest that the minima in the recovery curves at 37°C are associated with the progress of surviving cells toward division during the division-delay period induced by the first exposure.

To test this interpretation, as part of the experiment shown in Fig. 1, the effects of temperature changes during the course of recovery were also examined. In Fig. 3, curves *A* and *B* in Fig. 1 have been redrawn for reference. Curve *C* shows that the survival of cells which recover for 80 minutes at 37°C and are then cooled to room temperature does not drop to a minimum in 5 to 6 hours. The slow decline suggests that the minimum is considerably displaced to the right. In contrast, cells which are allowed to recover for 2 hours at room temperature and are then warmed to 37°C (curve *D*) drop in net survival fairly rapidly. Also, the minima in curves *A* and *D* appear to be displaced by about 2 hours.

With Chinese hamster cells, we have also found that surviving cells can undergo repeated cycles of damage and repair. Cells allowed to reach the maximum along curve *A*, Fig. 1, yield curves having the same shapes as curves *A* and *B* when the second recovery cycle is followed at 37°C and room temperature, respectively. By the term "second recovery cycle," we mean the variation in net survival as a function of time between a second and third exposure. The same is true of the second recovery cycles of cells allowed to reach the plateau in curve *B*. Further, cells which survive 433 rad, and which recover at room temperature have essentially the same survival curve at 100 minutes and at 5.5 hours after the conditioning dose. The curve has a clear and distinctly broad threshold.

A separation between intracellular recovery and cell kinetics appears to be brought about by changes in temperature. With this separation in mind, we examined the effect of metabolic inhibitors on the kinetics of survival after a conditioning dose. We report results with the RNA inhibitor actinomycin D here. Puromycin and 5-fluorodeoxyuridine, at concentrations yielding degrees of toxicity comparable to actinomycin D after the same conditioning dose, produce smaller and qualitatively different results.

In Fig. 4, all the incubations, after a conditioning dose of 542 rad, are at 37°C (the arrows indicate the appropriate abscissas). Curves *A*, *B*, and *C* show the toxicity produced by 0.005, 0.015, and 0.045 μg of actinomycin D per milliliter, added to the growth medium promptly after exposure. At the times shown the medium was removed, the cells were rinsed, and drug-free medium was returned to the dishes. The curve marked "control" is the reference recovery curve for two doses of 542 rad without the drug. Curves *A'*, *B'*, and *C'* correspond to the same concentrations of antibiotic as curves *A*, *B*, and *C* (drug present only between exposures), and are to be compared to the control curve for two doses.

The fact that the usual prompt increase in survival can be progressively suppressed during periods of exposure to the drug which produces a small

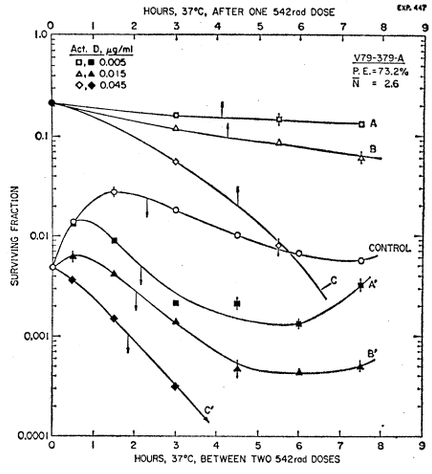

Fig. 4. The influence of actinomycin D on the survival of Chinese hamster cells to two-dose exposures of x-rays. Actinomycin was added to the medium promptly after the conditioning dose, 542 rad. At the times indicated along curves *A*, *B*, and *C* (upper abscissa), medium containing the drug was removed, cells were rinsed, and the drug-free medium was returned to the dishes for colony formation. At the times indicated along curves *A'*, *B'*, and *C'* (lower abscissa), after rinsing and before drug-free medium was added, cells received a second dose of 542 rad. The curve labeled "control" shows the net survival to two 542 rad doses in the absence of drug. All doses were given at room temperature and all incubations were at 37°C; $\bar{N}$ is the average multiplicity per colony-forming unit at the time of the first dose; other details as for Fig. 1.

concomitant toxicity—for example, 0.015 or 0.045 μg/ml for ½ hour—suggests that actinomycin D can suppress the amount or rate of recovery or both. And the fact that, at a drug concentration which produces only a moderate degree of toxicity (0.005 μg/ml), the recovery curve lies well below the "control" curve suggests that actinomycin may also make cells more sensitive.

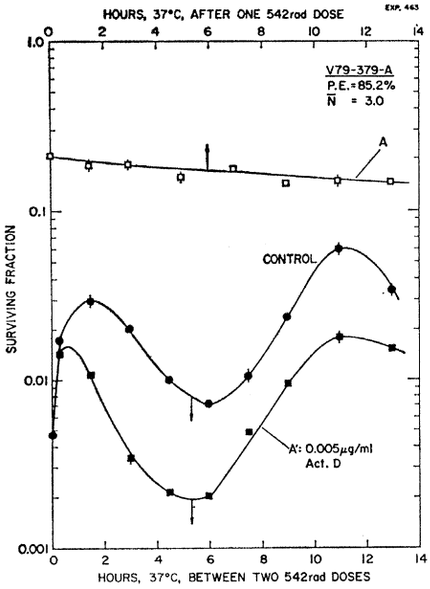

Fig. 5. The influence of 0.005 μg of actinomycin D per milliliter on the survival of Chinese hamster cells to two-dose exposure of x-rays. Details as for Figs. 1 and 4.

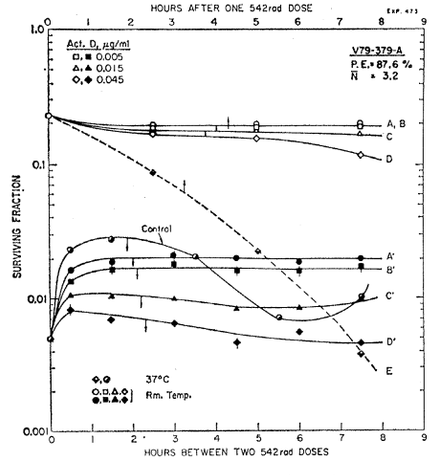

Fig. 6. The influence of reduced temperature plus actinomycin D on the survival of Chinese hamster cells to two-dose exposures of x-rays. Curves A and A' show the survival for cells incubated at room temperature (about 25°C) after one 542-rad dose (upper abscissa) and between two doses of 542 rad (lower abscissa), respectively. Similarly for curves B and B', C and C', and D and D' except that actinomycin was added and removed as for Fig. 4. Curve E compared to D shows the added toxicity of 0.045 μg of actinomycin per milliliter when cells are incubated at 37°C after the first dose of 542 rad. Other details as for Figs. 1 and 4.

The influence of 0.005 μg of actinomycin D per milliliter on the recovery curve over a longer interval between doses is shown in Fig. 5. Curve A' is displaced downward from the "control" curve after 1 hour. Thirteen hours after a dose of 542 rad, the number of surviving cells dropped by about 30 percent owing to drug toxicity, curve A. Therefore, in the region of the second maximum, a significant part of the reduced net survival may be due to drug toxicity alone. However, we have measured survival curves after single doses and found that 40 minutes after the delivery of 542 rad, the principal effect of actinomycin is to decrease the width of the threshold, that is, to reduce the value of $\tilde{n}$ compared to cells without drug. At 3 and 5 hours after a dose of 542 rad the main effect of actinomycin is to cause the survival curve to drop off more rapidly than it does when the drug is absent.

If actinomycin D can prevent or at least retard recovery processes, and reduced temperature can prevent or retard the progress of cells during the division-delay period, actinomycin plus reduced temperature between exposures should tend to flatten out the curve. We tested this as shown in Fig. 6. Curve A shows the survival resulting from incubation at room temperature after a dose of 542 rad, and curves B, C, and D show the added toxicity resulting from increasing concentrations of actinomycin D at room temperature. Curve E (0.045 μg/ml, 37°C) compared to curve D (0.045 μg/ml, room temperature) shows clearly that a reduced temperature decreases toxicity, an effect which may reflect the concomitant suppression of cell progression at room temperature. The effect of room temperature only on the recovery curve is seen from comparing curve A' with the "control" curve for 37°C. Compared to curve A', curves B', C', and D' show a progressive suppression of the plateau region of the two-dose curve not accompanied by the usually pronounced minimum around 5 to 6 hours; for example, Figs. 4 and 5.

Taken together, our results suggest the following: First, the kinetics of survival observed by the two-dose technique can be grossly divided into (i) repair of sublethal damage which, because of its rapidity and the lack of delay in its initiation, is largely independent of changes in survival response associated with the progression of cells toward division; and (ii) the progression toward division of recovering cells during which the surviving moieties present take on the survival responses which add up to a minimum in the recovery curve. Second, actinomycin D affects both parts of the two-dose survival pattern. Third, because actinomycin D is thought to inhibit specifically DNA-dependent RNA synthesis (*11*), recovery from acute x-ray damage at least involves the synthesis of RNA.

As noted, in our earlier studies (*1*, *7*) we found that cells surviving a moderate conditioning dose after the onset of division have a survival curve similar to that of the starting population. Still, over the relatively short intervals studied, we cannot be sure of the degree of recovery of all cells because asynchronous populations were used. Similarly, we cannot be sure that actinomycin D influences all cells in the same way or that it is equally effective in all cells. Studies with synchronized populations may be required to answer these questions. Nonetheless, the relatively large effects produced by the low concentrations of actinomycin used suggest that RNA (*11*) plays a significant role in recovery from x-ray damage, although possibly some other, perhaps as yet unknown, effect of actinomycin may be involved. While by no means assured, we note also the inference that RNA or RNA structures may be the sites or may be closely related to the sites inactivated in cell killing.

Lastly, in addition to the reported potentiation of single-dose cell inactivation (*12*), the capacity of actinomycin to potentiate the response to fractionated-doses can be of importance in radiation therapy (*13*).

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Excess Lactate: An Index of Reversibility of Shock in Human Patients

Abstract. *"Excess lactate," an indicator of oxygen debt, has been studied as a metabolic index of severity of the shock state in human patients. The levels of excess lactate correspond to severity of circulatory failure, and an excess of more than 4 millimoles per liter prognosticates a fatal outcome. The validity of this index was confirmed by studies on experimental hemorrhagic shock in dogs. It provides a parameter for measurement of "reversibility" and serves as an objective clinical guide.*

The fundamental defect in shock is failure of effective blood flow, and hence defective transport of vital nutrients (*1*). Functional impairment of cellular metabolism is followed by permanent cellular damage. Since the availability and delivery of oxygen are critical, the severity of oxygen deprivation and differential sensitivity of tissues to this lack are immediate determinants of survival.

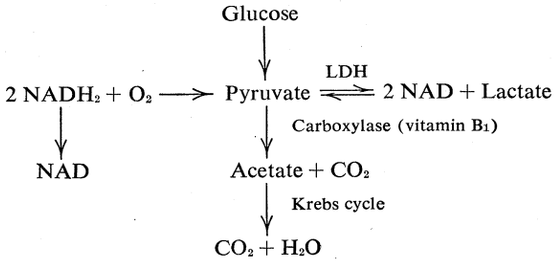

Fig. 1.

During periods in which metabolism is sustained by anoxic energy exchange, the metabolic fate of pyruvic acid is temporarily altered. Aerobic oxidation in the tricarboxylic acid cycle is blocked and pyruvic acid is converted to lactic acid. Nicotinamide-adenine dinucleotide ($NADH_2$) provides for this electron exchange. The transformation of $NADH_2$ to NAD during the conversion of pyruvic to lactic acid allows glycolysis to proceed without obligatory rejuvenation of $NADH_2$ by oxygen, according to the process shown in Fig. 1, where LDH is lactic dehydrogenase. The oxidation of $NADH_2$ may be blocked by cyanide or anoxia, for example. The accumulation of lactic acid accounts, in part, for the progressive acidosis of shock.

Increase in blood lactic acid concentrations is a feature not only of circulatory anoxia but also of ventilatory anoxia. Sometimes a profound lack of circulating hemoglobin or an enzymatic block (as in cyanide intoxication) reflect a defect in chemical transfer of oxygen but do not, in themselves, reflect hypoxia. During hyperventilation, infusion of glucose, or adrenal medullary stimulation, there is an increase in both blood lactate and pyruvate but no selective increase in lactic acid content. A series of studies on the relationship of blood lactic acid concentrations and oxygen deficiency have been made by Huckabee (*2*). He relates oxygen debt to an excess of lactate (XL), defined as

$$XL = (L_T - Lo) - (P_T - Po)\frac{Lo}{Po}$$

where L_T is lactate at time T, Lo is the "normal" lactate during the basal state, P_T is pyruvate at time T, and Po is the "normal" pyruvate during the basal state. Inherent in this assumption is a predictable increase in lactate with increase in pyruvate. Huckabee has provided theoretical and empirical justification for this formulation.

Several workers have recently provided theoretical objections to the derivation of the excess lactate measurement and empirical objections to excess lactate as a precise measure of oxygen debt (*3*). In our own work we have not measured oxygen debt because of the technical difficulties inherent in making such measurements in patients who are in shock. Thus we neither support nor refute the concept that excess lactate is a valuable index of oxygen debt. However, the empirical observations which are the subject of this report indicate the potential prognostic value of this index for patients who are in shock.

The role of oxygen debt as a limiting factor to survival in dogs has been defined by Guyton and Crowell (*4*), based on measurements of oxygen consumption prior to, during, and after hemorrhagic shock. They found a very close relationship between oxygen debt and severity of shock as reflected in mortality. We have made similar observations in dogs shocked with endotoxin (*5*). Techniques available for measuring oxygen debt in human patients are not sufficiently sensitive. However, the availability of a metabolic index for estimating oxygen debt has four advantages: (i) It has high sensitivity; (ii) it provides a guide to the cumulative oxygen debt; (iii) the laboratory determination has satisfactory chemical precision; and (iv) the test is easily adapted for routine use with the facilities available in general hospital laboratories.

In the study reported here, the excess lactate was measured serially in 56 patients with clinical signs of circulatory failure. Measurements were made at approximately 8-hour intervals during observation, which continued from admission to recovery or death. Only patients who survived 4 hours or more were included. Patients were observed for an average period of 36 hours, but occasionally for as long as 5 days. The highest value obtained is reported here since it is regarded as most descriptive of the largest oxygen debt measured. The patients were studied in the Shock