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Effects of ara A and fresh medium on chromosome damage and DNA double-strand break repair in X-irradiated stationary cells.

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Summary Evidence supports the view that double-strand breaks (dsb) in the DNA of X-irradiated mammalian cells are the lesions leading to chromosome aberrations and eventual cell death. The detailed kinetics of repair of dsb in Ehrlich ascites tumour cells over long repair intervals have therefore been studied and compared under conditions simulating procedures known to cause large changes in cell survival. These conditions are: holding cells in stationary phase for 7 h after X-irradiation, transference of cells to fresh growth medium immediately after X-irradiation, and treatment with the DNA synthesis inhibitor 9-β-D-arabinofuranosyladenine (ara A) for 30 min before, during and for 7 h after X-irradiation. These conditions have also been investigated for their effects on the frequencies of chromosome abnormalities (anaphase bridges and fragments). It is shown that conditions leading to both an inhibition of dsb repair (in the presence of ara A) as well as an acceleration of dsb repair (by fresh growth medium) lead to higher frequencies of chromosome abnormalities as compared to those for cells under stationary conditions for 7 h after irradiation. Holding dsb open for long periods with ara A may maximize the probability of formation of aberrations, however the data for fresh medium treatment show that it is not merely the rate at which dsb repair which determines the aberration frequency, and indicate the presence of other biochemical mechanisms in the cell which determine the frequency of conversion of dsb into chromosome aberrations.

Extensive evidence indicates that the major cause of death of mammalian cells after exposure to ionizing radiation is the formation of chromosome aberrations (e.g. Dewey *et al.*, 1970; Grote & Revell, 1972). Thus, in looking for primary lesions in the DNA, responsible for mammalian cell killing, we may regard the chromosome aberration as an intermediate step and therefore logically investigate those lesions which give rise to chromosome aberrations. Observations of metaphase cells following exposure to ionizing radiation have shown that chromosome aberrations of both the deletion and the exchange types are induced. In order to explain the origin of such aberrations, Bender *et al.* (1974) proposed a model in which unrepaired or irreparable double-strand breaks (dsb) in the DNA of G_0- or G_1-cells, give rise to deletions, and misrepaired or misjoined dsb give rise to exchanges. In a similar way, they proposed, exposure of cells to X-ray in the S- or G_2-phases would result in dsb yielding mainly chromatid aberrations of both deletion and exchange types. Strong evidence in favour of this view was provided by Natarajan and his colleagues (Natarajan *et al.*, 1980). These authors showed that increasing the number of dsb in irradiated and permeablized cells, by incision of the DNA at single-strand break sites using a single-strand specific endonuclease led to a corresponding increase in the yields, at first mitosis, of chromosome or chromatid aberrations, depending on the cycle stage of the cells at the time of irradiation. Preston (1980) has however proposed that chromosome aberrations may arise from base damage in the DNA, by conversion of this into strand breaks by cellular endonucleases. This also seems possible in the light of recent data showing that base damage in X-irradiated cells can be converted into dsb by cellular enzymes (Ahnström & Bryant, 1982).

It has therefore been appropriate to study the induction and repair kinetics of DNA dsb under conditions which cause strong alterations in the survival of cells, or the yields of chromosome aberrations.

The drug 9-β-D-arabinofuranosyladenine (ara A), a powerful inhibitor of DNA synthesis (e.g. Doering *et al.*, 1966) has been shown to remove the "shoulder" region from the survival curve of stationary mouse Ehrlich ascites tumour cells after X-irradiation, and to inhibit the repair of potentially lethal damage (Iliakis, 1980). It was proposed that the removal of the shoulder region from the X-ray survival curve resulted from the promotion by ara A of expression or fixation of

potentially lethal damage (PLD). Ara A was also shown to strongly increase the yield of chromosome abnormalities (anaphase bridges and fragments) arising from chromosome aberrations (Bryant, 1983).

Repair of dsb was found to be inhibited by ara A, more dsb remaining unrepaired in ara A treated cells which had been washed free of the drug and incubated for long intervals (Bryant & Blöcher, 1982). It seemed thus plausible that the inhibition of repair of dsb during ara A treatment caused the fixation of more of these breaks as chromosome aberrations of both the deletion and the exchange types.

It is also well known (e.g. Iliakis & Pohlit, 1979) that introduction of stationary mammalian cells into fresh growth medium (F-med) immediately after irradiation, leads to more cell killing. This procedure (so called "immediate plating") thus causes additional fixation or expression of PLD (Iliakis, 1980). It was reported however that contrary to expectations, the rate of repair of dsb in X-irradiated stationary cells, introduced immediately following irradiation into F-med, was about twice as fast as that for cells held after X-ray exposure under stationary conditions. It is clear therefore that the amount of damage fixed or expressed as cell killing is not related in a simple way to the *rate* of dsb repair.

The initial kinetics of dsb repair under stationary conditions, with ara A treatment, and in fresh medium have already been published previously (Bryant & Blöcher, 1980, 1982). In this paper the detailed repair kinetics of dsb repair over longer repair intervals under these conditions are examined. Also the effect of the immediate transference of cells to fresh medium on the frequency of chromosome abnormalities has been investigated and is compared with data under ara A treatment and after incubation of cells under stationary conditions for 7 h.

Materials and methods

Cell culture

A stable line of mouse Ehrlich ascites tumour cells which grew in suspension cultures was used. The conditions of culture and medium have been described previously (Iliakis & Pohlit, 1979). For experiment cells (which have a doubling time of ~ 10 h) were allowed to grow to stationary phase over a period of 3–4 days.

Radioactive labelling

Cells were labelled for dsb measurements with ^{14}C-thymidine at a concentration of $0.02\,\mu$Ci ml^{-1} and in the presence of $5\,\mu$mol l^{-1} of thymidine. Radioactive label was added to growing cultures over the last cell division, and cells then allowed to grow into stationary phase.

Irradiation

Suspensions of cells, concentrated to 2×10^7 cells ml^{-1} were irradiated in conditioned medium (C-med: medium derived from unlabelled, parallel stationary cultures), in air, at room temperature, in small plastic petri dishes, with X-rays of 150 kV, filtered with 0.8 mm aluminium, at a dose rate of $\sim$ 10 Gy min^{-1}. Doses were monitored by an ionization chamber and checked by a ferrous sulphate method.

Repair conditions

After X-ray exposure cells were diluted either with C-med or with F-med back to their original concentration (2×10^6 cells ml^{-1}) and incubated for 7 h at 37°C.

Ara A treatment

Ara A (Sigma) was added in the form of a 10 mmol l^{-1} solution in balanced salts solution to stationary cultures at a final concentration of $120\,\mu$mol l^{-1} for 30 min before, during and for 7 h after X-ray exposure. The pre-irradiation treatment had been found necessary to achieve the maximum effectiveness of the drug (Iliakis, 1980).

Chromosome abnormalities

The methods used to study anaphase bridges and fragments at the first mitosis after irradiation have been fully described previously (Bryant, 1983). In the additional experiments described here (treatment of cells under "immediate plating" conditions), cells were diluted immediately after X-ray exposure to 2×10^6 cells ml^{-1} for 7 h and then further diluted to 2×10^5 cells ml^{-1} for 24 h. Suspensions were incubated at 37°C. It was shown previously (Bryant, 1983) that the frequency of chromosome abnormalities in X-irradiated cells was not critically dependent on the fixation time between 17 and 27 h after irradiation.

Cells were scored as abnormal if they contained either chromosome bridges, fragments or both together. Approx. 400 anaphases were examined per dose point.

Measurement of repair of dsb.

DNA dsb were measured by the unwinding method (Bryant & Blöcher, 1980). The kinetics of repair of DNA strand breaks measured with the unwinding method, after the first 2 h of incubation, were found

to be identical to those for dsb repair measured by velocity sedimentation in neutral sucrose, so validating the unwinding method for the measurement of dsb repair kinetics (Bryant & Blöcher, 1980). However because of the uncertainty in measurement of the absolute numbers of dsb by the unwinding method, the damage registered (corresponding to dsb in the DNA) has been expressed as "remaining damage" in Grays. The number of dsb present in the DNA is proportional to this dose.

Results

Figure 1 shows the decrease with time of DNA damage, expressed in Grays, which corresponds to the repair of dsb, after X-ray exposure. The measurement of dsb repair kinetics was begun after 2 h because during the first 2 h the kinetics represent the disappearance of single strand breaks from the DNA (Bryant & Blöcher 1980). The results show that during treatment with ara A at 120 μmol l^{-1}, the repair of dsb is inhibited. After washing cells free of the drug at 7 h following irradiation, and resuspension in F-med, cells were able to repair their dsb. The repair of cells held under stationary conditions (open circles) proceeded at approximately the same rate as has been reported previously (Bryant & Blöcher, 1980), i.e.

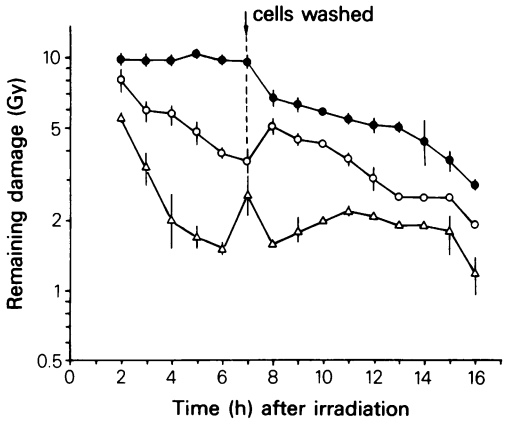

Figure 1 Damage (expressed in Grays) remaining in the DNA of X-irradiated stationary cells as a function of time after X-ray exposure. The dose was 50 Gy. (○) cells held under stationary conditions for 7 h after irradiation, (●) cells treated with ara A (120 μmol l^{-1}) for 30 min before, during and for 7 h after irradiation, (△) cells transferred immediately after irradiation to fresh growth medium. At 7 h after irradiation cultures (○ & ●) were washed and resuspended in fresh growth medium. All incubations were at 37°C. Vertical bars represent standard errors of mean values.

with a $t_{1/2}$ of ~ 4 h. After transference of these cells to F-med following a 7 h period in C-med, more damage appeared at first, but then was later repaired again. The reasons for this are not understood, but may be due to the disturbance of centrifugation and washing procedures which were performed at room temperature.

Cells which had been stationary at the time of irradiation, but were then transferred to F-med immediately after X-ray exposure (triangles) repaired dsb initially at about twice the rate of cells held under stationary conditions. This doubling of the dsb repair rate confirms previous measurements (Bryant & Blöcher 1980). Under these "immediate plating" conditions there appeared to be an increase in damage at 7 h and onwards. This probably represents unwinding points introduced into the DNA during the onset of replicative DNA synthesis.

In Figure 2 are shown dose-response curves for the percentage normal anaphase cells (cells without anaphase bridges or fragments). Two of the curves (replotted from Bryant, 1983) show the effect of holding cells after irradiation under stationary conditions, and the effect of treating cells both for 30 min before and for 7 h after X-ray exposure with ara A (120 μmol l^{-1}). Ara A leads to a synergistic enhancement of the effects of X-rays and yields an exponential dose-effect curve. Cells incubated immediately after irradiation in F-med also yielded more chromosome abnormalities than incubation under stationary conditions; a curve intermediate between that for stationary conditions and that for ara A treatment was obtained.

Discussion

The data show that more chromosome abnormalities arose in cells treated in one case with ara A and the other case with F-med immediately after irradiation than in controls under stationary conditions (Figure 2). These treatments have been shown to yield dose-effect curves for cell killing of similar form (Iliakis, 1980) and thus support the view that chromosome aberrations are a major cause of cell death after exposure to X-rays.

The repair rates of dsb in the presence of ara A or F-med, were however very different. In the presence of ara A, dsb were not repaired at all during the first 7 h, up to the time of removal of the drug, whereas in F-med dsb were repaired at double the rate of that in C-med (stationary conditions). It is clear from these experimental results, that there is no simple relationship between the *rate* or the extent of dsb repair and the occurrence of chromosome abnormalities. A similar conclusion was drawn from a comparison of dsb

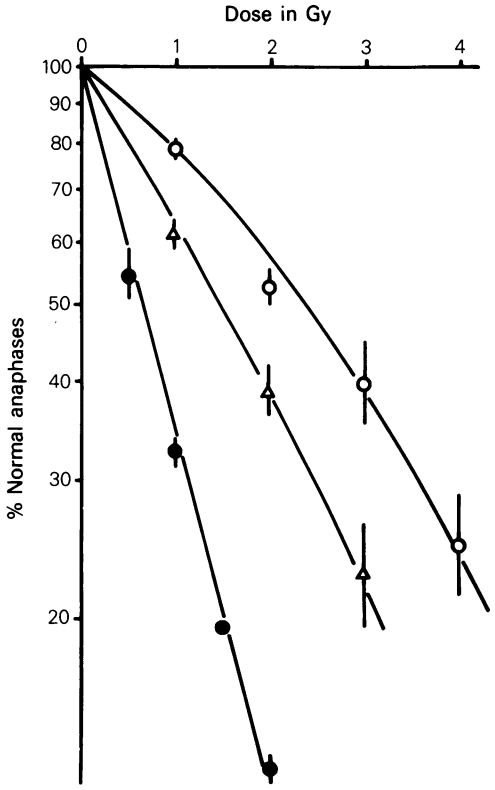

Figure 2 Percentage of cells showing normal anaphases (without chromosome bridges or fragments) as a function of X-ray dose. (○) Stationary cells were irradiated in conditioned medium and held under stationary conditions for 7 h, (△) stationary cells transferred immediately after X-ray exposure into fresh growth medium, (●) stationary cells treated with ara A ($120\,\mu mol\,l^{-1}$) for 30 min before, during and after irradiation for 7 h. At 7 h after irradiation, cultures (○ & △) were washed and diluted in fresh growth medium. All cultures were then incubated for 24 h (up to the first mitosis. Approximately 400 anaphases were examined per point. Vertical bars represent standard errors of mean values. The data represented by the symbols ○ & △ have been redrawn from previously published data (Bryant, 1983).

repair and cell killing in yeast under immediate plating or liquid holding conditions (Frankenberg-Schwager *et al.*, 1980). In this case a higher survival was obtained for cells held under liquid holding conditions where a slow repair of dsb occurred, compared to the immediate introduction of cells into fresh growth medium where lower survival corresponded with a faster repair of dsb.

It seems therefore that the rapid repair of dsb under growth conditions leads to a higher frequency of deletions and exchanges (Figure 2), however the mechanisms by which this occurs are not understood. The mechanism by which ara A enhances the frequency of chromosome abnormalities is similarly not understood, although it seems possible that the increased number of chromosome fragments could arise by more dsb remaining unrepaired up to the first mitosis (Bryant & Blöcher, 1982). One possibility is that the incorporation of ara ATP into the DNA may act as a chain terminator at certain repair sites in the DNA, e.g. those opposite poly dT regions. However such chain termination was not observed in cells undergoing semi-conservative replication in the presence of ara A (Müller *et al.*, 1975).

The anaphase bridges measured in these experiments are assumed to arise from asymmetrical chromosome exchanges, although it is possible that some could have arisen from sister chromatid unions as a result of dsb remaining unrepaired in cells entering S-phase. It seems possible that the increase in exchanges caused by ara A may arise from holding essentially all dsb open for 7 h after irradiation. Under these conditions it might be expected that dsb would have the highest probability of interacting to form exchanges.

Another possibility is that by holding cells in the presence of ara A for long periods, additional dsb form from base damage in the DNA, or base damage opposite a single-strand break so that an endo-nuclease nick can lead to a dsb (Ahnström & Bryant, 1982). It was shown that in Chinese hamster cells additional dsb are generated during repair after X-irradiation, and led to an increasing number of dsb present per cell with time. This effect was however not observed in Ehrlich ascites tumour cells (Blöcher & Pohlit, 1982), even when treated with ara A, (see Figure 1 and Bryant & Blöcher, 1982). The reasons for this difference are not understood, but it may reflect a difference in the rates of endonucleolytic incision of the DNA in the two cell lines.

Preston (1980) found that chromosome aberrations of both deletion and exchange types in X-irradiated unstimulated human lymphocytes, increased with time of treatment with ara C (1-β-D-arabinofuranosylcytosine), an arabinoside with a similar action to ara A on repair of DNA strand break repair and PLD (Iliakis & Bryant, 1983). From this and other results Preston postulated that chromosome aberrations arise from base damage in the DNA via the formation of strand breaks. It was also shown that only deletion type aberrations accumulated in cells which were in G_2-phase at the time of X-ray exposure, suggesting that ara C inhibited all repair, even that required for the mis-joining of dsb to form exchanges (Preston, 1980).

From our own results it seems likely that an

early step in the mis-joining process already takes place in the presence of ara A, since removal of the drug from cells and incubation in C-med without ara A did not lead to repair of PLD (Iliakis & Bryant, 1983).

Thus, although it seems likely from the evidence provided by Natarajan *et al.* (1980), that chromosome aberrations result from dsb occurring in the DNA of irradiated cells as proposed by Bender *et al.* (1974), our results show that paradoxically either an inhibition of repair of dsb, or rapid repair in fresh growth medium immediately after irradiation, both lead to an enhancement in the aberration yields. This indicates the possible involvement of other cellular processes in the determination of the frequency of mis-joining, for example changes in the super-coiling properties of the DNA as cells are passaged from stationary to growth conditions. The biochemical effects of these treatments are subjects of current research.

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