High Frequency of Simple and Complex Chromosome Aberrations Detected in the Tokai-mura Survivor four and Five Years after the 1999 Criticality Accident

Natalia SUMPTION1, Liz AINSBURY2, Dudley GOODHEAD3, Toshiyuki HIRAMA4, Makoto AKASHI5, Manami MONOBE6, Koichi ANDO5 and Rhona ANDERSON7*

24-colour karyotyping/Radiation-induced chromosome aberrations/Complex aberrations.

In September 1999 a criticality accident occurred in a uranium processing plant in Tokai-mura, Japan. During the accident, three workers (A, B and C) were exposed to high acute doses of neutrons and γ-rays: workers A and B fatally and worker C to an estimated whole body absorbed dose of 0.81 Gy neutrons and 1.3 Gy γ-rays. We obtained fixed peripheral blood lymphocytes (PBL) preparations from worker C approximately four and five years after the accident and assayed by 24 colour karyotyping (M-FISH) to determine the frequency and complexity of chromosome aberrations present. We observed a high frequency of simple reciprocal translocations, which we used to provide a rough estimation of dose and, in addition, for the assessment of the emergence of any clinically-relevant clonal exchanges. We did not observe any evidence of clonality but did find some evidence suggesting chromosome 1 as being preferentially involved in exchanges in stable cells. We also detected a relatively high frequency of damaged cells containing complex chromosome aberrations, of both the stable and unstable types. Qualitatively these complex aberrations were consistent with those observed to be induced after exposure to low doses of high-LET radiation or moderate doses of low-LET radiation, supporting the suggestion that heavily damaged cells can be quite long-lived in vivo.

INTRODUCTION

In September 1999 a criticality accident occurred in a uranium processing plant in Tokai-mura, Japan. During the accident, three workers (A, B and C) were exposed to high acute doses of neutrons and γ-rays and all, suffering a variety of immediate health consequences, were within five hours transported to the National Institute of Radiological Sciences (NIRS), Chiba. Upon admission, a range of medical interventions were carried out including the sampling of peripheral blood for biodosimetric purposes. Cytogenetic analysis were carried out after in vitro culture times totalling 48 hours providing initial estimates of > 20, 7.4 and 2.3 GyE’ using PCC-ring analysis and 24.5, 8.3 and 3.0 GyE’ by conventional dicentric assays, for workers A, B and C, respectively.1) Physical estimates based on the specific activity of 24Na in the blood and the predicted neutron/γ-ray ratio gave estimated whole body neutron doses of 5.4 Gy, 2.9 Gy and 0.81 Gy. γ-ray whole body absorbed doses were estimated using calculations based on either environmental measurements of ambient dose after the accident (8.5, 4.5 and 1.3 Gy) or data from the IAEA Technical Report No. 211 (13, 6.9 and 2.0 Gy) for workers A, B and C, respectively.2) Unfortunately, given the severity of exposure and despite extensive medical efforts workers A and B died 82 and 210 days after the accident. Worker C was treated to minimise gastrointestinal infection and to aid bone marrow recovery; however no haemopoietic stem cell transplantation was carried out.3) He was discharged from hospital in December 1999 and continues to be medically assessed.

Multiplex fluorescence in situ hybridization (M-FISH), first described by Speicher4) enables the simultaneous visualization of all chromosomes in a single hybridization. The technique, which essentially paints the entire genome in multiple colors, combines the significant advances that had been made in fluorescence probe labeling strategies,9) digital
imaging fluorescence microscopy and image processing capability, to create a revolutionary karyotypic tool for the analysis of structural and numerical abnormalities. The power of M-FISH lies in its ability to identify all cytogenetically visible chromosomal interchanges throughout the whole genome (with the exception of homologous rearrangements) and therefore to resolve even quite complex karyotypes within non-clonal populations with a high degree of confidence. M-FISH is therefore the technique of choice for the detailed cytogenetic assessment of mixed populations of heavily damaged cells such as those observed after exposure to ionising radiation. For instance, it is well established that the complexity of aberrations induced is dependent on quality and dose of exposure whereby complex aberrations (involving three or more breaks in two or more chromosomes) are characteristically induced after low doses of high but not low linear energy transfer (LET) radiation. Damaged chromosomes can be resolved as occurring in a single rearrangement or multiple different rearrangements within each damaged cell and correlated with exposure e.g. a cell with four damaged chromosomes may be visible as a single complex aberration typical of high-LET exposure or as two independent simple aberrations typical of low-LET exposure. Further, the stability of the cell can be predicted based on the stability of all aberrations detected, including whether the aberration is clonal and/or whether there is evidence of karyotypic evolution indicative of genomic instability. Thus, data can be generated from M-FISH karyotypes that have the potential to be informative for retrospective dosimetry, determination of long-term stability of damaged cells and the possible emergence of clinically-relevant aberration types.

The purpose of this study was to extend the cytogenetic studies thus far carried out on the surviving worker (worker C) to provide a more detailed study of the chromosome aberrations present. To do this we obtained fixed peripheral blood lymphocytes (PBL) from worker C approximately four (P1) and five (P2) years after the accident and assessed for the frequency, type and complexity of chromosome aberration present by M-FISH.

**MATERIALS AND METHODS**

**Lymphocyte culture and chromosome preparation**

Whole blood was sampled on two separate occasions from Worker C approximately four years (June 30, 2003) (P1) and five years (June 28, 2004) (P2) after the accident. We obtained consent from Worker C and agreement from the internal ethics committee before blood sampling at the NIRS hospital, Chiba. Peripheral blood T-lymphocytes (PBL) were stimulated to divide by the addition of 0.1 mg/ml purified phytohaemagglutinin (HA15, Murex) in culture medium (RPMI containing 15% heat inactivated foetal bovine serum (Gibco), 2 mM sodium pyruvate (Sigma), 100 U/ml penicillin (Sigma), 0.1 mg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma) for 48 hours including a 2 h colcemid (50 ng/ml) block. After this time, PBL were centrifuged, re-suspended in pre-warmed hypotonic solution (0.075 M KCl) for 20 minutes at 37°C then fixed as standard with fresh 3:1 methanol:acetic acid. PBL preparations were subsequently fixed three further times before being transported at room temperature to MRC Radiation Genome and Stability Unit where they were re-fixed and dropped onto clean glass slides.

**Multiplex fluorescence in situ hybridisation (M-FISH)**

Fresh slides of metaphase cells were hardened (3:1 methanol:acetic acid for 1 h, dehydrated through an ethanol series (2 min each in 70%, 70%, 90%, 90% and 100%), baked at 65°C for 20 min, then immersed for 10 min in acetone) and pretreated with RNase A (100 μg/ml in 2 × SSC) at 37°C for 1 h. After washing in 2× (saline-sodium citrate) SSC and phosphate buffered saline (PBS), the cells were treated with pepsin (1:20 × 103 in 10 mM HCL) at 37°C for 5–10 min then washed twice in PBS, 50 mM MgCl2/PBS, 50 mM MgCl2/1% formaldehyde/PBS then PBS before finally dehydrating through an ethanol series. For hybridisation, cells were denatured in 70% formamide/2 × SSC at 72°C for 3 min and dehydrated for 1 min each in 70/90/100% ethanol. In parallel, an aliquot of SpectraVision™ Assay (Vysis, UK) 24-colour paint cocktail was denatured at 73°C for 6 min. Cells and probe were then mixed and left to hybridise for 36–48 h at 37°C before being washed in 0.4 × SSC/0.3% Igepal (Sigma, UK) at 71°C for 2–3 min and in 2 × SSC/0.1% Igepal at room temperature for 10 sec. Cells were counterstained using 4’,6-diamidino-2-phenylindole (DAPI) (Vysis III) (Vysis, UK), sealed and stored in the dark at –20°C.

**M-FISH analysis**

Chromosome aberrations were analysed as previously described. In brief, metaphase chromosomes were visualised using a 6-position Olympus BX51 fluorescent microscope containing individual filter sets for each component fluor of the SpectraVision (Vysis (UK) Ltd) probe cocktail plus DAPI. Digital images were captured for M-FISH using a charged-coupled device (CCD) camera (Photometrics Senys CCD) coupled to and driven by Genus (Applied Imaging, UK). In the first instance, cells were karyotyped and analysed by enhanced DAPI banding. Detailed paint analysis was then performed by assessing paint coverage for each individual fluor down the length of each individual chromosome, using both the raw and processed images for each fluor channel. A metaphase spread was classified as being apparently normal if all 46 chromosomes were observed by this process, and subsequently confirmed by the Genus M-FISH assignment, to have their appropriate combinatorial paint composition down their entire length.
Classification of chromosome aberrations

Chromosomal abnormalities were identified as colour-junctions down the length of individual chromosomes and/or by the presence of chromosome fragments. The M-FISH paint composition was used to identify the chromosomes involved in the abnormality and assignment of a breakpoint to a specific chromosomal region (pter, p, peri-centromere, q or qter) was based on the DAPI banding pattern at the M-FISH colour junction, the location of the centromere and the size of the painted material on each rearranged chromosome. A detailed assignment of breakpoints to chromosome bands was not possible due to limits in DAPI resolution and no attempt was made to consider intra-chromosomal events such as inversions. Chromosome abnormalities were described in detail using a modified version of the mPAINT classification system.15)

Exchange aberrations involving 3 or more breaks in 2 or more chromosomes were classed as complex. The size and complexity of each complex aberration was determined by the number of different chromosomes and the minimum number of different breaks required to produce the visible complex. The potential transmissibility of each complex was also detailed; where a stable (transmissible) complex is defined as a complete exchange with no evidence of unstable elements e.g. dicentric or acentric fragments.

Exchange aberrations involving only two breaks in one or two chromosomes were classified as Simple. Simple exchanges were further classified as:

- Stable reciprocal translocations - 2B
- Incomplete stable reciprocal translocation - inc2B
- Unstable dicentric plus acentric fragment - 2A
- Incomplete dicentric - inc2A
- Centric ring - CR

Chromosome breaks not involving additional chromosomes were classed as Break-only. Abnormalities were classified as clonal if the same chromosome aberration, involving breakpoints in the same regions, was observed in two (or more) spreads. Metaphase spreads were categorised as stable only if all of the abnormalities detected within that spread were classified as stable.

Statistical analysis

Statistical analyses were carried out using Microsoft Excel and the Dose Estimate cytogenetics data analysis program developed at the Health Protection Agency.16) The chi-squared test for homogeneity between groups, Students t-test and the z-test were used, where appropriate, to compare proportions or frequencies of aberrations in P1 and P2 and subsets of these data (e.g. types of aberrations) with, for example, group mean values.

RESULTS AND DISCUSSION

We obtained fixed PBL preparations from the surviving worker of the Tokai-mura accident approximately four (P1) and five (P2) years after exposure and assayed by M-FISH 24 colour karyotyping to determine the different chromosome aberration types present and their frequency of occurrence. In addition, in an effort to assess for any emerging clinically-relevant aberrations we have identified the participating chromosomes and relative breakpoint positions of all chromosome exchanges and categorised these based on the transmission potential of all damaged cells.

For this study, only a small total number of metaphase spreads were analysed, yet we observed an extremely high frequency of spreads containing chromosomal type damage (43 and 48% of total PBL pool sampled for P1 and P2 respectively) (Table 1). This is consistent with the frequency of damaged cells (60%) observed in a radiation accident victim 25 years after exposure17) but is far in excess of that detected in workers with significant plutonium body burdens, unexposed nuclear workers or normal healthy volunteers.14,18) Of the varying types of chromosomal damage, simple-type exchanges dominated and the majority of these were stable (2B) as expected given the length of sampling time after the accident (Table 2).19) Thus, the majority of unstable simple exchanges have disappeared from the PBL pool by this time; indeed based on the painting pattern of the incomplete types (2Binc), we suggest that these are most likely artefacts of the M-FISH technique whereby the ‘missing element’ of the exchange is below the limit of paint resolution.20–22)

In contrast to simple aberrations, many of the complex chromosome aberrations detected in P1 and P2 were not

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<th>Table 1. Chromosome-type aberrations observed in Worker C four (P1) and five (P2) years after the accident.</th>
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<tr>
<td>Total cells analysed</td>
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<tr>
<td>Frequency (f) of cells damaged</td>
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<tr>
<td>No. Complex (f)</td>
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<td>No. Simple (f)</td>
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<td>No. Break-only (f)</td>
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<th>Table 2. Simple exchange types observed in Worker C four (P1) and five (P2) years after the accident.</th>
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<td>CR</td>
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classified as being of the stable transmissible type (Figs. 1 and 2). We did observe a non-significant trend in reduction in the frequency of all complexes with time (between P1 and P2) and a reduction in the amount of unstable damage (all aberration types) was detected in P2 compared to P1 \((p = 0.026)\), suggesting this decline was primarily due to a decline in unstable complexes as might be predicted. However the occurrence of unstable complex aberrations four and five years after the accident is striking and does demonstrate the longevity of heavily damaged cells in vivo. Complex aberrations are known to be effectively induced after exposure to high-LET radiation including neutrons\(^7,23,24\) and are also induced after exposure to low-LET \(\gamma\)-rays at frequencies of 0.038 and 0.117 after exposure to 1 and 2 Gy, respectively.\(^15\) However, only a small fraction of these initially-induced complex aberrations (1–2%) are expected to be stable through repeated cell division.\(^14,25,26\) The possibility that the unstable complex aberrations observed in this study are a consequence of recently irradiated PBL due to internalised radionuclide contamination is unlikely given the negligible amount of radioactivity that was produced and released during the initial criticality burst.\(^27\) Therefore the most likely explanation for the presence of non-clonal unstable complex aberrations is that damaged PBL are capable of surviving for extended periods by remaining in a quiescent, essentially 1st interphase stage, after receiving the damage. This argument is supported by observations of unstable complex aberrations in nuclear workers, accident victims and thorotrast patients many years after exposure.\(^18,28–30\) Thus, heavily damaged cells appear to be quite long lived in vivo.

The frequencies of translocations given in Table 2 can be used to give a rough estimate of dose. Ishigure et al. (2001) suggested that neutron energies between 1 and 4 MeV were produced in the accident, with an average energy of 1 MeV incident on the whole body of the three exposed workers.\(^2\) Kojima et al. (2001) recorded \(\gamma\)-radiation between 0 and 2 MeV, from a variety of sources, while the NIRS final report on the accident gives peak neutron and \(\gamma\)-ray energies at approximately 1 MeV for both.\(^31,32\) For \(\gamma\)-irradiation, the HPA Co 60 curve for 1.2 MeV gamma rays \((y = 0.0005 (\pm 0.0005) + 0.0165 (\pm 0.0090) D + 0.6280 (\pm 0.0046) D^2)\) which is similar to previously published curves,\(^33\) was used. The closest HPA neutron calibration curve available is for 7.6 MeV neutrons curve \((y = 0.0005 (\pm 0.0005) + 0.4820 (\pm 0.0400) D)\). Both curves were prepared for dicentrics, however the length of time since the accident means that the dicentrics given in Table 2, measured at the time of blood sampling, would have been significantly reduced compared to the original frequency and so are of little use for practical retrospective biodosimetry. It has been shown after low-LET irradiation that simple translocations in stable cells are not diluted in time, and that the translocation frequency at any given date following irradiation exposure should approximate the dicentric frequency immediately after irradiation.\(^34\) With the simplifying assumption that this applies also to high-LET irradiation, which is of only limited validity because it ignores single-track correlations between aberrations within a cell, the HPA calibration curves can be used for the stable translocations (2B) given in Table 2. Indeed Hayata et al. (2001) reported a dicentric + centric ring frequency of 0.637 in Worker C 48 hours after the accident.

![Fig. 1. Number of chromosomes involved in each complex exchange in sample P1. Grey bars represent fraction classified as stable.](image1)

![Fig. 2. Number of chromosomes involved in each complex exchange in sample P2. Grey bars represent fraction classified as stable.](image2)

<table>
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<th>Type of damage</th>
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<td>Single simple exchange</td>
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<td>Single complex exchange</td>
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<td>Total</td>
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### Table 4. mPAINT descriptor of all aberrations observed in cells classified as stable in samples P1 and P2

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<th>Cell</th>
<th>P2</th>
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Chromosome abnormalities detected in each damaged cell are described according to a modified version of the mPAINT system. In brief, the origin of each rearranged chromosome, including centromere and breakpoint region, is described within each parenthesis. A linked series of parenthesis describe individual simple or complex exchanges. Additional abnormalities within the same damaged cell are identified by a comma break.
using solid staining techniques while we observed a simple (mainly stable 2B) frequency of 0.490 and 0.480 in samples P1 and P2 respectively (Table 1). Using an average neutron to γ-ray kerma ratio of 1:2.1, the iterative criticality calculations give a neutron dose of 0.47 (95% Poisson Confidence Interval, CI: 0.42–0.52) Gy and a γ-ray dose of 0.99 (95% CI: 0.90–1.07) Gy. It should be noted that the above represents only a very rough estimate, as it is derived in part from the HPA 7.6 MeV neutron calibration curve. The LET spectrum of recoil protons is very different for 1 and 7.6 MeV neutron energies and these will produce substantially different radiobiological effects. Further, this estimate is

<table>
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<th>Table 5. mPAINT descriptor of all aberrations observed in cells classified as unstable in samples P1 and P2</th>
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Chromosome abnormalities detected in each damaged cell are described according to a modified version of the mPAINT system. In brief, the origin of each rearranged chromosome, including centromere and breakpoint region, is described within each parenthesis. A linked series of parenthesis describe individual simple or complex exchanges. Additional abnormalities within the same damaged cell are identified by a comma break.
based on several simplifying assumptions and on a small
number of translocations (~30) observed in a small number
of cells (98). Lastly, the confidence intervals only reflect
the uncertainty in the doses due to the standard errors on the
translocation numbers and disregard the errors in the yield
curve coefficients, in the estimation of neutron and γ-ray
ergies or of the n:γ ratio. Incorporating all of these sources
of error would substantially increase the size of the confi-
dence limits.

M-FISH analysis allows all visible damage in each dam-
aged metaphase spread to be scored on a cell-by-cell basis.
Each ‘independent’ chromosome aberration (complex,
simple or single chromosome break) can then be assigned as
occurring on its own in the damaged cell or in combination
with one or more other aberrations in the same damaged
cell. This is useful for two reasons. Firstly it enables the dis-
tribution of aberration types within each damaged cell to be
correlated with radiation quality (of particular relevance if in
the 1st cell division after exposure) and secondly, as is rele-
vant here, it enables a more accurate estimation of the long-
term transmission potential of each damaged cell. Thus
in terms of cellular stability, 0.224 and 0.347 of all metaphase
spreads analysed in P1 and P2, respectively, were theoreti-
cally capable of long-term transmission (Table 3). These
damaged cells contained either a simple or a complex or
stable combinations of both. Table 4 details the mPAINT
notation for stable simple and complex translocations
observed in stable cells. Consequently, these aberrations are
theoretically capable of long-term transmission, while Table
5 details all those aberrations observed in spreads classified
as being unstable (Table 5 and Fig. 4). Relative breakpoint
positions to one of five regions (pter, p, cen, q or qter) were
assigned for each damaged chromosome and included in the
mPAINT notation for each aberration. This enabled assess-
ment for the occurrence of clonal aberrations and also, the
determination of individual chromosome involvement
between samples P1 and P2 (Tables 4 and 5). The total
number of breakpoints was considered too low to perform a
statistical assessment of breakpoint distribution or an ana-
lysis of chromosome-chromosome exchange partners,
however there is a statistical indication that chromosomes 1
(p = 0.018) and 12 (p = 0.046) were involved in more stable
aberrations in P2 compared to P1 (Table 4 and Fig. 4). In
addition, a review of the different chromosome exchanges
shows that chromosome 1 accounts for 30% of all stable
aberrations in stable cells in P2, while chromosomes 8, 9 and
10 are involved in a further 47% (Table 4). We detected no
evidence of clonality and we saw no evidence of ongoing
genomic instability as measured by the detection of an
excess of chromatid-type aberrations, therefore these find-
ings could be suggestive of a non-random persistence of sta-
ble aberrations. Alternatively, the frequent involvement of
chromosome 1 in particular, in exchanges involving many
different exchange partners, could suggest ongoing genomic

Fig. 3. Number of abnormalities (all types) involving each chro-
mosome in stable (grey bars) and unstable (black bars) cells in sam-
ple P1.

Fig. 4. Number of abnormalities (all types) involving each chro-
mosome in stable (grey bars) and unstable (black bars) cells in sam-
ple P2.

instability. Chromosome 1 has been observed to be prefer-
entially involved in random reciprocal exchanges after high
dose acute exposures in vitro and in vivo and additionally
contains regions termed as fragile sites that are associ-
ated with breakage and rearrangement. A further follow-
up examination of the frequencies and types of chromosome
aberration currently detected in worker C would provide an
in-sight into the clinical relevance of these findings.

In conclusion, we have assayed PBLs sampled from
worker C four (P1) and five (P2) years after the Tokai-mura
criticality accident using the 24-colour technique of M-
FISH. We observed damaged cells containing both simple
and complex chromosome aberration types. In the main, the
simple exchanges were stable reciprocal translocations how-
ever a large proportion of the complex aberrations detected
were classified as being of the unstable type. These com-
plexes were qualitatively similar to those induced after exposure to low doses of high-LET radiation or medium doses of low-LET radiation\(^{13,14,18}\) and support the suggestion that heavily damaged PBL can be quite long-lived in vivo.

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**REFERENCES**


