Analysis of chromosome loss and chromosome segregation in cytokinesis-blocked human lymphocytes: non-disjunction is the prevalent mistake in chromosome segregation produced by low dose exposure to ionizing radiation

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The aim of the present work was to examine in human lymphocytes, firstly, whether in vitro γ-rays as compared with X-rays also induce chromatid malsegregation and at higher frequencies than chromosome loss and, secondly, whether the cytokinesis-blocked micronucleus assay combined with fluorescence in situ hybridization might be useful for the biomonitoring of individuals exposed to ionizing radiation. After irradiation, the relative frequencies of centromere-positive micronuclei decreased from 39.2% at 0.1 Gy to 21.63% at higher doses. There was no statistically significant increase in MNCen+ frequencies at doses below 1 Gy (0.1, 0.25 and 0.5 Gy), but a statistically significant increase at 1 γ (P < 0.05) and 2 Gy (P < 0.001) was observed for all the donors. No significant differences in baseline and γ-ray-induced non-disjunction frequencies for chromosomes 1 (P = 0.9) and 17 (P = 0.8) between individuals were detected. For radiation-induced non-disjunction, lower doses (0.1, 0.25 and 0.5 Gy) of γ-rays did not induce a statistically significant increase in non-disjunction frequencies whereas 1 Gy and above clearly induced a statistically significant increase in the total non-disjunction frequencies for all the donors (P < 0.05 at 1 Gy and P < 0.0001 at 2 Gy). The aneugenic effect of radiation is less clearly dose dependent at the lower doses, suggesting an apparent threshold below which no change could be demonstrated. At high radiation doses the major mechanism for γ-ray-induced aneuploidy is related to chromosome loss through non-disjunction, as has been demonstrated using X-rays, and not through the formation of micronuclei.

Introduction

The hazards from radiation exposure of most concern in the past have been those arising from relatively high radiation exposure but to those must now be added the possible consequences from exposure to low radiation doses as in diagnostic radiology, occupational activities or in nuclear medical investigations.

Evidence that ionizing radiation (IR) induces chromosome breakage is well known and all data reported either in human lymphocytes (Lloyd et al., 1988), human fibroblasts (Cornforth and Goodwin, 1991) or in mouse cells (Miller et al., 1992) agree fairly well. Besides its major clastogenic effect IR can also induce aneuploidy. Aneuploidy is the condition whereby cells or organisms suffer the loss or gain of one or more whole chromosomes and is found in both hereditary disorders and human malignancy. Indeed, aneuploidy plays a major role in birth defects (Hetch and Hetch, 1987). Oshimura and Barret (1986) found that aneuploidy is a direct causal event in some forms of neoplasia. Moreover, irradiation appeared to be a contributing factor in the case of triploid abortuses (Alberman et al., 1972). The aneugenic activity of IR has been demonstrated by Natarajan and co-workers using fluorescence in situ hybridization (FISH) in both mouse (Boei and Natarajan, 1995) and human populations accidentally exposed to IR (Natarajan et al., 1991, 1994, 1996).

Many distinct mechanisms can contribute to the production of aneuploidy cells at mitosis, e.g. chromatid non-disjunction and chromosome loss. When the failure of sister chromatids to separate properly and to move to opposite poles during cell division leads to one nucleus with a missing chromosome and one nucleus with an extra chromosome, the event is called non-disjunction. However, when entire chromosomes which lack attachment to the spindle apparatus lag behind during mitosis and are excluded from the main nucleus in the daughter cells, the event is called chromosome loss. In the latter case, after nuclear membranes form around these chromosomes, they appear as micronuclear bodies in the cell cytoplasm where they can be identified.

In the case of low LET radiation and its effects upon the induction of aneuploidy, most of the work has dealt with the effects of X-rays both in vivo (Natarajan et al., 1993; Boei and Natarajan, 1995) and in vitro (Darroudi et al., 1996; Kirsch-Volders et al., 1996) and relatively little data are available on γ-rays. Uchida et al. (1975) observed that mitotic non-disjunction of lymphocyte chromosomes could be induced by exposure in vivo to low doses of 137Cs γ-rays (the doses were 0.1, 0.2 and 0.3 Gy). Their conclusion has been supported by the cytogenetic analyses of Natarajan et al. (1991), who showed aneuploidy events in blood lymphocytes of victims of 137Cs radiation exposure in Goiania. More recently, studies performed with thyroid cancer patients treated with radiodine have revealed a 13¹I-mediated aneugenic effect in vivo (Ramirez et al., 1997). However, a systematic study of radiation-induced aneuploidy by γ-rays in the low dose range is still lacking.

Several cytological methods have been devised in the past to detect mitotic aneuploidy in mammalian cells, such as metaphase chromosome counts (Danford, 1984), differential staining of chromosomes and spindle fibres (Parry et al., 1982; Kirsch-Volders, 1986) and the characterization of micronuclei in bone marrow cells (Heddle et al., 1983; Vanderkerken et al., 1989; Van Hummelen et al., 1992). There have been increased efforts in the area of test development for aneuploidy detection after 1986, when the lymphocyte cytokinesis block assay (Fenech and Morley, 1986) was implemented. Using a pan-centromeric DNA probe, Elhajouji et al. (1995) showed the sensitivity of centromere probing in micronuclei and recommended it as a basic technique to detect chromosome

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loss. Unlike the study of chromosome loss that can be determined on micronuclei (MN), non-disjunction requires analysis involving the main nuclei. Zijno et al. (1994) have shown that fluorescence in situ hybridization can be effectively used to detect aneuploidy in cultured interphase human lymphocytes arrested with cytochalasin B by following the reciprocal products of chromosome distribution in daughter nuclei and MN of the same binucleated cell. The in vitro micronucleus assay, when combined with FISH for specific chromosome or/and centromere regions, now allows discrimination between clastogenic events, chromosome loss and chromosome non-disjunction induced by known spindle poisons (Elhajouji et al., 1997; Kirsch-Volders, 1997).

The objectives of this study were to examine: (i) whether low LET γ-rays induce chromatid non-disjunction and at the same frequencies as chromosome loss using FISH applied to cytokinesis-blocked human lymphocytes as described for X-rays (Kirsch-Volders et al., 1996); and (ii) whether this non-disjunction may be useful for the biomonitoring of workers exposed to IR.

To evaluate the inter-individual variation, the responses of five healthy donors (three males and two females) were compared for chromosome loss and non-disjunction after in vitro exposure of lymphocytes to 0.1, 0.25, 0.5, 1 and 2 Gy 60Co γ-rays. Therefore, the combination of the cytokinesis-blocked MN assay (Fenech and Morley, 1986; Van Hummelen and Kirsch-Volders, 1990; Darroudi et al., 1992; Farouqi et al., 1993) with FISH for either pan-centromeric (to assess chromosome loss) or specific centromeric chromosome regions (to detected chromatid non-disjunction) (Elhajouji et al., 1995, 1997) was applied.

Materials and methods

Irradiation protocol

Human venous blood was drawn into heparin-containing vacutainers from five healthy non-smoking donors, three males (donor 1, 26 years; donor 2, 28 years; donor 3, 58 years) and two females (donor 4, 24 years; donor 5, 30 years). The whole blood samples were irradiated in vitro at 37°C in a water bath with 60Co γ-rays at 1 Gy/min to doses of 0.1, 0.25, 0.5, 1 and 2 Gy. Dosimetry was performed at the position of the samples with a NE2571 cylindrical ionization chamber and NE2570 dosimeter (Nuclear Enterprises, Reading, UK) applying the IAEA 1987 Code of Practice.

Cytokinesis-blocked micronucleus assay

The lymphocytes were stimulated with 2% phytohaemagglutinin (PHA 16; Wellcome Diagnostics, Dartford, UK) and treated with cytochalasin B (6 µg/ml) for 44 h. After 72 h cells were subjected to a cold hypotonic treatment (0.075 M KCl), immediately centrifuged and the supernatant was removed. The cells were fixed in methanol/acetic acid 3:1. The fixed cells were dropped on to the slides, air dried and stored at ~20°C for the in situ hybridization procedures.

In situ hybridization with pan-centromeric probe

A 30 nt oligomer shown to hybridize to the conserved region of the α satellite DNA present at the centromeres of all human chromosomes (Meyne et al., 1989) was used. This 30mer oligonucleotide (SO-α-AllCen, Synthetic oligomer, α-All Centromeres) was synthesized with a Gene Assembler Plus (Pharmacia, Netherlands). The probe was 3’-end labelled by terminal deoxynucleotidyl transferase ( Gibco, USA) with biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany). The cells were pretreated with pepsin (Sigma, Bornem, Belgium) (0.005% in 10 mM HCl). Cells and probe were denatured simultaneously on a hotplate at 80°C for 3–4 min. Following overnight hybridization, immunofluorescence detection of the probe was performed by means of avidin–FITC (Vector Laboratories, Burlingame, CA) and biotinylated goat-anti-avidin (Vector Laboratories). After dehydrating in an ethanol series, the slides were counterstained with propidium iodide (5 µg/ml) and p-phenylenediamine antifade solution.

In situ hybridization with centromeric chromosome-specific probes

FISH with probes for centromeric regions of chromosomes 1 (pUC17) and 17 (D17Z1) was applied. The probes were labelled by nick translation according to the instructions of the suppliers (Life Technologies BRL, Belgium). FISH was performed as described by Elhajouji et al. (1997). Slides were treated with RNase (Sigma) (0.1 mg/ml in 2× SSC) for 1 h and pepsin solution for 10 min at 37°C in a water bath. The same denaturation step as described for FISH with pan-centromeric probes (Elhajouji et al., 1995) was performed. Following an overnight hybridization at 37°C, the slides were washed with 50% formamide in 2× SSC at 42°C. Detection of the biotinylated probe for chromosome 17 was performed by means of avidin–FITC (fluorescein avidin D; Vector Laboratories) and biotinylated goat anti-avidin antibodies (Vector Laboratories, allowing signal amplification. The digoxigenin-labelled probe (chromosome 1) was detected using a mouse anti-digoxigenin antibody (Boehringer Mannheim) followed by a Texas Red-conjugated sheep antimouse antibody (Amersham, Little Chalfont, UK). After dehydrating in an ethanol series (50, 75 and 100%), the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Boehringer Mannheim) in a phenylenediamine antifade solution (Johnson and Nogueira Araujo, 1981).

MN analysis

Generally two cultures per dose were analysed. The cytochalasin B-blocked binucleated cells (CB) were examined for the presence of one, two or more MN. All slides were coded and analysed with a Zeiss microscope (Carl Zeiss, Oberkochen, Germany) at a magnification of 1000×.

FISH analysis

For FISH analysis, in general two cultures per dose and per donor were analysed and as many CB as possible were scored with a maximum of 1000 CB per culture. The MN in binucleated lymphocytes were examined for the presence of one or more spots and are classified as centromere-positive (MN+Cen+) or centromere-negative (MN+Cen−), which do not have centromeres present. The standard scoring criteria for MN (1/3 diameter, no overlap, shape) were used. MN+Cen+ (%) gives the number of MN+Cen+/1000 binucleated cells.

For chromosomal non-disjunction, to restrict the scoring to the first mitosis after irradiation and exclude artefacts, only binucleated cells having the diploid number (two spots in each of the two nuclei for each of the two probes) of hybridization signals were detected and analysed. The distribution of signals for both probes between the binucleated cells was scored as 2/2, 1/3 and 0/4 for, respectively, two spots in each of the two nuclei, one spot in one nucleus and three spots in the second, no spots in one nucleus and four spots in the second. Both the 1/3 and 0/4 combinations were scored as a single non-disjunction cell. NDCB (%) gives the number of non-disjunction events observed per 1000 binucleated cells. The events involving chromosome 1 were scored independently of those involving chromosome 17 but recorded in parallel per cell. The preparations were examined with a Zeiss Axioscop microscope (Carl Zeiss) equipped with a filter (filter block 9; Carl Zeiss) to visualize the fluorescein/Texas Red labelled probe and the orange-red ethidium bromide or DAPI counterstaining.

Statistics

The χ2 test was used to compare each irradiated sample with its matched untreated control. The distributions of individual frequencies of baseline and γ-ray-induced MN+Cen+ and non-disjunction were analysed for the possible existence of inter-individual variation by the Kruskal–Wallis test.

Results

Inter-individual variation of chromosome loss and chromosome non-disjunction

Frequencies of centromere-positive versus centromere-negative micronuclei in binucleated lymphocytes. The specificity of the probes (pan-centromeric and chromosome-specific) was evaluated using metaphase cells.

The dose–response curves for MN+Cen+ obtained for γ-rays up to 2 Gy in five donors are shown in Figure 1. These data were generally obtained from two repeated experiments for each donor and for each dose. No significant differences in baseline and γ-ray-induced MN+Cen+ frequencies between individuals were detected (P = 0.22, Kruskal–Wallis test). For radiation-induced MN+Cen+, doses below 1 Gy (0.1, 0.25 and 0.5 Gy) do not show a statistically significant increase in MN+Cen+ frequencies, whereas there is a statistically signi-
For radiation-induced non-disjunction, lower doses (0.1, 0.25 and 0.5 Gy) of γ-rays did not induce a statistically significant increase in non-disjunction frequencies for donors 1, 2, 3 and 5, whereas 1 Gy and above clearly induced a statistically significant increase in the total non-disjunction frequencies (Table I) ($P < 0.01$ at 1 Gy and $P < 0.0001$ at 2 Gy).

**Comparison of chromosome loss and non-disjunction after irradiation with γ-rays**

To use the actual frequencies of MNCen+ as a measure of γ-ray-induced chromosome loss in cytokinesis-blocked human lymphocytes, all frequencies were related to 1000 binucleated cells, which allowed subtraction of spontaneous background frequencies from those observed in irradiated cells. Table I compares the frequencies of MNCen+ (%) and the frequencies of chromosomal non-disjunction (Total ND (%)) for the total genome. It was found that non-disjunction frequencies are much higher (by a factor of 10–20) than those of chromosome loss (Figure 3).

**Discussion**

In our previous studies (Elhajouji et al., 1995; Tallon et al., 1997) it was shown that the spontaneous MNCen+ yield (34 and 55%, respectively) is in relatively good agreement with the data reported by Eastmond and Tucker (1989) and Migliori et al. (1991), who found that 50% of the spontaneous MN were centromere/kinetochore-positive. The data presented here (29–50%) confirm the results obtained by us earlier with the same probe. However, using different pan-centromeric probes, ~50–81% of spontaneous MN are MNCen+ (Noppra et al., 1993; Thierens et al., 1999). These differences between laboratories might be due to differences in the centromere-positivity of spontaneous MN with the probes used by different authors.

No evidence for inter-individual variation in IR-induced chromosome loss was observed in the dose range analysed (0.1–2 Gy) and therefore no differences in donor radiosensitivities for γ-rays. This might be due to the limited number of donors without adjustment for confounding factors such as gender and age.

On the other hand the results reported in this work for γ-rays confirm previous ones obtained for X-rays (Kirsch-Volders et al., 1996), that for the dose range analysed (0.5, 1 and 2 Gy) IR induce chromosome loss (MNCen+) and non-disjunction as well as acentric chromosome fragments (MNCen–). The clastogenic effect is clearly dose dependent and higher than the aneugenic effect demonstrated by the decrease in the ratio of MNCen+ versus MNCen–.

The data reported in Table I show that the frequencies of spontaneous non-disjunction are quite high, especially when total frequencies were estimated (7.29–11.37‰); these data are close to those previously published by our laboratory of 18.4% (Kirsch-Volders et al., 1996) and 7.04–15.39‰ (Elhajouji et al., 1997). In this work, however, no differences between non-disjunction for chromosome 1 as compared with chromosome 17 were observed in both control and irradiated cells. These observations disagree with non-disjunction frequencies for the same chromosomes and same cell type in a previous work on X-ray-irradiated cells (Kirsch-Volders et al., 1996) but are in a good agreement with the work of Elhajouji et al. (1997) on spindle tubulins. These discrepancies might be due to the higher inter-individual variations found by Kirsch-Volders et al. (1996) for spontaneous non-disjunction. However, in this work
### Table I.

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- ‡Number of cytokinesis-blocked lymphocytes scored in two separate cultures per dose and per donor. The CB were scored using fluorescence in situ hybridization with a pan-centromeric probe for the assessment of chromosome loss.
- §Total number of MN per 1000 CB.
- †Frequencies of MNcen+ (%) as a measure of chromosome loss.
- ‡Number of cytokinesis-blocked lymphocytes scored in two separate cultures per dose and per donor. The CB were scored using fluorescence in situ hybridization with centromere-specific probes for chromosomes 1 and 17 to assess chromosomal non-disjunction.
- §Frequencies of non-disjunction for chromosomes 1 and 17 examined per thousand CB.
- †Chromosomal non-disjunction for the total genome. To obtain these frequencies, the frequencies in column 6 were multiplied by 23/2, assuming that non-disjunction occurs at random and independently for each chromosome.
- ‡Only one culture was available.
- §P < 0.05 compared with the respective control frequency of MNcen+ or ND; †P < 0.001; ‡P < 0.0001. The statistical significance of the results was analysed with the χ² test.

No significant inter-individual differences were observed for chromosome non-disjunction.

To determine whether non-disjunction may be the major effect of aneuploidy-inducing γ-rays, as was seen for X-rays (Kirsch-Volders et al., 1996), we have used FISH for the same chromosomes (chromosomes 1 and 17). The analysis of dose-effect for non-disjunction showed that γ-rays induce a slight increase in the frequencies of non-disjunction of chromosomes 1 and 17 at 0.5 Gy, but it was not statistically significant (except for donor 2, for whom a statistically significant increase was observed at 0.25 Gy), whereas they induce a dose-related increase at 1 Gy and above (P < 0.001) for all the donors. The corresponding frequencies induced by ionizing radiation are quite high compared with those for chromosome loss (by a factor of 10–20) (Table I and Figure 3). Therefore, when aneuploidy is considered as a whole, the contribution of chromosome non-disjunction (assuming that non-disjunction occurs at random) seems to be more important than chromosome loss. The major mechanism for γ-ray-induced aneuploidy is related to chromosome loss through non-disjunction, as has been demonstrated using X-rays, and not through the formation of MN.

The reason why the aneugenic effect is less clearly dose dependent at lower doses might be ascribed to a possible threshold effect on the spindle tubulins or other non-DNA targets. In fact, since aneuploidy is induced as a result of chromosomal loss or malsegregation, structures that are thought to be involved in chromosomal segregation should be the major targets. The possible interaction of IR with the spindle is related to their capacity to generate free radicals. Indeed,
Ionizing radiation induced non-disjunction

Fig. 3. Frequencies of non-disjunction for the total genome, chromosome loss and total aneuploidy (spontaneous and induced by γ-rays) for the five donors.

Frequencies of chromosome loss were obtained from data of FISH centromere probing on micronucleated cytokinesis-blocked lymphocytes related to 1000 CB. To obtain the frequencies of non-disjunction (ND) for the total genome, the added frequencies of ND for chromosomes 1 and 17 were multiplied by 23/2, assuming that ND occurs at random and independently for each chromosome.

IR might alter, by means of free radicals, the disulphydryl bridges which are important for microtubule assembly. It has been shown that at high doses radiolysis of proteins containing SH and S-S groups leads to fragmentation of the peptide chains. These effects are oxygen dependent and inhibited under anaerobic conditions (Schuessler and Herget, 1980; Schuessler and Schilling, 1984). Such a mechanism might be possible in the low dose range of ionizing radiation.

Ionizing radiation may also damage the structural elements of the chromosome necessary for normal disjunction (Bond and Chandley, 1983). Such damage might lead to DNA adducts or DNA–protein cross-linking, which can increase chromosome loss in yeast (Mormiter et al., 1981). It is unknown whether or not the same mechanism for aneuploidy induction exists in mammalian cells.

On the other hand, premature separation of centromeres of sister chromatids may also conceivably cause aneuploidy (Vig, 1984). A problem that has to be overcome for separation of chromatids is that newly replicated daughter DNA molecules are interwound, and an enzyme such as topoisomerase II is
needed to disentangle them (Hsieht, 1990). Radiation-induced alterations in the activity or expression of topoisomerase II could provide an answer, but this question is still controversial. Warters et al. (1989) reported that 100 Gy X-irradiation had no detectable effect on total cellular topoisomerase II activity, while Goswani et al. (1992) reported that synthesis of topoisomerase II is suppressed as cells accumulate in G2 following irradiation.

If it is assumed that multiple events are required to lead to chromosome loss or malsegregation, the existence of a threshold is probable. However, we cannot conclude from our study whether or not a threshold does exist because a higher number of donors and a still more detailed study in the low dose range (more doses) is needed for the analysis.

Concerning the use of non-disjunction events as biomarkers for occupational exposure to IR, it is clear that they are not detectable at γ-ray and X-ray doses lower than 1 and 0.5 Gy, respectively. These doses are higher than the detection limits observed for stable translocations and dicentrics (Thierens et al., 1999). However, the survival of aneuploid cells may be better than those with dicentrics and therefore should be followed in a biomonitoring study (e.g. for chronic exposure) before drawing definitive conclusions.

The following general points may be drawn from this study:

1. The confirmation of the possible occurrence of a threshold for both chromosome loss and non-disjunction for ionizing radiation needs a more detailed study in the low dose range in accordance with the effect (the lower the dose, the higher number of cells);
2. The results of our study permit the conclusion that non-disjunction is the prevalent aneuploidy-inducing mechanism of low LET energy;
3. The use of the cytochalasin B-blocked MN test in combination with FISH as an adequate biomarker in the low dose range for risk evaluation for chromosome loss and chromosome non-disjunction is not supported by the data obtained in this small sample (five donors) of exposed blood cultures in vitro.

Additional studies on chronic in vivo exposure are necessary to draw further conclusions.

References


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