Loss of telomeres in the progeny of human lymphocytes exposed to energetic heavy ions

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Abstract
We have used cross-species multi-color banding (RxFISH) combined with telomere FISH probes, to measure chromosomal aberrations in the progeny of human peripheral blood lymphocytes exposed to ionizing radiation. Accelerated iron particles (energy 1 GeV/nucleon) induced many more terminal deletions than the same dose of γ-rays. We found that truncated chromosomes without telomeres could be transmitted for at least three cell cycles following exposure, and represented about 10% of all aberrations observed in the progeny of cells exposed to iron ions. High energy heavy ions generate the most significant health risk for human space exploration and the results suggest that telomere loss may be the leading mechanism for their high efficiency in the induction of late effects.

Report

DNA breaks induced in human cells by ionizing radiation are processed within a few hours of exposure, and are either rejoined, leaving normal chromosomes, or misrejoined, leading to structural chromosomal aberrations (*1*). However it is unclear if a fraction of the initial breaks remain unrejoined indefinitely. After extended incubation times, the yield of breaks in prematurely condensed chromosomes (PCC) usually reach a plateau level above the control (*2*) but these residual breaks may include interstitial deletions (intra-arm asymmetrical intrachanges), as well as "open" DNA breaks. Most studies using peptide nucleic acid (PNA) telomeric probes show that terminal deletions or incomplete exchanges (leading to a truncated chromosome) are very rare at the first mitosis following exposure to either γ- (*3*) or X-rays (*4*). In contrast, high-energy heavy ions are very effective in the induction of chromosomal aberrations in human lymphocytes (*5*), and the fraction of both residual PCC breaks (*6*) and true incomplete exchanges (*7*) is much higher than after sparsely ionizing radiation. However, it is unknown if truncated chromosomes without a telomere, can be transmitted through the cell-cycle to the progeny of irradiated cells and this could be significant because telomere dysfunction has been identified as a primary mechanism involved in the chromosomal instability observed in cancer cells (*8*). Loss of telomeres can indeed elicit sister chromatid union and the prolonged breakage/fusion/bridge (B/F/B) cycles (*9*) that have been observed in mouse (*10*) and human (*11*) tumours.

All comparisons of chromosomal damage induced by sparsely and densely ionizing radiation are complicated to some extent by the technique employed for the analysis. It is necessary to use different cytogenetic methods in order to visualize different aberration types, and sparsely and densely ionizing radiation can produce substantially different patterns of aberrations: for instance, α-particles induce a higher fraction of complex rearrangements (*12*) and intrachromosomal exchanges (*13*) compared to γ-rays. We utilized rainbow cross-species FISH (RxFISH), that comprises of flow-sorted, differentially labelled gibbon chromosomes (*14*), to assess chromosome damage in human lymphocytes exposed to accelerated heavy ions. This method can be used to identify inter- as well as intra-chromosomal exchanges, along with terminal deletions. Owing to the extensive homology between human and gibbon DNA, and the many chromosomal rearrangements that have occurred during evolution, RxFISH results in a specific color banding for each human chromosome. The gibbon DNA probes are labelled with three different fluorochromes (Cy3, Cy5, and FITC), generating seven different colors and approximately 90 bands in the human haploid genome. We applied RxFISH to human peripheral blood lymphocytes exposed *in vitro* at

the NASA Space Radiation Laboratory at Brookhaven National Laboratory to either ^{137}Cs γ-rays or 1 GeV/n ^{56}Fe-ions with linear energy transfer (LET) of about 147 keV/μm. Lymphocytes were stimulated to grow immediately after exposure and chromosomes were harvested after 144 h (*15*) in culture. Over 90% of the cells had reached at least 3rd mitosis by this collection time, and any remaining 1st and 2nd division cells were excluded from the analysis using differential replication staining. Anderson *et al.* (*16*) cultivated human lymphocytes for much longer, and they observed no significant differences in the yield of stable radiation-induced chromosome aberrations from day 7 to 41 in culture. Fig. 1a shows an RxFISH painted cell from the population originally exposed to 3 Gy iron ions. The karyotype of this cell, which includes a reciprocal translocation, is displayed in panel 1b. Examples of intra-chromosomal exchanges visualized by RxFISH in the progeny of cells exposed to Fe-ions are shown in the panel 1c. Although some interchanges and intrachanges will remain undetected using RxFISH technique, the aim was to compare the results for heavy ion and γ-ray exposed samples rather than provide absolute numbers.

Dose-response curves for the induction of each chromosome aberration-type are reported in Fig. 2 (crude data are in Table S1). We observed aberrant karyotypes in 1% of the unirradiated cells, whereas around 60% of karyotypes were aberrant in the progeny of cells exposed 3 Gy of Fe ions. Although the analysis was restricted to cells reaching 3rd cell division or later after exposure, we still observed a fraction of unstable, asymmetrical aberrations that accounted for about 10% and 40% of the aberrant cells from the populations exposed to γ-rays or Fe-ions, respectively. These unstable aberrations included dicentrics that were involved in either simple asymmetrical interchanges or in complex-type exchanges, along with centric rings, and terminal deletions (Fig. 2D). Most of the interchanges were reciprocal translocations (Fig. 2A). Stable intrachanges included interstitial deletions, as well as pericentric and paracentric inversions (Fig. 2B). Complex-type exchanges (Fig. 2C) included insertions, non-reciprocal exchanges, and multi-break rearrangements involving both inter- and intra-changes. Complexes accounted for only 27% and 17% of the inter-chromosomal exchanges induced by 3Gy of Fe-ions or γ-rays, respectively. This compares to approximately 70% and 24 % complex exchanges measured at the first cell-cycle after exposure, to 3 Gy Fe ion and γ-rays respectively, when human lymphocytes were assessed using multi-fluor FISH (*17*). This suggests that most of the complex-type exchanges induced by energetic heavy ions are unstable, and lead to cell death within the first three replicative cell-cycles. The relative biological effectiveness (RBE) of iron ions for the induction of interchanges in the progeny of exposed cells is much lower in comparison to the values measured in cells directly after exposure to heavy-ions, where an RBE>4 has been determined using FISH after low dose (*5, 17*). Iron particles were only slightly more effective than γ-rays in the induction of stable intrachanges (Fig. 2B), confirming previous observations using multicolour banding (mBAND) in lymphocytes exposed to Fe-ions and analyzed at the first cell-cycle after exposure (*18*). The RBE is higher for complexes although, as noted above, clearly many complex exchanges induced by Fe-ions are lost after three or more cell-cycles. Interestingly, many more truncated chromosomes, apparently terminal deletions, were observed in the progeny of the population exposed to Fe- ions compared with population exposed to γ-rays (Fig. 2D). Terminal deletions accounted for about 10% of all aberrations observed in the progeny of cells exposed to Fe-ions, whereas only two events were positively identified as terminal deletions in the 84 aberrant cells from the population exposed to γ-rays. In order to confirm that these chromosomes were indeed missing a telomere, we re-painted the slides using telomere PNA probes, and this resulted in a positive identification of telomere loss, as well as identification of interstitial deletions (Fig. 3).

The data prove that high-energy heavy ions induce "frank" terminal deletions, and that chromosomes lacking a telomere can be transmitted through the cell-cycle. Although it has been shown that radiation can induce terminal deletions, especially in repair-deficient cells (*19*), this is the first evidence that these types of aberrations can be transmitted through the cell cycle. It is likely that the cells containing telomere deficient chromosomes will either senesce, or undergo B/F/B cycles, promoting genetic instability. Late morbidity associated with exposure to heavy ions

is one of the major health concerns for manned interplanetary space missions (*20*). The frequency of chromosomal aberrations in the progeny of cells exposed to radiation may represent a useful surrogate endpoint of latent health risks to astronauts (*21*). However, information is also needed on specific types of aberrations that have been correlated with mechanisms of carcinogenesis. Our results show that RBE for heavy ions is lower for the daughters of irradiated normal human cells than in the population originally exposed to radiation. This is caused by the loss of cells carrying complex-type exchanges, which predominant after exposure to heavy ions. Terminal deletions are the only aberration-type that present the very high RBE in the progeny of cells exposed to Fe-ions that is consistent with the expected values for late endpoints such as cancer (*22*). Since terminal deletions have been directly linked to genomic instability in yeast (*23*), these results may explain why heavy ions are so efficient in inducing chromosomal instability in human cells (*24*). In addition, telomere shortening is notoriously associated with aging in normal human cells (*25*). Heavy ions are particularly effective in inducing endpoints related to accelerated aging, such as cataractogenesis (*26*) and central nervous system damage (*27*). In fact, high-LET heavy ions are so effective in inducing accelerated aging-effects that a RBE can hardly be defined, given the lack of effects after low doses of sparsely ionizing radiation (*20*). Our results suggest therefore that terminal deletions and transmission of telomere-free chromosomes are the key event in determining late effects after exposure to heavy ions.

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Figure captions

Fig. 1. Aberrations in human lymphocytes, visualized by RxFISH. (**A**) A metaphase cell from the progeny of the population exposed to 3 Gy Fe-ions. (**B**) Karyotype of the cell shown in panel A, showing a reciprocal translocation involving chromosomes 14 and 17. (**C**) Examples of inter- and intra-arm intra-chromosomal exchanges in the progeny of the lymphocytes exposed to Fe-ions or γ-rays. The panel includes pericentric inversions in chromosomes 2, 7, and 16, and paracentric inversions in chromosomes 3 and 14.

Fig. 2. Dose-response curves for the induction of chromosomal aberrations in the progeny of cells exposed to γ-rays (O) or Fe-ions (■). Bars represent standard errors of the mean values (see Table S1 for the original data set). Lines are guides for the eye. (**A**) Stable interchanges (translocations); (**B**) stable intrachanges (interstitial deletions or inversions); (**C**) complex-type exchanges; (**D**) terminal deletions.

Fig. 3. Identification of terminal deletions using telomeric PNA probes. (**A**) A typical metaphase painted with the PNA probe. (**B**) A daughter cell from the population exposed to Fe-ions, carrying two deletions in chromosomes 1 and 11. The lack of banding on 11p makes it impossible to positively classify the deletion as terminal or interstitial, whereas the deletion in 1q appears terminal because the last colour band in 1q is missing. (**C**) Telomere painting by PNA probes allows positive identification of the deletion in chromosome 1 as terminal and the deletion in chromosome 11 as interstitial.

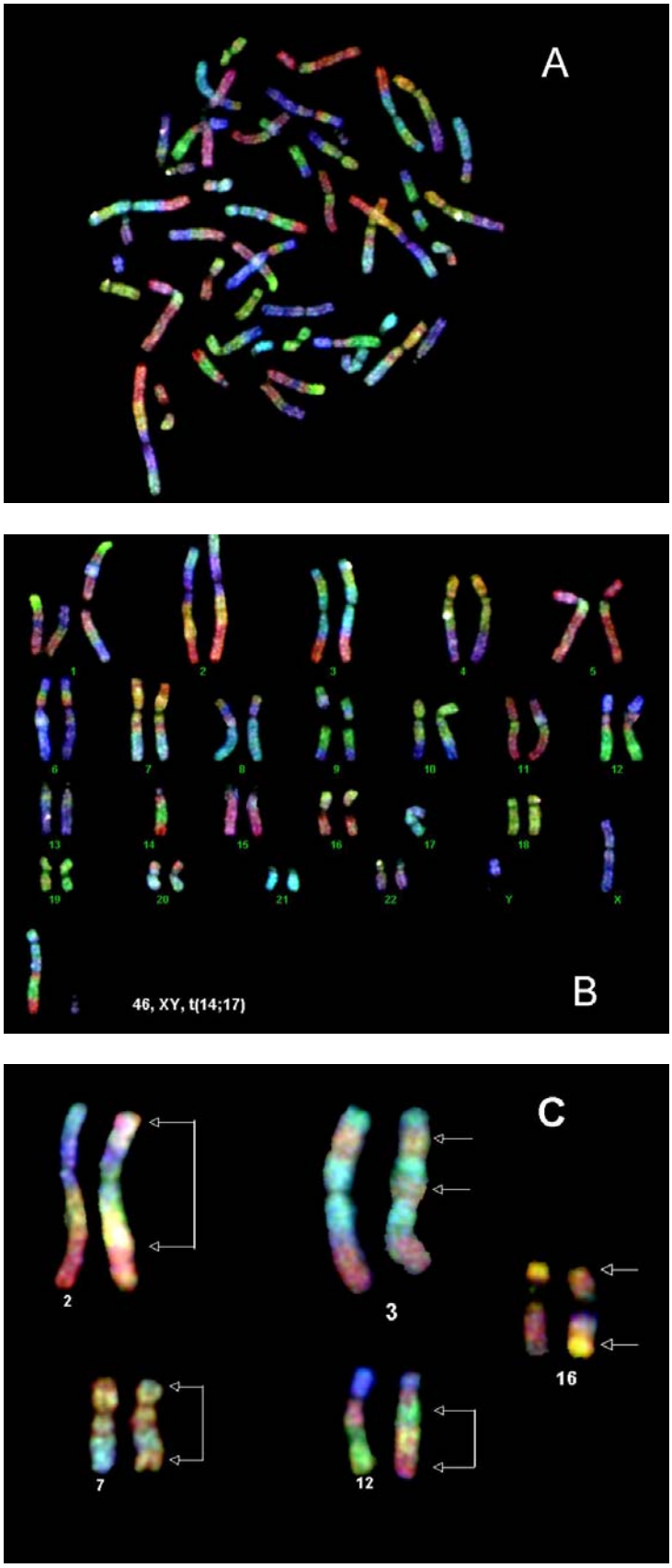

Figure 1

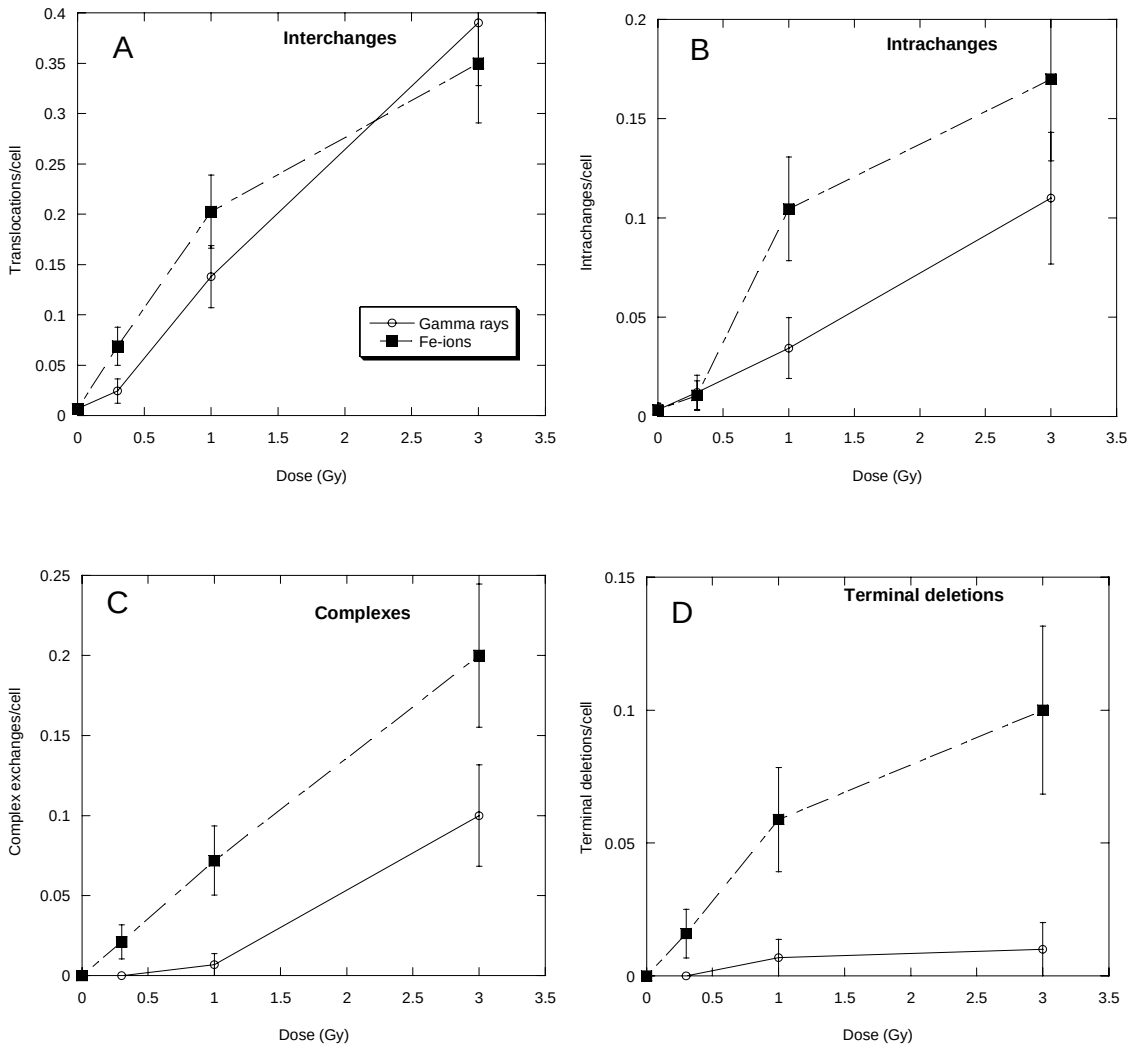
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Figure 2

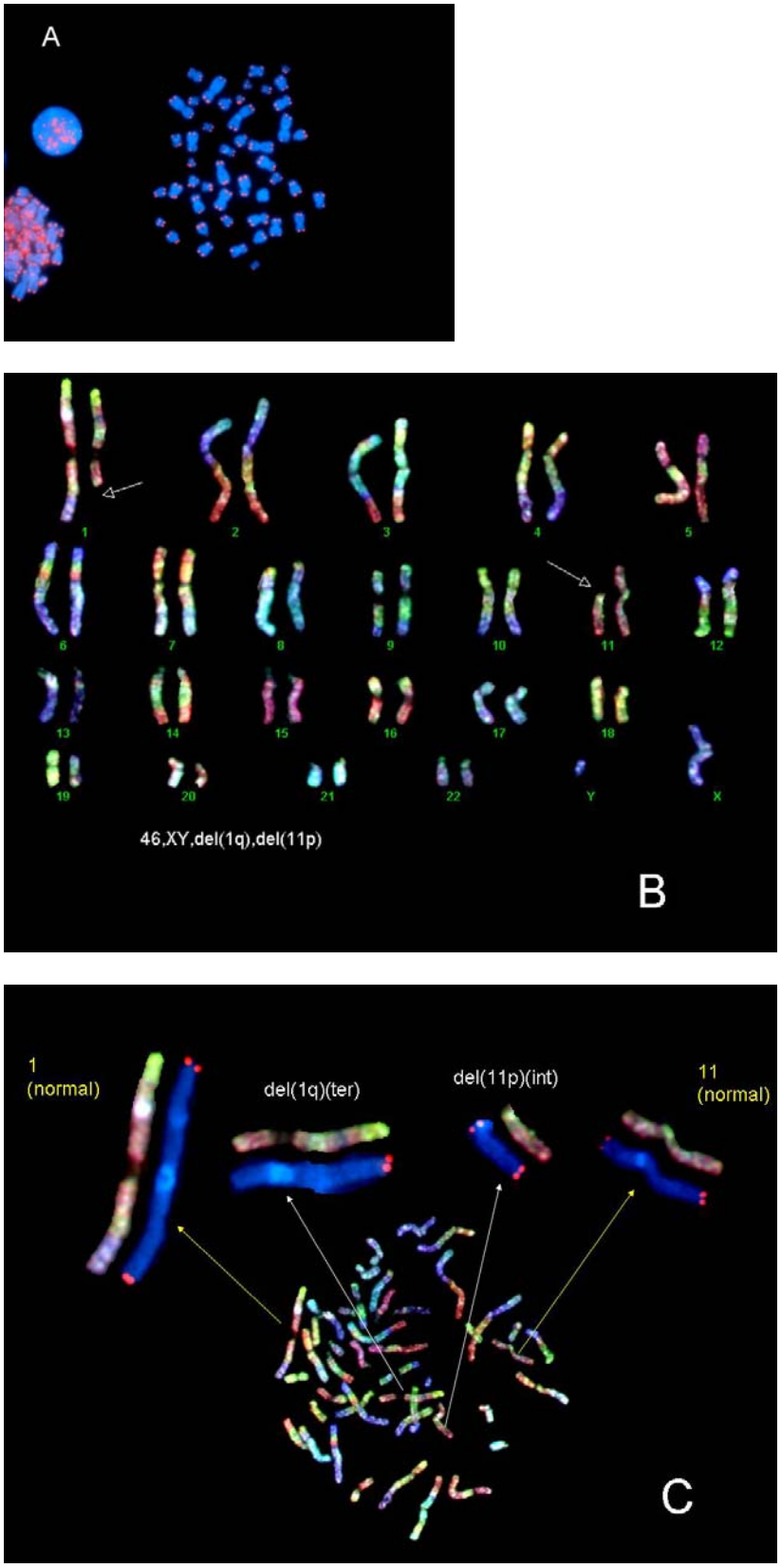
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Figure 3

Supporting online material

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Materials and Methods

Blood samples

Venous blood from a healthy male volunteer was drawn into a sodium-heparinized vacutainer. The volunteer gave informed consent for his blood sample to be used in these experiments, and the protocol was approved by IRB at Brookhaven National Laboratory. The blood was transferred into 15-ml Falcon conical centrifuge tubes and irradiated within 2 h of the blood draw.

Irradiations

Whole blood was exposed at room temperature to γ-rays using a ^{137}Cesium source, or to accelerated ^{56}Fe ions using the NASA Space Radiation Laboratory facility at Brookhaven National Laboratory (Upton, NY). Dose-rate was approximately 1 Gy/min in both experiments and doses were 0.3, 1, or 3 Gy (±5%). Physical characteristics and dosimetry of the 1 GeV/n ^{56}Fe beam have been described in detail by Zeitlin *et al.* (*S1*). The dose-average LET of this beam is approximately 147 keV/μm, which is around the peak of effectiveness for charged particles *(5)*. Samples were processed within 1 h of exposure, as described below.

In vitro growth

Blood was diluted 1:20 in RPMI 1640 medium (Gibco-BRL, Grand Island, NY), supplemented with 20% calf serum, 2% phytohaemaglutinin, 1% L-glutamine, 1% penicillin/streptomycin, 0.1% sodium heparin (stock 176.2 units/mg), 5 μg/ml bromodeoxyuridine (BrdU) (Sigma), 5 μg/ml deoxycytidine (Sigma) and incubated in 25 cm^2 tissue culture flasks at 37 °C in vertical position in a humidified atmosphere with 5% CO_2. The cultures were shaken gently every day. After 146 h incubation, colcemid (Gibco-BRL, Grand Island, NY) was added to the cultures at a final concentration of 0.1 μg/ml and the cells were incubated for a further 2 h at 37 °C.

Chromosome spreads

Blood cultures were transferred into 15 ml centrifuge tubes (Falcon) and spun for 5 min at 2000 rpm. The pellet was carefully resuspended in 8 ml of 75 mM KCl and incubated for 20 min at 37 °C. Two milliliters of freshly-prepared fixative solution (methanol:acetic acid = 3:1) was then slowly added to the solution, and the tube was centrifuged again. The pellet was resuspended in fixative and left for 20 min on ice. After centrifugation, the cells were resuspended in 14 ml fixative, centrifuged, and stored at - 20 °C in fixative.

Slide preparation and aging

After two further washes in methanol:acetic acid solution, cell pellets were resuspended in a small volume of fresh fixative, and cells were dropped onto a humid slide kept at 37 °C. Slides were air-dried, then treated for 5 min at 37 °C in 0.005% pepsin. Slides were then washed in PBS, fixed for 2 min in 1% formaldehyde, washed again in PBS and dehydrated in 70%, 85%, and 100%

ethanol, 2 min each. After air-drying, slides were aged in the dark for 2-3 days at room temperature before denaturation.

RxFISH hybridization

Cells were hybridized with Harlequin*FISH™ probes (Cambio Ltd, Cambridge, UK), containing gibbon DNA, following the basic protocol recommended by the manufacturer. Briefly, slides were denatured in 70% formamide for 2 min at 65 °C, while 10-ml of the Star*FISH probe was denatured for 10 min at 68 °C. The probe was pre-annealed at 37 °C for 10 min and then applied to the target area of the slide (22x22 mm) on a slide warmer at 37 °C. The coverslip was sealed with rubber cement and the slide incubated overnight at 37 °C in a humid incubator. Slides were then washed in a 50% formamide solution at 45 °C, and finally processed for immunostaining. First, a layer containing Cy5-avidin and rabbit anti-FITC antibodies was added, and the slide incubated 20 min at 37 °C. After washing in a 2xSSC/0.05% tween-20 detergent at 45 °C, the slide was hybridized with goat anti-rabbit FITC antibody and incubated again for 20 min at 37 °C. After washing three times (5 min each) in detergent at 37 °C, the slide was counterstained in DAPI II (Vysis, Downers Grove, IL) and stored at -20 °C prior to the analysis.

Karyotyping

Hybridized slides were visualized with the PowerGene™ RxFISH system (Applied Imaging, Houston, TX), connected to a Zeiss Axioplan fluorescent microscope. The slide was scanned with a 40x immersion objective using a triple bandpass filter. Spreads with long, well separated chromosomes were located, the coordinates on the microscope translator noted, and the image visualized with a 100x objective. Four images were saved using the Cy3, Cy5, FITC, and DAPI filters in order. Karyotypes were analyzed off-line as described below.

Classification of chromosome aberrations

RxFISH has been used in clinical cytogenetics to identify cryptic aberrations that are hard to classify by other methods (*S2, S3*). Unlike 23-color FISH (mFISH) (*S4*), RxFISH technique can be used to visualize intrachanges as well as interchanges. However, RxFISH has too few colors to resolve very complex interchanges involving several different chromosomes, such as those observed in cells at the first mitosis following exposure (*S5*). Whereas, RxFISH has lower resolution than multicolour banding (mBAND) for the analysis of intrachromosomal exchanges (*S6, S7*), it has the advantage of a full karyotype analysis, while mBAND is restricted to one single chromosome pair.

We divided aberrations into the categories shown in Table S1, i.e. translocations, dicentrics, rings, terminal deletions, interstitial deletions, pericentric and paracentric inversions. We did not find any acentric fragments in the progeny of the exposed cells. Terminal and interstitial deletions thus refer to shortened chromosomes, and were distinguished based on the banding pattern. Further verification of deletions was performed using telomere probes (see below). Complex-type exchanges were classified according to Savage's definition *(S8)* of all configurations with "2 or more breaks in 3 or more chromosomes", and events involving both intra- and inter-chromosomal exchanges were included in this category.

Telomere detection

Slides with cells containing chromosome deletions were washed in 2xSSC/0.05% Tween-20 for 15 min at 65 °C and rinsed in Tris-buffered saline (TBS). Slides were then hybridized with the telomere PNA FISH probe kit/Cy3 (DakoCytomation, Glostrup, Denmark), following the protocol recommended by the manufacturer. Briefly, slides were fixed in 3.7% formaldehyde, washed in TBS and then incubated 10 min in proteinase K. After rinsing in TBS and dehydrating in a cold ethanol series (70%, 85%, 100%), the PNA probe was added to the target area and the slide was

incubated 5 min at 80 °C and then 30 min at room temperature. Slides were then washed 5 min at 65 °C, dehydrated in ethanol, and counterstained with DAPI I (Vysis). The cells were analyzed using the same microscope used for RxFISH, and the Probe module of the PowerGene™ system (Applied Imaging, Houston, TX). The images were acquired with Cy3 and DAPI filters.

Differential replication staining

In order to exclude cells at 1st or 2nd mitosis, slides were washed by incubation in 2xSSC/0.05% Tween-20 for 20 min at 37 °C and harlequin staining was completed as described previously (*S9*). Briefly, slides were stained in Hoechst 33258 (Sigma), and then exposed to UV light for 20 min. After washing in PBS and dehydration in an ethanol series, cells were counterstained in DAPI and the cells analyzed previously were re-located. Spreads at the 1st (uniform painting) or 2nd (one chromatid dark and the other light) were excluded from the analysis. Positive discrimination of metaphases in 3rd, 4th or higher mitosis was not possible with this method.

Table S1. Chromosomal aberrations scored in human lymphocytes harvested 144 h after exposure to radiation.

Radiation	Dose (Gy)	Cells scored	Aberrant cells (stable)[1]	Aberrant cells (unstable)[2]	Translo-cations	Dicentrics[3]	Terminal deletions	Interstitial deletions	Inversions[4]	Rings	Complex exchanges[5]	Total aber-rations
-	0.0	297	3	0	2	0	0	1	0	0	0	3
Gamma rays	0.3	164	6	0	4	0	0	1	1	0	0	6
	1.0	145	18	2	20	2	1	3	2	0	1	29
	3.0	100	46	9	39	9	1	5	6	1	10	71
Iron ions	0.3	189	11	9	13	6	3	1	1	0	4	28
	1.0	153	29	21	31	11	9	9	7	1	11	79
	3.0	100	40	24	35	20	10	8	9	2	20	104

Notes

1. Karyotypes containing transmissible aberrations only
2. Karyotypes containing non-transmissible aberrations
3. Including complex-type dicentrics
4. Including inter-arm and intra-arm
5. Including inter- plus intra-chromosomal complex rearrangements

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