INHIBITION OF REPAIR OF DNA SINGLE STRAND BREAKS IN MOUSE LEUKEMIA CELLS BY ACTINOMYCIN D

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SUMMARY: The effects of Actinomycin D, cytosine arabinoside and temperature shifts on the repair of single strand breaks produced in murine leukemia cell DNA by ionizing radiation have been studied. A recently introduced modification of the alkaline sucrose sedimentation methods was used, allowing breaks to be demonstrated following clinical range irradiation doses. The results contrast to previous data using standard gradient procedures and indicate that low concentrations of Actinomycin D can inhibit single strand break repair, while cytosine arabinoside is ineffective. Inhibition can also be demonstrated by temperature shifts to 3° but not 24°, paralleling previous results from cellular repair studies (Elkind-Sutton repair). The results are consistent with the hypothesis that the accumulation of sublethal radiation damage in mammalian cells may be based on residual non-repaired single strand breaks.

The mechanisms by which mammalian cells repair the damage caused by ionizing radiation have been analysed at several levels of cellular organization (1). These include recovery of cell proliferability (2), repair replication (3), unscheduled DNA synthesis (4), rejoining of single strand DNA breaks (5) and the restitution of chromosome aberrations (6). The relationships between these various types of repair functions is not as yet clear. For example (1), single strand break rejoining and repair replication are inhibited by low temperature but not by Actinomycin D (Act D), while Elkind-Sutton repair (recovery of cell proliferability) is strongly inhibited by Act D but is considerably less sensitive to a suboptimal temperature shift (7). Since it is inhibition of cancer cell proliferability which is desired *in vivo*, it is important that drugs be developed which inhibit the Elkind-Sutton repair processes. On the other hand, evidence has

steadily been accumulating which describes repair of damaged DNA in terms of a relatively simple set of enzymes which involves endonucleolytic production of 3' OH and 5' phosphoryl termini, insertion (in some cases) of a new nucleotide, followed by strand rejoining by a ligase (8). Since in vitro ligase activity can be demonstrated in the absence of three nucleoside triphosphates (9), it is likely that nucleotide substitution is not a necessary correlate of repair of all strand breaks. While it now appears that two or more DNA polymerase activities may be involved (10), this enzyme model appears essentially acceptable in terms of the available information save for the discrepancies cited above. We report here the effects of Act D, cytosine arabinoside (Ara-C) and sub-optimal temperatures on single strand rejoining as measured by a modified alkaline sucrose method described recently by McBurney, Graham and Whitmore (11). This technique reduces artifactual shearing of the DNA being studied and allows demonstration of strand breakage and rejoining at radiation doses below 500 rads (12). The results strongly suggest that inhibition of Elkind-Sutton repair may well be grounded in the inhibition of repair of single strand breaks in cellular DNA.

MATERIALS AND METHODS

All experiments reported here utilized log phase murine L-1210 leukemia line grown in RPMI 1630 medium (Associated Biomedic System Inc., Buffalo, N.Y.) in suspension culture. The cells were grown for at least one generation in ^{3}H-thymidine (0.1 μci/ml) to obtain labelled DNA. Irradiation was accomplished using a Cobalt-60 source with a dose rate indicated in the figures. Alkaline sucrose density gradient methods were exactly those described recently by McBurney et al, (12), as modified from the original method (5). Act D or Ara-C were dissolved in Hank's salt solution and added at the times indicated in the figures. Temperatures were controlled by constant temperature water baths circulating throughout the periods of irradiation and post-radiation repair period as indicated.

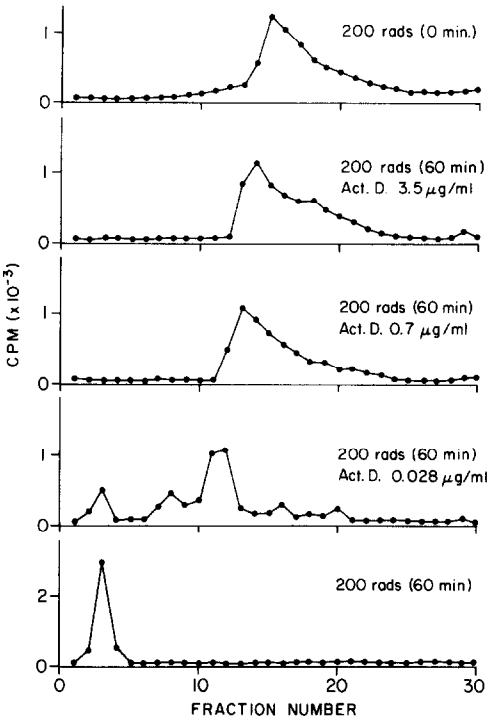

Fig. 1: Effect of Actinomycin D on Single Strand Break Rejoining.

Cells were grown for 24 hours in RPMI 1630 medium (Associated Biomedic System, Inc., Buffalo, N.Y.) containing 0.1 μCi/ml ^{3}H-thymidine (specific activity 18 Ci/mmole). The labelled culture was divided into 5 equal portions and incubated with or without Actinomycin D (Act D) as shown in each panel. The cells were centrifuged after 60 min. incubation, and resuspended in Gey's balanced salt solution (Grand Island Biological Corp., Grand Island, N.Y.) at 4° prior to irradiation (200 rads) at a dose rate of 500 rads per min. Following irradiation, the cells were again centrifuged at 4°, resuspended in the growth medium containing the various Act D concentrations shown, and incubated for 60 min. One culture (top panel) was collected immediately after the irradiation. An aliquot of cell suspension (5 - 10 x 10^{4} cell in 0.05 - 0.1 ml of Tris-HCl buffer, 0.05 M, pH at 7.4) was then placed on top of 2 ml of 2% sucrose in water prelayered over a 30 ml 10 - 30% linear sucrose density gradient made up in 0.3 N NaOH, 0.5 M NaCl, and 0.01 M EDTA (McBurney et al., 1971). Each tube also contained a 2 ml cushion of 60% sucrose. The gradients were stored for 16 hours at 4° prior to centrifugation in a Beckman SW 25.1 rotor for 4 hours at 24,000 rpm and 4°. The contents of each centrifuge tube were fractionated from the bottom and the acid precipitable material of each fraction collected on glass fiber filters (Whatman GF/C) for scintillation counting. The direction of sedimentation is from right to left.

RESULTS

The effect of Act D on post-radiation single strand rejoining is shown in Fig. 1. It can be seen that in the absence of Act D, restoration of

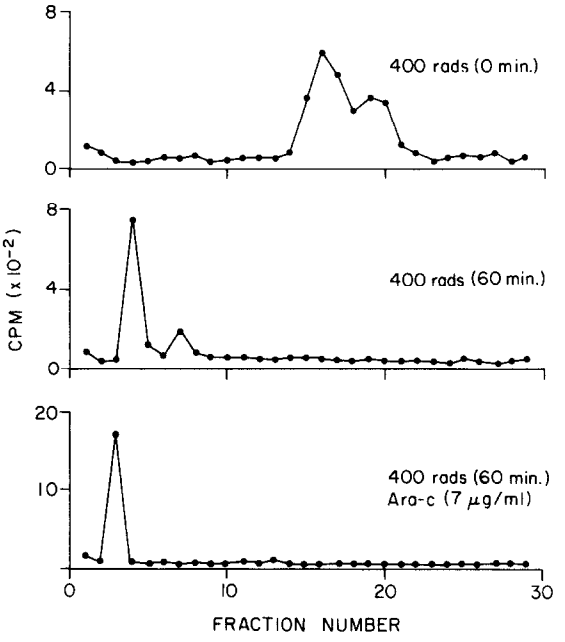

Fig 2: Effect of Cytosine Arabinoside on Single Strand Break Rejoining.

Aliquots of labelled cells were centrifuged, resuspended in Gey's balanced salt solution at 4°, and irradiated (400 rads) at a dose rate of 500 rads per min. Following irradiation, the cells were centrifuged at 4°, resuspended in the growth medium and incubated with or without cytosine arabinoside (Ara-C; 7 μg/ml) for 60 min; one culture (top panel) was collected immediately after the irradiation. Aliquots of cell suspension were then placed on the alkaline sucrose gradients. Other experimental conditions were the same as described in the legend of Fig. 1.

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molecular weight is virtually complete by 60 minutes. The presence of Act D strongly inhibits this rejoining at concentrations as low as 0.028 μg/ml, about one-twentieth the concentration previously reported not to affect rejoining measured by the standard alkaline sucrose method (1). It is of interest that this concentration is similar to that obtained in humans during clinical cancer therapy. Cytosine arabinoside has no effect on the repair of single strand breaks (Fig. 2).

The effect of sub-optimal temperatures are shown in Fig. 3 (3° and 24°C). These temperatures were chosen because they are the same as those employed by Elkind et al, in their studies on recovery of proliferability

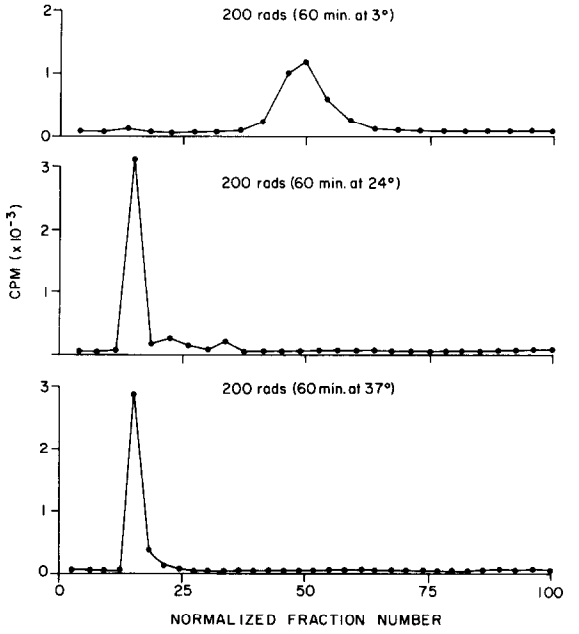

Fig. 3: Effect of Sub-optimal Temperatures on Single Strand Break Rejoining

Aliquots of labelled cells were centrifuged, resuspended in Gey's balanced salt solution at 4° and irradiated (200 rads) at a dose rate of 500 rads per min. Following irradiation, the cells were centrifuged at 4° again, resuspended in the growth medium maintained at three different temperatures shown in each panel and incubated for 60 min. Aliquots of cell suspension were then placed on the alkaline sucrose gradients. Other experimental conditions were the same as described in Fig. 1.

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(13). Single strand rejoining is strongly inhibited at 3° but is only slightly affected at 24°.

DISCUSSION

These experiments suggest that the effects of Act D and temperature shifts on Elkind-Sutton repair may be based on the net inhibition by these modalities of the complex of enzymes involved. In a recent study, Donlon and Norman showed that repair of breaks in single strand DNA was very rapid in human lymphocytes; under ideal conditions they estimated that each cell could repair 10^4 breaks per minute (14). McBurney et al.,

using the same technique we employed here, have found that while the bulk of single strand break repair is very rapid there is a minority fraction whose repair is delayed with persistant breaks being still demonstrable after one hour (12). We have also noted this but only in some gradients, suggesting that resolution is still not ideal despite the improvements made by the McBurney et al. procedure. Since resolution of single strand breaks by this method requires a shift in the molecular weight of between 1 and 5% of the labelled DNA, the presence of any residual damage at one hour following a dose of 500 rads suggests that between 50 and 250 breaks could still be present, but be non-detectable (based on an estimated 10 breaks/cell/rad).

In a related recent study, Brent and Wheatley showed that non-semi-conservative replication can be demonstrated in G-1 HeLa cells following doses of less than 1,000 rads (15). Results such as this imply that assays of single strand rejoining such as we have used here necessarily involve repair of a variety of lesions, some requiring only ligase activity, others the entire series of enzymatic steps. The time course of repair in the system of Brent and Wheatley was rapid but decayed exponentially; significant repair was still demonstrable after 3 to 4 hours. In this context the effect of a sub-optimal temperature shift to 24° is interesting (Fig. 3) in comparison to the delay in the repair of cellular sublethal injury of the same temperature (13). In each type of assay, the repair of single strand breaks reported here and the restoration of colony-forming ability previously reported by Elkind et al. (13), one sees a slight but definite inhibition. Donlon and Norman have shown that the temperature dependence of a single strand break rejoining by human lymphocytes is characteristic of an enzymatic process; their Arrhenius plot of repair suggests that the ligase activity is reduced to about 30% maximum rate at 24°. However, their data also suggests that under optimum conditions repair of single strand breaks after 500 rads would be complete in less than 5

minutes since the enzyme content of lymphocytes can rejoin 10^4 breaks/min./cell at full activity. With such high efficiency it is therefore not surprising that a 60 minute shift to 24° shows only slight inhibition. It is evident, however, that repair is still not complete and that additional (as yet unidentified factors) must be acting to inhibit completion of the repair process. We interpret all of these data as suggesting the following: (a) alkaline sucrose gradient methods monitor a variety of repair levels but these are all probably grounded in the enzymatic processes cited in the introduction; (b) even under optimal conditions the time course of repair of these lesions is variable depending on the number of steps involved; (c) the current levels of resolution of sucrose gradients may not detect breaks still present after several hours; (d) the repair of some forms of single strand breaks is delayed for hours and this delay can be exaggerated by non-optimal conditions, particularly the presence of drugs like Act D which can affect the quaternary structure of the DNA substrate; (e) accumulation of sublethal damage noted in cellular assays using split-fraction radiation may yet be based on residual single strand breaks; (f) a useful class of radiosynergizing drugs will have inhibition of single strand break repair as their primary mode of action. These hypotheses are all subject to the reservation that the techniques employed here measure true single strand breaks, an assumption which must be viewed in the light of recent studies on high molecular weight DNA complexes which may also be involved (17). From the pragmatic point of view of inhibition of repair of sublethal damage, however, this distinction is less important.

In a recent communication we showed that inhibition of non-semi-conservative repair replication could be quantitatively studied in a relatively simple fashion by blocking normal replication with either hydroxyurea or cytosine arabinoside and then measuring the effect of a second agent on net exogenous ^{3}H-thymidine incorporation (16). The data in Fig. 2 show clearly that Ara-C has no effect on the terminal step

in radiation DNA repair, i.e., strand rejoining. From the viewpoint of future developments in applied radiation pharmacology it is clear that a separate assay for inhibitors of the polynucleotide ligase will be required.

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