Neutron-induced Adaptive Response Studied in Go Human Lymphocytes Using the Comet Assay

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This study demonstrates that cells adapted to ionizing radiation developed reduced initial DNA damage when compared to non-adapted cells. The results were obtained by subjecting in vitro irradiated whole blood from 10 healthy volunteers (including 2 A-bomb survivors carrying 1.5-2 Gy in vivo exposure) in an unstimulated condition (G0) using the comet assay. The intensity of DNA damage was assessed by computing the ‘tail moment’. Adaptive response (AR) was noticed in only donor 3, as indicated by reduced tail moment when the blood samples received priming + challenging doses over a 4 h interval. The priming dose was either 0.01 Gy 137Cs γ-rays or 0.0025 Gy 252Cf neutrons. The delivered challenging dose was either 1 Gy 60Co g-rays or 0.25 Gy 252Cf neutrons. The irradiation was conducted using the HIRRAC facility. A prior exposure to 0.0025 Gy 252Cf neutrons nullified the excess tail moment caused by 0.25 Gy neutrons given during a 4 h gap. In a similar way, 0.01 Gy 137Cs γ-rays offered a cross-adaptive response to the neutron challenging dose. The tail moment of A-bomb survivors after in vitro irradiation was less than that of the age-matched control and, at the same time, was not influenced by the priming dose. An altered subset and the immunological status of blood after A-bomb exposure were cited as possible factors. Because AR can affect the outcome of RBE, its individual variability only emphasizes the need to have individual biodosimetry for better risk assessment, especially in planning for a long space voyage.

INTRODUCTION

The health effects of neutron exposure have been of a great concern ever since “Little
Boy” was detonated over Hiroshima in 1945. This idea has gained momentum in tune with further space research. Cosmic radiation creates high-energy secondary radiation consisting mainly of neutrons, protons and γ-radiation. The potential radiation risks associated with high-altitude flight by users have been examined by NCRP, which has also recognized the need for further research in the radiobiology of neutrons. An increase of chromosomal aberrations (CA) in peripheral lymphocytes and elevated incidents of cancer among aircrew members have been well-documented. Obe et al. predicted a radiation hazard of 40 dicentrics to occur in every 1,000 cells of T-lymphocytes during the 975 days of a space mission to Mars. At the same time, the radiation risk is modified to some extent by an adaptive response (AR) as a result of previous exposure. Radioadaptation is evident from a recent study conducted on flight engineers who were exposed to cosmic neutrons. The radioadaptive phenomenon is generally noticed with low-LET radiation, and only scarce information exists for neutrons. AR is known for non-specific cross-protection. The response of human tissue to low doses of low-LET radiation has been shown to reduce the spontaneous occurrence of chromosomal aberrations in human lymphocytes in vitro. An in vivo AR has been reported in the lymphocytes of chronically exposed workers and in children exposed to fallout radiation in the Ukraine following the Chernobyl accident. People living in high background-radiation areas have displayed lower mortality from cancer, though it was not statistically significant from a control.

The exact mechanism of a cellular adaptive response is yet to be fully explored. Conflicting opinion still persists concerning the inducibility of AR in the G₀ phase of the lymphocyte cell cycle. It was postulated that cells need to pass through the S phase between the exposure of adapting and challenging doses in order to show AR. On the other hand, AR was found in G₀ lymphocytes irradiated in vitro. AR in human lymphocytes is generally identified by a reduced frequency of chromosomal damage. The suggested logic sequel was to find a relationship between adaptation and either lowering the initial DNA damage and/ or faster repair. AR without imposing changes in the repair kinetics, while at the same time reducing the frequencies of chromosomal damage has been recorded. Here, we have attempted to study AR by measuring the kinetics of the initial DNA damage in non-stimulated human peripheral blood lymphocytes using single-cell gel electrophoresis (commonly known as ‘comet assay’). This is made possible because of the availability of the Hiroshima University Radiobiological Research Accelerator (HIRRAC) facility and a unique cohort of A-bomb survivors present in Hiroshima. For this study, the comet assay is particularly suitable because the initial DNA damage at the individual cell level can be analyzed rapidly. In addition to being a sensitive method, doses as low as 1.02 mGy can be studied. Fingerprints left in exposed cells by neutrons differing only by narrow energy ranges could be detected by the comet assay. Also, the study can include a vast majority of the cell population displaying different radiosensitivity, which otherwise would fall outside the scope of AR by conventional CA and MN analyses.
MATERIALS AND METHODS

Blood donors

Heparinised blood samples of ten healthy non-smoking volunteers from Hiroshima Prefecture were obtained by venepuncture after informed consent. The medical history revealed that the volunteers were not exposed to therapeutic irradiation, viral vaccination, drug intake or diagnostic X-irradiation during a 6-months period prior to sampling. Donors 4 and 10 were A-bomb survivors (Table 1) who had been exposed to A-bomb radiation of 1.5 to 2 Gy.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Sex</th>
<th>Age</th>
<th>History of A-bomb exposure (including parental exposure)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>25</td>
<td>Nil</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>22</td>
<td>Nil ’young’</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>27</td>
<td>Nil</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>72</td>
<td>1.5-2 Gy</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>73</td>
<td>Nil</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>80</td>
<td>Nil</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>74</td>
<td>Nil ’aged’</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>77</td>
<td>Nil</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>79</td>
<td>Nil</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>74</td>
<td>1.5-2 Gy</td>
</tr>
</tbody>
</table>

Irradiation

Blood samples were incubated at 37°C until irradiation and were used within 30 min of collection. The ‘whole blood’ irradiation was carried out at ambient temperature with a 252Cf source (3.1-mg on 1 April 1988) housed in the HIRRAC facility at Research Institute for Radiation Biology and Medicine, Hiroshima. The HIRRAC facility also houses 137Cs- (11.1 GBq as on March 1986) and 60Co- (111 TBq as on Feb. 1992) gamma sources. The 252Cf beam and irradiation system were described previously. The 252Cf beam contained ~33% γ-ray and 67% fission neutrons. The measurement of the neutrons and γ-rays was discussed elsewhere. The control sample received an identical treatment, except for radiation. 252Cf neutrons of 0.0025 Gy and 0.25 Gy were delivered at dose rates of 0.2656 cGy/min, and 1.62 cGy/min, respectively. When 137Cs was used in an AR study, a 0.01 Gy adaptive dose was delivered at a rate of 0.001 Gy/min. 60Co γ at 0.1 Gy/min was used either as a challenge dose (1 Gy) in an AR study or to compare the radioresponse (2 Gy) among ‘aged’ and ‘young’ cohorts. An interval of 4 h was maintained between the adaptive (priming) and challenging doses. During this period, blood samples were incubated at 37°C. Samples after the final irradiation were transferred on ice for a brief period before subjecting them to the comet assay.
All samples whether irradiated or sham-treated followed all other steps uniformly and to-

gather.

Adaptive response study

Blood samples from donors 1 to 9 were subjected to a priming dose of 0.01 Gy $^{137}$Cs $\gamma$, and were treated after 4 h with 1 Gy $^{60}$Co $\gamma$ as the challenging dose. The experiment was repeated three times and the sampling was done in 2-day intervals. $^{252}$Cf neutrons of 0.0025 Gy and 0.25 Gy served as the adaptive and challenging doses, respectively.

Radioresponse of ‘aged’ and ‘young’

The volunteers were divided into 2 groups. Donors 1–3 formed the ‘young’ cohort (22–
27 years) and donors 5–9 were grouped as ‘aged’ (73–80 years). The blood samples from the 2 cohorts were irradiated to 2 Gy of the $^{60}$Co $\gamma$ dose. At the end of irradiation the ‘whole blood’ samples were subjected to the comet assay.

Radioresponse of A-bomb survivors

The samples of donors 4 and 10 were subjected to an AR study using $^{252}$Cf neutrons and compared with donor 3. The priming dose was 0.0025 Gy and the challenging dose was 1 Gy. For ethical reasons, blood sampling was done only once from each survivor. The results for a statistical analysis were obtained by repeating the comet assay out of these samples.

Single-cell gel electrophoresis (comet assay)

The initial DNA damage was determined by an alkaline comet assay, as described by Singh et al.\textsuperscript{12)\textsuperscript{,}} with minor modifications. About $10^4$ cells either in a 5 $\mu\text{L}$ blood sample or a 10 $\mu\text{L}$ blood sample (A-bomb survivors) were mixed with 75- or 80-$\mu\text{L}$ of warm low-melting-point agarose (Gibco BRL: Gaithersburg, USA) at 0.75%, 37$^\circ$C, in a microfuge tube and spread over a fully frosted microscope slide pre-coated with 200 $\mu\text{L}$ of 0.1% agarose by layering a cover slip. The cover slips were gently removed after placing the slides on ice for 5 min. Slides were immersed in a jar containing a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10) to which were added 1% Triton X-100 and 10% DMSO just before use. The lysis was done at 4$^\circ$C for 1 h in the dark.

After lysis, the slides were subjected to DNA unwinding for 20 min in a buffer (0.3 M NaOH, 1 mM Na$_2$EDTA, pH >13) and subsequently electrophoresed at 300 mA (15 V) for 20 min, 24$^\circ$C, under dim yellow light. Slides were washed twice for 5 min in a neutralizing buffer (0.4 M Tris, pH 7.5) and stained with 75 $\mu\text{L}$ of propidium iodide (20 $\mu\text{g/}\text{ml}$). They were stored in a moist chamber at 5$^\circ$C and analyzed within 3 h under a fluorescent microscope with excitation at 530–560 nm, detection > 580 nm, coupled with an intensified target camera with a self-designated image analysis system (Olympus, Tokyo). The ‘comets’ images were analyzed using software in BAS 1500 (Fuji Co., Tokyo). The intensity of the initial DNA damage was measured as ‘tail moment’, which was calculated by multiplying the tail length by the amount of DNA in the tail. The comet tail was set to be the area from the edge of the head to the end of the tail. Relative units were used in a graphic presentation. For each
sample about 80–100 images were analyzed randomly. Each data point of the initial DNA damage in the graphic presentation represents the mean ± SD of at least 3 individual experiments, unless otherwise stated.

Statistical analysis

A statistical analysis was carried out using the INSTAT GRAPHPAD program. The results are provided as mean ± SD of 3 individual experimental values. Student’s t-test was employed to assess the statistical significance.

RESULTS

Blood samples from 9 donors were initially screened for in vitro AR after delivering 0.01 Gy and 1 Gy $^{137}$Cs γ-rays in 4-h intervals. The initial DNA damage at the individual cell level was assessed by single-cell gel electrophoresis. A clear response was observed only in donor 3 (data not included for others), while donor 4 (A-bomb survivor) displayed reduced tail moment irrespective of the pretreatment (adaptive dose) when compared with the age-matched control. A similar observation was also made in donor 10 (A-bomb survivor) later. The samples of donor 3 were used further for an AR study. Whole blood pretreated with either 0.01 Gy $^{137}$Cs γ-rays or 0.0025 Gy $^{252}$Cf neutrons significantly reduced the level of the initial DNA damage in response to the respective challenging dose (Fig.1). One-tailed P val-

![Graph showing adaptive response of human lymphocytes towards ionizing radiation (donor 3)](image)

Fig. 1. Adaptive response of human lymphocytes towards ionizing radiation (donor 3)

*Represents the mean of each experimental group obtained by subtracting the control value from those experimental values that happen to fall at the maximum over the values of the control group (which equals to the mean plus 95% confidence limit). Error bar represents (±) SD of the mean.
ues of 0.0441 and 0.0261, respectively, are considered to be significant. In general, a pre-exposure to the adaptive dose resulted in a reduction of nuclear damage (Fig. 2). $^{137}$Cs $\gamma$-rays as the priming dose offered comparable cross protection to neutron radiation. A one-tailed $P$
value of 0.0074 is considered to be very significant.

The tail moment of A-bomb survivors after \textit{in vitro} irradiation was less than the A-bomb unexposed samples (data not shown). The radioresponse of the two age groups, ‘young’ and ‘old’, after irradiating the blood samples to 2 Gy $^{60}$Co $\gamma$-rays was compared for any age-related differences (Fig. 3). No marked difference in tail moment was observed between the two cohorts (the one-tailed P value, 0.058, is considered not to be very significant), indicating that the radioresistance noted in A-bomb survivors’ blood was not related to their age factor. We also compared the \textit{in vitro} radioresponse of A-bomb survivors with that of an A-bomb unexposed healthy volunteer who displayed an adaptive response (Fig. 4). \textit{In vitro} irradiation of 1 Gy $^{252}$Cf neutrons caused a lower tail moment in the A-bomb survivors’ blood when compared to that of donor 3 in the absence of the adaptive dose. However, a pretreatment of 0.0025 Gy neutrons as the adaptive dose caused a significant reduction in the tail moment only in the latter case (the one-tailed P value is 0.0095 which is considered to be very significant).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{fig4.png}
\caption{\textit{In vitro} radioresponse of A-bomb survivors

*Represents the mean of each experimental group obtained by subtracting the control value from those experimental values that happen to fall at the maximum over the values of the control group (which equals to the mean plus 95\% confidence limit).
CD: Samples received ‘challenging dose’ (1 Gy neutrons)
A+CD: Samples received ‘adaptive + challenging dose’ (0.0025 Gy + 1 Gy neutrons)}
\end{figure}

\section*{DISCUSSION}

The radiobiology of neutrons is gaining importance amidst the prevailing nuclear arsenals (including H-bomb), nuclear power plants and the unique A-bomb exposed cohorts in Hiroshima and Nagasaki. Since 1945, more than 2,050 nuclear tests have been conducted, 26\% of which were in the atmosphere\textsuperscript{16,17}. The recent criticality incident at Tokai Mura\textsuperscript{18}
also emphasizes the need for evaluating the biological response. In donor 3, 0.01 Gy \(\gamma\)-ray pre-irradiation cross-adapted to 0.25 Gy neutrons. It is evident from the present study that the biological effective dose of a neutron-dose exposure can be modified by a prior exposure to very low doses of \(\gamma\)-rays or neutrons. It is in support of previous observations\(^{19}\) that 20 cGy of d(4)-Be neutrons could produce an adaptive resistance to subsequent 1 Gy challenge doses of X-rays, and thus adaptation was at least the same phenomenon as that induced by a 20 cGy X-ray dose.

In our experiment, a clear difference in the occurrence of initial DNA damage between adapted and non-adapted neutron exposed cells was noticed. This is further strengthened by an earlier report that described a comet assay in which either faster DNA repair kinetics or reduced initial damage was possible in adapted cells of the peripheral blood lymphocyte culture\(^{20}\). However, the present investigation is unique as a study, which was made with unstimulated human cells involving whole blood under the \textit{in vitro} condition, and thus minimizing the artifact. Using the comet assay, a reduction in the initial damage after 1 Gy \(^{137}\)Cs \(\gamma\)-rays has been reported in blood samples from those patients who had undergone \(^{131}\)I radiotherapy in relation to those who had not received \(^{131}\)I therapy\(^ {21}\). Other experimental results also substantiate our findings that the adaptive dose can result in reduced damage evoked by the challenge dose\(^ {11}\). Its molecular mechanism is not yet clearly understood. However, these results are independent of the opinion that a cell needs to pass through the S phase to express AR\(^ {9}\). Probably, intracellular free radicals produced by low doses of radiation may act as a trigger for the induction of a radical scavenging substance\(^ {22}\) or structural modifications of pre-exposed DNA to resist further damage\(^ {23}\). On the other hand, Ikushima et al.\(^ {24}\) failed to observe any such differences in the initial DNA damage in V79 cell lines. Unlike lymphocytes, cell lines generally represent a tampered cell-cycle control by which cells accumulate at the G1 stage. Moreover, the cell phase at which the adaptive and challenging dose (gamma-rays) given was different and their applied method was a neutral comet assay which predominantly detects the double-strand breaks of DNA.

In this study, AR was found in 1 of 8 healthy volunteers, excluding the A-bomb survivors. Although AR is understood to be a universal phenomenon, it has a high probability to vary with individuals\(^ {9}\) and to operate under more than one mechanism (Fig.5). Pereira Luis and Povo\(^ {25}\) have observed that 4 donors out of 6 did not develop any significant AR. The donor’s constitution seems to have an important bearing on AR. Our results also support the variability in the individual response to the adaptive dose, which would be an important factor in a genetic risk assessment as well as in radiotherapy. Because the cohort under this study was unique (3 out of 10 volunteers were ‘young’), it is of interest to look into the ‘age’ factor in AR. It is evident from our study that the A-bomb survivors exhibited less susceptibility to \textit{in vitro} irradiation when compared to the age-matched controls. The exact mechanism behind such an observation is at present not clear. That the extent of DNA migration was similar in young and elderly subjects has been reported earlier\(^ {26}\). Since the observation was based on limited samples, it should be treated as preliminary until a large database is analyzed. However, the existing evidence does not reject such an observation, even though Ban et al.\(^ {27}\) did not find a disproportionately large number of either radioresistant or radiosensitive persons...
among the A-bomb survivors. However, it has also been suggested that the population of A-bomb survivors may not include radiosensitive persons, because they died of acute infections while young\(^{28}\). An altered composition of T and B cells in the peripheral blood of A-bomb survivors has been observed, which raises the possibility that A-bomb radiation may affect the developmental process of T and B cells\(^{29}\). Furthermore, significant late effects of A-bomb radiation have been found in certain immunological functions and in the number of CD5\(^{+}\) mature T cells in peripheral blood lymphocyte\(^{30}\). Chromosome aberrations (CA) and translocations were observed in both peripheral blood lymphocytes and bone-marrow cells of heavily exposed survivors many years after the bombing\(^{31}\). These findings strongly suggest that the constitution of the hematolymphoid system changed as the result of exposure to radiation, which possibly altered the radioreponse.

We are able to show that AR can influence the outcome of neutron exposure. AR gains importance in biodosimetry or accidental risk assessment for occupational workers and radiotherapeutic patients, which are assayed on CA, MN, mutations as end points. In the current high-altitude commercial flights, both the passengers and crews are subjected to very low doses of neutrons. About 500 million people cross the transcontinental borders every year. The possibility of more people being exposed will increase in the coming millennium. Hence, long-term neutron exposure at low level needs deeper study.
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