Frequencies of X-ray induced chromosome aberrations in lymphocytes of xeroderma pigmentosum and Fanconi anemia patients estimated by Giemsa and fluorescence *in situ* hybridization staining techniques

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Abstract

Blood lymphocytes from xeroderma pigmentosum (XP) and Fanconi anemia (FA) patients were assessed for their sensitivity to ionizing radiation by estimating the frequency of X-ray (1 and 2 Gy)-induced chromosome aberrations (CA). The frequencies of aberrations in the whole genome were estimated in Giemsa-stained preparations of lymphocytes irradiated at G_0 or G_2 stages. The frequencies of translocations and dicentrics involving chromosomes 1 and 3 as well as the X-chromosome were determined in slides stained by fluorescence *in situ* hybridization (FISH) technique. An increase in all types of CA was observed in XP and FA lymphocytes irradiated at G_0 when compared to controls. The frequency of dicentrics and rings was 6 to 27% higher (at 1 and 2 Gy) in XP lymphocytes and 37% higher (at 2 Gy) in FA lymphocytes than in controls, while chromosome deletions were higher in irradiated (30% in 1 Gy and 72% in 2 Gy) than in control XP lymphocytes and 28 to 102% higher in FA lymphocytes. In G_2-irradiated lymphocytes the frequency of CA was 24 to 55% higher in XP lymphocytes than in controls. In most cases the translocation frequencies were higher than the frequencies of dicentrics (21/19).

INTRODUCTION

Ionizing radiation induces chromosome aberrations in G_0 human peripheral lymphocytes which can be classified as unstable (dicentrics, centric rings and acentric fragments) or stable (various kinds of translocations) (Natarajan *et al.*, 1992, 1994). Unstable aberrations are lost during successive cell divisions whereas the stable ones can persist for a long time (Natarajan *et al.*, 1991; Lucas *et al.*, 1992; Boei *et al.*, 1994). The frequencies of structurally aberrant chromosomes in peripheral lymphocytes of persons accidentally exposed to ionizing radiation have been used since the 1960s to estimate absorbed radiation dose (Bender and Gooch, 1966; Sasaki and Miyata, 1968; Natarajan, 1984).

According to classical models of formation of chromosome exchange aberrations, the ratio of radiation-induced symmetrical exchanges (reciprocal translocations, inversions) and asymmetrical exchanges (dicentrics, rings) should be 1:1 (Evans, 1962). A change in this ratio in favor of translocation frequencies has been observed (Natarajan *et al.*, 1992; Schmid *et al.*, 1992; Nakano *et al.*, 1993), and it has been suggested that the higher estimate of translocations in these studies may be due to the misclassification of exchange aberrations. This problem could be overcome by combination of whole chromosome marking with centromere specific DNA probes (Weier *et al.*, 1991). Straume and Lucas (1993) found a close correlation between dicentrics and reciprocal translocations even after the application of these techniques. Finnon *et al.* (1995) also observed that the yield of radiation-induced translocations was not significantly higher than that of dicentrics. Natarajan *et al.* (1996) explained this by suggesting that if one confines scoring only to reciprocal translocations, ignoring other types such as terminal and interstitial translocations, then the frequencies of dicentrics and translocations are almost equal. In general, the frequency of translocations has been found to be higher than the frequency of dicentrics. This shift may be dependent upon the structure of the chromosomes, position of the centromere (Natarajan *et al.*, 1996), and the nature of the chromatin at the time of irradiation (Vyas *et al.*, 1991). The formation of these two types of aberrations (translocations and dicentrics) may be the result of different mechanisms (Natarajan *et al.*, 1996).

Fanconi anemia (FA) and xeroderma pigmentosum (XP) are rare repair-deficient, mutagen-sensitive, autosomal recessive human disorders with chromosome instability, which are cancer prone. Cells derived from FA patients have more spontaneous chromosomal damage than those of other instability syndromes (Schroeder *et al.*, 1964, 1989; Natarajan *et al.*, 1989). There exist inter- and intra-individual variations specifically with regard to susceptibility to cross-linking agents and X-rays (Sasaki and Tonomura, 1973).

XP is characterized by high sensitivity to sun exposure, susceptibility to skin cancer, cutaneous pigmentation, impaired DNA repair and, in some patients, neurological degeneration (Kraemer and Slor, 1985). These patients have a greater than 1000-fold-increased frequency of UV-induced skin cancer (Cleaver and Kraemer, 1989) when compared with the normal population.

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Several studies (Bigelow *et al.*, 1979; Arlett and Harcourt, 1980; Parshad *et al.*, 1983) have shown increased radiosensitivity of XP and FA cells in the G_2 phase of the cell cycle, but in other studies an increase in chromosomal aberrations was not observed in FA cells (Evans *et al.*, 1978; Sasaki, 1978) and XP fibroblasts (Darroudi *et al.*, 1995).

The present study was designed to estimate the frequencies of X-ray-induced chromosome aberrations in lymphocytes of XP and FA patients.

MATERIAL AND METHODS

Culture conditions

Venous blood drawn in lithium heparin tubes was set up for whole blood cultures in Ham's F10 medium supplemented with 15% heat inactivated fetal calf serum (Gibco), phytohemagglutinin and antibiotics. The cultures were incubated at 37°C in a 5% CO_2 atmosphere. The FA blood sample was obtained from the Academic Hospital, Leiden, and XP from the Institute of Human Genetics, Budapest, Hungary; the complementation groups of these patients are not known.

X-Rays were generated by an ENRAF machine, operating at 200 kV, 6 mA at a dose rate of 2 Gy/min.

Treatment

G_0 irradiation

Normal, XP and FA blood lymphocyte cultures were irradiated with a dose of 1 or 2 Gy and harvested 48 h after initiation of the cultures. Colcemid (Sigma; 0.3 µg/ml) was added to the cultures 2-3 h before harvest. 5-Bromo-2-deoxyuridine (BrdU, Sigma; 10 µM) was added to all the cultures after irradiation for identification of first and second division cells. Scoring of chromosomal aberrations was restricted to cells at first mitosis.

G_2 irradiation

Lymphocyte cultures were set up and grown for 69 h and then irradiated with doses of 0.5 or 1 Gy. Colcemid was added to these cultures 0.5 h after irradiation and the lymphocytes were harvested and fixed after 2.5 h. For harvesting the cultures, cells were subjected to hypotonic shock (0.075 mM KCl) for 25 min and fixed in acetic acid:methanol (1:3). Appropriate controls were prepared.

For G_0 irradiation studies, air-dried preparations were stained by the fluorescence plus Giemsa (FPG) technique (Perry and Wolff, 1974). Two hundred metaphases were analyzed for the presence of dicentrics, rings and chromosome fragments for each radiation dose. For G_2 irradiation studies, the slides were stained with 5% Giemsa solution. Chromatid gaps, chromatid breaks, isochromatid breaks and chromatid exchanges were scored in 200 cells at each dose.

Fluorescence *in situ* hybridization

Slides to be processed for *in situ* hybridization were stored dry at -20°C. Chromosome specific libraries were obtained from blue-scribe plasmids. A mixture of three different chromosomes (1, 3 and X) was used. The *in situ* hybridization method routinely used in our laboratory (Natarajan *et al.*, 1992) was adapted. In order to analyze the aberrations in the first mitosis, the slides were stained with Hoechst 33258 (Sigma), exposed to UV light and processed for *in situ* hybridization (Boei *et al.*, 1994).

Triple-color hybridization was performed using biotin 11-dUTP (chromosome number 1; Sigma), digoxigenin 11-dUTP (chromosome number 3; Boehringer Manheim, Germany) and fluorescein 12-dUTP (X-chromosome; Boehringer). The labelled DNA representing the library was combined with competitive (human cot 1 DNA) DNA followed by denaturation, then hybridized *in situ* (overnight at 37°C) with metaphase preparation.

For immunological detection, after hybridization the slides were washed successively in 50% formamide/2X SSC (42°C), 0.1X SSC (60°C) and 4X SSC/0.05% Tween 20, pH 7, at room temperature. The first incubation was carried out with immunological buffer (NFDM) for 30 min at room temperature. After four washes with SSC, 0.05% Tween 20 and TNT (0.1 M Tris-HCl, 0.15 M NaCl and 0.05% Tween 20), the second incubation with antibodies avidin-FITC and mouse anti-digoxigenin diluted in TNB (0.1 M Tris-HCl, 0.15 M NaCl and 0.5% Boehringer blocking agent) was performed at 37°C for 30 min. The third incubation was carried out with goat-anti-avidin D and sheep anti-mouse dig diluted in TNB for 30 min at 37°C. The fourth incubation was carried out with avidin-FITC and sheep anti-dig TRITC for 30 min at 37°C. Each incubation was followed by washing with TNT. After the final wash with PBS the slides were counter-stained with DAPI (4,6-diamidino-2-phenylindole; Sigma) diluted in an anti-fading agent, vectashield (Vector Laboratories) (Figure 1).

In order to detect translocations, dicentrics and complex exchanges involving chromosome numbers 1 and 3 and the X-chromosome, 300 to 500 metaphases were analyzed for each radiation dose using a Zeiss fluorescence microscope equipped with triple filters. Due to variations in mitotic index the number of metaphases scored by FISH varied for each experimental point. The translocations and dicentrics were scored using the same slide. The background frequencies were subtracted to obtain the induced frequency of translocations and dicentrics.

The genomic translocation and dicentric frequencies were calculated using the formula for correction of the probed fraction of the genome (Lucas *et al.*, 1992). This formula relates the translocation frequency measured by FISH, Fp, to the genomic translocation frequencies, F_G, through the fraction of the genome covered by the probes, fp, as follows:

$$F_G = \frac{F_p}{2.5 \times fp\ (1 - fp)}$$

RESULTS AND DISCUSSION

Spontaneous chromosomal aberrations in normal, XP and FA lymphocytes

The baseline aberration frequencies in normal and XP lymphocytes were in the range of 2 to 3%, whereas in FA the frequency was 23% (Table I). Darroudi *et al.* (1995) also observed a similar spontaneous aberration frequency in the range of 2 to 3% in the normal and XP-C cell lines, and 16 to 22% in FA cells, which is known to be characteristic of FA patients (Schroeder *et al.*, 1964; Natarajan *et al.*, 1989). However, the frequency of spontaneous aberrations in XP lymphocytes was similar to that of normal lymphocytes.

G_0 radiation induced CA in normal, XP and FA lymphocytes

To compare the aberration frequencies in XP, FA and normal lymphocytes the background aberration frequencies were subtracted from the induced aberration frequencies. The frequency of dicentrics and rings in XP and in FA lymphocytes was markedly high. There was a high frequency of chromosome deletions with a 30-72% increase in XP lymphocytes after 1 and 2 Gy of X-rays and a 28-102% increase in deletions in the FA lymphocytes as compared with normal lymphocytes. The ratio between the frequencies of dicentrics and chromosome deletions was 1:0.6 and 1:0.8 in normal lymphocytes, 1:1.9 and 1:1.6 in XP lymphocytes and 1:3.8 and 1:1.8 in FA lymphocytes (1 and 2 Gy, respectively). The frequencies of chromatid type aberrations in FA lymphocytes were more than 30 times higher when compared with XP lymphocytes and no chromatid aberration was observed in normal lymphocytes, while chro-

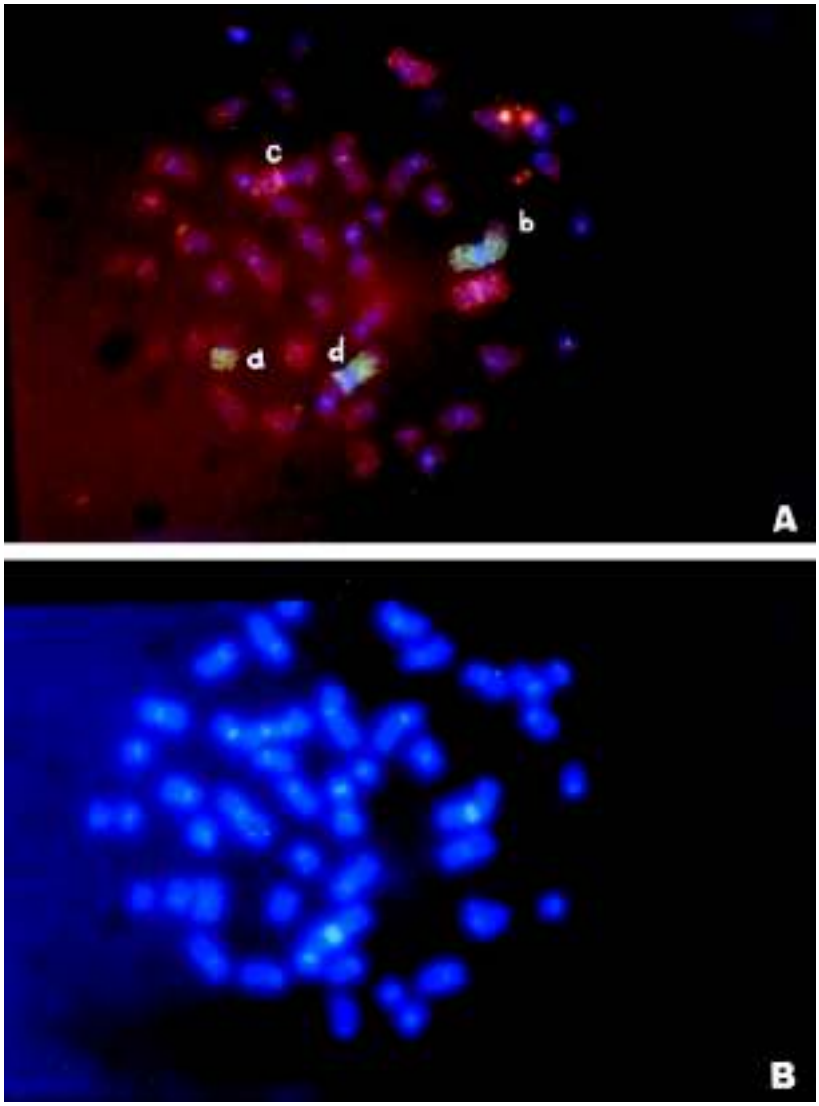

Figure 1 - A. Metaphase spread of an irradiated lymphocyte painted with libraries for chromosomes 1, 3 and X showing translocations (**a**, **b**) involving chromosome 1 and dicentrics involving chromosome 3 (**c**) and chromosome 1 (**d**). **B.** The same cell counter-stained with DAPI (4,6-diamidino-2-phenylindole).

matid exchanges were only observed in FA lymphocytes, a known characteristic of these cells. G_0 radiation produced chromatid breaks/gaps in FA lymphocytes (0.37 and 0.36 per cell in 1 Gy and 2 Gy, respectively) and were zero in normal lymphocytes and 0.01 in XP lymphocytes, which further confirms a positive defect in repair of radiation damage in FA cells.

G_2 radiosensitivity in normal and XP lymphocytes

The frequency of X-ray-induced total CA aberrations in XP lymphocytes was 0.79 and 2.17 per cell for 0.5 Gy and 1 Gy, respectively, while in the normal lymphocytes it was 0.55 and 1.62, indicating an increase (24-55%) in XP lymphocytes (Table II). The frequency of chromatid and isochromatid breaks in XP lymphocytes was 0.72 and 2.03 for 0.5 and 1 Gy, respectively, while in normal lymphocytes the frequencies were 0.54 and 1.6, indicating an increase (18-43%) in the XP lymphocytes when compared to normal lymphocytes. The frequency of interchanges was seven times (6% at 0.5 Gy and 12% at 1 Gy) higher in the XP lymphocytes than in the controls. Isochromatid breaks induced by X-rays were observed only in XP lymphocytes and were dose related (0.15 and 0.22 per cell for 0.5 and 1 Gy, respectively). Even though Taylor (1978) observed an increase in chromatid aberrations in XP cells irradiated in G_2, it was not as significant as it was in the case of ataxia telangiectasia cells. In XP-C lymphocytes an increase in ionizing radiation-induced chromosome aberrations has been observed in the G_2 phase of the cell cycle (Parshad *et al.*, 1983).

Table I - G_0 radiosensitivity of human peripheral lymphocytes in normal, xeroderma pigmentosum (XP) and Fanconi anemia (FA) individuals estimated by Giemsa staining*.

X-ray dose (Gy)	Chromosome aberrations per cell (n = 200)			
	Chromatid break/gap	Interchange	Dicentric + ring	Chromosome deletion
Normal (46, XX)				
Control	-	-	-	0.02
1	-	-	0.15	0.10
2	-	-	0.31	0.25
XP (46,XX)				
Control	-	-	0	0.03
0.5	0.01	-	0.15	0.15
1	0.01	-	0.21	0.40
2	-	-	0.58	0.97
FA (46,XY)				
Control	0.16	0.06	0.07	0.23
1	0.37	0.04	0.07	0.38
2	0.36	0	0.68	1.27

*Induced aberrations have been corrected for spontaneous aberrations.

Table II - G_2 radiosensitivity of human peripheral lymphocytes in normal and xeroderma pigmentosum (XP) individuals estimated by Giemsa staining*.

X-ray dose (Gy)	Aberrations per cell (n = 200)		
	Chromatid break/gap	Interchange	Isochromatid break
Normal (46,XX)			
Control	-	-	-
0.5	0.54	0.01	-
1	1.6	0.02	-
XP (46,XX)			
Control	0	0	0.02
0.5	0.57	0.07	0.15
1	1.81	0.14	0.22

*Induced aberrations have been corrected for spontaneous aberrations.

Estimation of radiation-induced dicentrics and translocations in normal, XP and FA lymphocytes using FISH

A. Genomic translocation frequency

The genomic translocation frequency (including reciprocal and terminal ones) calculated for individual chromosomes (1, 3, X) represents about 20% of the total genome. The translocation frequencies for chromosomes 1, 3 and the X-chromosome followed the order expected on the basis of their DNA contents in normal lymphocytes. The DNA contents of chromosomes 1, 3 and the X-chromosome are 8.15, 6.63 and 5.08% of the total genomic value, respectively (Mendelsohn *et al.*, 1973). The translocation frequencies in individual chromosomes of XP lymphocytes when compared with those of the chromosomes of normal lymphocytes showed an increase of 73.2% in the X-chromosome (2 Gy) and 48.5% in chromosome 3 (at 2 Gy). At 1 Gy the translocation frequency was 3.2% and 10.3% higher than those of XP lymphocytes for chromosome 3 and the X-chromosome respectively (Table III). In FA lymphocytes, the translocation frequencies for chromosomes 1, 3 and the X-chromosome followed the order based on their DNA content. The translocation frequencies in individual chromosomes of FA lymphocytes were almost equal to the respective chromosomes in normal lymphocytes except for the X-chromosome (27.5% higher at 1 Gy).

Mean translocation frequency of the mixture of chromosomes 1 and 3 and the X-chromosome in normal, XP and FA lymphocytes

At 1 Gy the mean translocation frequency in XP lymphocytes was similar to that of normal lymphocytes, while at a dose of 2 Gy the mean frequency was about twice that of normal lymphocytes. At 1 Gy the mean frequency of translocation in FA lymphocytes was lower than that of normal but at 2 Gy it was almost similar to normal (Table IV).

B. Genomic dicentric frequency

The genomic dicentric frequencies calculated using the same formula for calculating genomic translocation frequencies, for chromosomes 1, 3 and the X-chromosome in normal and XP lymphocytes at 2 Gy followed the order based on their DNA contents (Table III). The dicentric frequencies in individual chromosomes of XP lymphocytes when compared with the chromosomes from normal lymphocytes showed an increase of 15.1% for chromosome 1 (2 Gy) and almost equal for chromosomes 3 and X. In FA lymphocytes, the dicentric frequencies for individual chromosomes (1, 3 and X) followed the order based on their DNA contents.

Ratio between translocation and dicentric frequencies

The frequency of translocations in control, XP and FA lymphocytes increased 8.4, 10.8 and 10.6% at 1 Gy and 3.8, 53.9 and 25.9% at 2 Gy, respectively, over dicentrics (Table V). This confirms the observation of Natarajan *et al.* (1996) that the frequency of translocation is higher than the frequency of dicentrics, indicating that two different mechanisms may be involved in the production of translocations and dicentrics as observed in normal humans.

C. Complex exchanges and centric rings

In XP and FA lymphocytes a dose-dependent increase in the frequency of complex exchanges and centric rings was observed, ranging from 0.2-3.3%. These values were not used in our analysis.

CONCLUSIONS

The frequency of CA produced by G_0 and G_2 irradiation in XP and FA lymphocytes was higher than that of normal lymphocytes. The deletion type of chromosomal aberrations observed in XP and FA lymphocytes was several fold more abundant than in the normal lymphocytes, reflecting a possible defect in their capacity to repair dam-

Table III - Genomic frequency of translocation and dicentrics in normal, xeroderma pigmentosum (XP) and Fanconi anemia (FA) lymphocytes following X-irradiation as estimated by FISH.

Chromosome number	X-ray dose (Gy)	Normal				XP				FA			
		No. of cells	TT	RT	Dic	No. of cells	TT	RT	Dic	No. of cells	TT	RT	Dic
1	0.5	300	-	-	-	290	11.4	1.9	4.6		-	-	-
1	1.0	300	33.4	06.6	26.5	500	34.5	4.8	12.6	583	23.9	09.1	05.2
1	2.0	300	72.9	19.9	66.3	159	112.5	35.5	81.4	483	74.1	35.2	29.3
3	0.5	300	-	-	-	290	2.7	0.0	2.7		-	-	-
3	1.0	300	16.1	00.0	08.1	500	12.9	4.8	17.7	583	11.0	06.9	01.9
3	2.0	300	53.0	16.1	48.4	159	101.5	35.5	45.6	483	33.4	15.0	19.5
X	0.5	300	-	-	-	290	7.1	3.5	4.0		-	-	-
X	1.0	300	30.9	08.1	20.6	500	20.6	12.4	5.2	583	3.4	03.4	00.0
X	2.0	300	30.9	08.1	30.9	159	104.1	51.8	29.3	483	37.2	12.4	20.0

TT - Total translocation, RT - Reciprocal translocation, Dic - Dicentric.

Table IV - Mean genomic translocation and dicentric frequencies detected using a mixture of chromosomes 1 and 3 and the X-chromosome in normal, xeroderma pigmentosum (XP) and Fanconi anemia (FA) lymphocytes as estimated by FISH.

X-ray dose (Gy)	Normal		XP		FA	
	Translocation	Dicentric	Translocation	Dicentric	Translocation	Dicentric
0.5	-	-	7.1	17.6	-	-
1.0	26.8	18.4	22.6	11.8	12.8	02.2
2.0	52.3	48.5	106.0	52.1	48.2	22.3

Table V - Ratio between genomic translocation and dicentric frequencies in normal, xeroderma pigmentosum (XP) and Fanconi anemia (FA) lymphocytes following X-irradiation as estimated by FISH.

X-ray dose (Gy)	Normal			XP			FA		
	Chrom No. 1	Chrom No. 3	Chrom No. X	Chrom No. 1	Chrom No. 3	Chrom No. X	Chrom No. 1	Chrom No. 3	Chrom No. X
0.5	-	-	-	2.5	1.0	1.8	-	-	-
1.0	1.26	1.99	1.5	2.7	0.7	3.9	5.2	5.8	3.4
2.0	1.1	1.1	0.7	1.4	2.2	3.6	2.7	1.7	1.9

Chrom No. - Chromosome number.

aged DNA, while the frequency of translocations was higher than the frequency of dicentrics in normal, XP and FA lymphocytes, indicating that more unrepaired breaks were available for interaction in these syndromes in comparison to normal cells. Though XP cells are known to be defective in repairing bulky DNA lesions, increased sensitivity to ionizing radiation has been observed, which may indicate that ionizing radiation induces several classes of DNA lesions and/or XP cells are defective not only in nucleotide excision repair but also in some other minor repair pathways. Similarly, though FA cells are known to be sensitive to cross linking agents, implying a defect in the repair of cross links, they are also known to be sensitive to several classes of clastogenic agents (Sasaki and Tonomura, 1973).

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RESUMO

Linfócitos sanguíneos de pacientes com xeroderma pigmentosum (XP) e anemia de Fanconi (FA) foram avaliados quanto à sensibilidade à ionização radiante estimando-se a freqüência de aberrações cromossômicas (CA) induzidas por raios-X (1 e 2 Gy). As freqüências de aberrações no genoma inteiro foram estimadas em preparações de linfócitos irradiados nas fases G_0 e G_2 coradas com Giemsa. As freqüências de translocações e dicêntricos envolvendo os cromossomos 1 e 3 e o cromossomo X foram determinadas em lâminas coradas por hibridização fluorescente *in situ* (FISH). Um aumento em todos os tipos de CA foi observado em linfócitos XP e FA irradiados na fase G_0 quando comparados a controles. A freqüência de dicêntricos e anéis foi 6-27% maior (com 1 e 2 Gy) em linfócitos XP e 37% maior (com 2 Gy) em linfócitos FA do que em controles, enquanto que as deleções cromossômicas foram mais freqüentes em linfócitos XP irradiados (30% com 1 Gy e 72% com 2 Gy) do que em controles e 28-102% mais freqüentes em linfócitos FA. Em linfócitos irradiados na fase G_2 a freqüência total de CA foi 24-55% mais elevada em linfócitos XP do que em controles. Na maior parte dos casos as freqüências de translocações foram maiores do que as freqüências de dicêntricos (21/19).

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