DNA Replication and Repair in Human Cells Exposed to Thermal Neutrons

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The degree of inhibition of DNA replication and the level of DNA repair synthesis induced by thermal neutron irradiation was determined in human lymphoblastoid cell lines. Dose response experiments revealed that, when compared with equivalent doses of ⁶⁰Co-γ-irradiation, thermal neutrons gave rise to similar levels of inhibition of DNA replication but induced relatively low levels of DNA repair synthesis. The amount of repair synthesis after exposure of cells to thermal neutrons, was about ten-fold lower than that observed after γ-radiation. Reasons for this difference are discussed in terms of the type and incidence of damage induced by thermal neutrons.

INTRODUCTION

Exposure of mammalian cells to γ or x-irradiation results both in a depression in the rate of DNA replication¹,² and a dose dependent increase in DNA repair synthesis³⁻⁵. A marked decrease in DNA replication is observed at low doses (200–500 rads) which appears to be due to inhibition of initiation of replication⁶. At higher radiation doses DNA synthesis continues to decrease but at a reduced rate. It seems likely that single strand breaks in DNA, and possibly base modification, induced by ionizing radiation interfere with the replication process. The removal of these lesions from DNA by repair enzymes apparently allows replication to proceed at normal rates⁷. Repair of ionizing radiation damage is largely complete in mammalian cells 4 hours postirradiation⁸.

While the effects of γ and x-irradiation have been well described, the effects of thermal neutrons on DNA replication and their ability to induce DNA repair have not been investigated. Thermal neutrons do not cause excitations or ionizations of atoms but can be captured by nuclei and give rise to nuclear reactions⁹. Disruption of chemical bonds in DNA has been reported to be caused by the nuclear reaction ³¹P(n,γ)³²P arising from thermal neutron capture¹⁰. In this report we have investigated the effect of thermal neutrons on DNA replication
in human lymphoblastoid cells, and determined the level of DNA repair synthesis induced by neutron irradiation. Comparison is made with $^{60}$Co-$\gamma$-irradiation.

**MATERIALS AND METHODS**

**Cell culture**

Epstein-Barr Virus transformed lymphoblastoid cell lines were maintained in suspension in RPMI 1640 (Gibco) medium supplemented with 10% heat inactivated foetal calf serum, penicillin (10$^5$ units/L) and streptomycin (60 mg/L) at 37°C in a humidified atmosphere of 5% CO$_2$. Cell lines were routinely checked for mycoplasma contamination.

**Irradiation**

Thermal neutron irradiation was carried out in MOATA, a 100 KW Nuclear Reactor located at the Australian Atomic Energy Establishment, Lucas Heights, Sydney. At the exposure position used in these experiments 88% of the neutrons had an energy level less than 0.14 eV. Neutron flux was determined by gold foil activation and was 1.39 x 10$^{10}$ neutrons/cm$^2$/sec. The dose rate was estimated to be 4.83 rads/sec and irradiations were carried out for 30 min. Contamination from $\gamma$-rays was reduced to approximately 10% of the total dose, using a lead shield around the sample.

$\gamma$-irradiation was carried out using a $^{60}$Co source (Gamma Cell 220, Atomic Energy of Canada Ltd.). The dose rate varied between 6.2 and 5.5 krads/min during the project.

**DNA repair synthesis — NaI gradient analysis**

This method has been described in greater detail by M. R. James. Parental DNA was prelabelled by growing two plates of cells to stationary phase in Eagle's MEM (F$_{15}$) medium which contained 1.0 $\mu$Ci/ml $[^{32}$P]orthophosphate. They were harvested along with 6 other plates of cells grown to stationary phase in unlabelled F$_{15}$ and subcultured into fresh RPMI 1640(H$_{18}$), 18 hours prior to the experiment. One hour before irradiation, cells were subcultured into H$_{18}$ medium containing FdUrd 10$^{-6}$ M and BrdUrd 10$^{-5}$ M. Cells were irradiated under aerobic conditions with either a $^{60}$Co source or with thermal neutrons and were then grown for 4 hours in FdUrd, BrdUrd and $[^{3}$H]thymidine (10 $\mu$Ci/ml) to determine both DNA replication and DNA repair synthesis. After this time cells were harvested, centrifuged, washed once in saline-EDTA, resuspended, and the DNA samples were prepared as described by Smith and Hanawalt.

An initial neutral NaI gradient centrifugation was used to separate replicated DNA from the light density parental DNA. A typical radioactivity profile of DNA from cells which had been "repair-labelled" by the standard repair protocol
and centrifuged in a neutral NaI gradient for 24 hours is shown in Fig. 1. The parental DNA (dotted line), identified as the $[^{32}P]$ peak region from Cerenkov counting of the neutral gradient fractions, was rebanded on an alkaline NaI gradient. Those fractions that were pooled for rebanding are indicated by the "reband" arrow in Fig. 1. This improved resolution and ensured covalent attachment of $[^3H]$thymidine to parental DNA. Neutral gradients were centrifuged at 42,000 rpm for 18 hours, and alkaline gradients at 42,000 rpm for 36 hours in a 50Ti rotor in an L2-65B Beckman Ultracentrifuge.

Gradients were fractionated, counted and repair synthesis was determined from the $[^3H]$thymidine incorporation in the $[^{32}P]$ region of the gradient. These values were then expressed as $[^3H]$ dpm/µg DNA after determination of the specific activity ($[^{32}P]$ dpm/µg DNA) of the DNA$^{12}$.

Fig. 1. Density gradient profile of lymphoblastoid DNA in a neutral sodium iodide gradient. Cells were irradiated with 20 krads and DNA extracted and prepared for centrifugation as described in Methods.
DNA replication

The extent to which irradiation affects DNA replication can be determined from the initial neutral NaI gradient centrifugation. This centrifugation step separates semiconservatively replicated DNA (hybrid density (HL) DNA) from the light parental (LL) DNA as shown in Fig. 1. The amount of semiconservative synthesis which occurred during the 4 hour period after irradiation was calculated as [³H] dpm/μg DNA in the HL region of the gradient. Radioactivity in this region was very largely tritium (unbroken line — Fig. 1).

RESULTS

The extent of inhibition of DNA replication with increasing radiation dose is described in Fig. 2. A rapid decline in DNA synthesis is observed after exposure

![Graph showing inhibition of DNA replication](image)

Fig. 2. Inhibition of DNA replication by γ-radiation (●) and thermal neutron irradiation (■). DNA replication was calculated from the heavy light peak on sodium iodide gradients. Error bars represent standard error of the mean (S.E.M.) for pooled duplicate determination of three lymphoblastoid cell lines (C1ABR, C2ABR and C6ABR).
of cells to 10 krads of γ-rays. The same dose of thermal neutrons gave rise to a similar level of inhibition. Irradiation with thermal neutrons was not extended above 20 krads because of the time taken to deliver this dose.

A number of studies indicate that ionizing radiation inhibits initiation of DNA replication by introducing breaks into DNA. Since thermal neutrons cannot ionize atoms it seems likely that neutron capture gives rise to lesions which are responsible for the marked inhibition of DNA synthesis observed in these studies (Fig. 2). In order to investigate this further we have determined the amount of repair synthesis induced in cells exposed to both forms of radiation. The results obtained demonstrate a dose-dependent increase in DNA repair synthesis after exposure of cells to γ-radiation (Fig. 3). Repair synthesis increases rapidly over the dose range 0—20 krads levelling off between 20 and 40 krads. Exposure of cells to similar doses of thermal neutrons leads to a low level of repair synthesis (Fig. 3). After a dose of 10 krads the amount of repair synthesis is about

![Diagram showing dose response of DNA repair synthesis induced by γ-radiation (●) and thermal neutron irradiation (■). Repair synthesis values are presented as dpm/µg of DNA and are the mean ± S.E.M. for pooled duplicate determinations on three lymphoblastoid cell lines (C1ABR, C2ABR and C6ABR).]
15% of the value obtained with the same dose of $\gamma$-radiation. At 20 krad the value is 7% of that obtained after a corresponding dose of $\gamma$-radiation. The time course of DNA repair synthesis up to 8 hours after irradiations reveals a similar pattern for both forms of radiation (Fig. 4). However, the amount of repair synthesis after exposure of cells to thermal neutrons, is about ten-fold lower than that observed after $\gamma$-radiation.

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**Fig. 4.** Time course of DNA repair synthesis over the periods 0–4 hr and 4–8 hr after irradiation of the same cell lines as in Figure 3 with $\gamma$-radiation (a) and thermal neutron irradiation (b). Error bars represent S.E.M.
DISCUSSION

The results obtained in this study describe a marked effect of thermal neutrons on DNA replication in mammalian cells. The extent of inhibition with increasing dose is comparable with that observed in cells exposed to γ-radiation over the same dose range. Inhibition of replication after γ-irradiation appears to result largely from changes to chromatin structure after the introduction of breaks into the phosphodiester backbone of DNA\textsuperscript{14}. A similar pattern of inhibition of DNA replication after exposure of cells to thermal neutrons (Fig. 2) would also appear to implicate interruptions in DNA structure. However it is also possible that inhibition is due to changes to the nuclear membrane or its association with chromatin. Breaks in DNA would not result from ionizations either directly at the level of DNA or indirectly due to radiolysis of water since this form of radiation does not cause ionizations or excitations of molecules\textsuperscript{9}. It seems more likely that neutron capture gives rise to nuclear recoil reactions which in turn lead to bond rupture. Akaboshi \textit{et al.}\textsuperscript{15} have shown that when DNA is irradiated with thermal neutrons, recoiled \textsuperscript{32}P nuclides derived from the \textsuperscript{31}P(n,γ)\textsuperscript{32}P reaction lead to localized breakage of chemical bonds. Approximately 50% of the [\textsuperscript{32}P] appearing in DNA after thermal neutron irradiation was released by alkaline phosphatase\textsuperscript{15}. It has been estimated that 75% of \textsuperscript{31}P(n,γ)\textsuperscript{32}P reactions lead to breakage of the phosphodiester backbone of DNA\textsuperscript{10}.

Further data to support a role for thermal neutrons in causing breakage in DNA is obtained from experiments in which bleomycin and actinomycin D were used in combination with radiation to provide a sensitizing effect\textsuperscript{16}. Bleomycin, which gives rise primarily to single strand breaks in DNA\textsuperscript{17}, was used at a concentration which had a negligible effect on cell survival. Increasing dose of thermal neutrons had a marked sensitizing effect on cell survival in the presence of bleomycin. It was suggested that single strand breaks produced by bleomycin are converted to double strand breaks by the action of thermal neutrons\textsuperscript{16}. It is generally accepted that double strand breaks introduced into DNA are more effective in cell killing.

Damage to DNA caused by neutrons is effective in inhibiting DNA replication but induces only a low level of DNA repair synthesis when comparison is made with similar doses of γ-radiation. Kawai \textit{et al.}\textsuperscript{10} have estimated that the number of \textsuperscript{31}P(n,γ)\textsuperscript{32}P reactions per cell irradiated with \(9.1 \times 10^{13}\) neutrons/cm\(^2\) is 2.73. This result demonstrates that a small number of strand breaks per cell induced by thermal neutrons is very effective in cell death. In our experiments we have exposed cells to \(2.5 \times 10^{13}\) neutrons/cm\(^2\) which would give rise to approximately one \textsuperscript{31}P(n,γ)\textsuperscript{32}P reaction per human cell. Therefore it is not surprising that the level of DNA repair synthesis is low in cells exposed to thermal neutrons (Fig. 3). Indeed it seems likely that most of the repair synthesis observed after neutron irradiation is due to γ-ray contamination.
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