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Cytogenetic effects of irradiation on somatic and germ cells

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Abstract

This paper summarizes the results obtained in two of the research projects carried out in our laboratory within the radiation protection programs of the Consejo de Seguridad Nuclear and the European Union. These two research lines are fundamentally interconnected, since the analysis of the cytogenetic effects of radiation on somatic cells studies the consequences of occupational or accidental exposure to radiation for the individual, especially from the point of view of developing some type of malignancy, while the studies carried out in germ cells evaluate the risk of exposure for future generations, through the transmission of chromosome abnormalities via affected spermatozoa.

In both cases these studies, which were mainly carried out during the last six years, in addition to providing basic data for the assessment of the consequences of radiation exposure and defining the steps to be taken to prevent the transmission of chromosome anomalies to the offspring in cases of therapeutic exposure, have also been fundamental in developing more effective techniques for the evaluation of the cytogenetic consequences of exposure to radiation.

Resum

Aquest treball resumeix el resultats obtinguts en dos dels projectes de recerca desenvolupats en el nostre laboratori dins del marc dels programes de protecció radiològica del Consejo de Seguridad Nuclear i la Unió Europea. Aquestes dues línies de recerca estan fonamentalment interconnectades, en el sentit que l'anàlisi dels efectes citogenètics de les radiacions en cèl·lules somàtiques estudia en el mateix individu les conseqüències de l'exposició ocupacional o accidental a la radiació, especialment des del punt de vista del desenvolupament d'alguns tipus de neoplàsia, mentre que els estudis en cèl·lules germinals avaluen el risc de l'exposició per a futures generacions, a través de la transmissió d'anomalies cromosòmiques per via d'espermatozoides afectats.

En ambdós casos, aquests estudis, que s'han dut a terme principalment en els darrers sis anys, a més d'aportar dades bàsiques per avaluar les conseqüències de l'exposició a radiacions i de definir les mesures a prendre per tal de prevenir la transmissió d'anomalies cromosòmiques a la descendència en casos d'exposició terapèutica, han estat també fonamentals per desenvolupar noves i més efectives tècniques per a l'avaluació de les conseqüències citogenètiques de l'exposició a les radiacions.

Key words: Chromosome aberrations, somatic cells, germ cells, ionizing radiation.

Cytogenetic studies on somatic cells

Studies on chromosome instability in cancer patients

The relationship between the presence of a high frequency of chromosomal aberrations and a predisposition to cancer has been well established in the so-called chromosomal instability syndromes (ataxia telangiectasia, Fanconi's anemia and Bloom's syndrome). Chromosomal instability, albeit with a lower frequency, is also present in peripheral blood lymphocytes of patients affected by some types of cancer, such as retinoblastoma [1-3], renal carcinoma [4], skin cancers [5], primary neoplasias [6], pre-cancerous and cancerous lesions of cervix uteri [7], familial polyposis of the colon [8] and in one case of breast carcinoma [9]. Furthermore, Wurster-Hill et al. [10,11] reported an increased frequency of structural chromosomal aberrations in families with a high incidence of neoplasias, affecting both cancer patients and individuals with no evidence of malignancy.

Table 1 shows the results of a cytogenetic study on peripheral blood lymphocytes from 6 Hodgkin, 13 bladder and

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Table 1. Cytogenetic study on lymphocytes from cancer patients before any treatment.

	Controls	*Hodgkin*	*Controls*	*Bladder*	*Controls*	*Breast*
Number of subjects / metaphases	5 / 591	6 / 417	7 / 824	13 / 393	10 /1141	10 /448
Aberrant metaphases(%)	27 (4.6)	46 (11)	32 (3.9)	41 (10.6)	43 (3.8)	33 (7.4)
Structural aberrations (t, dic, del, inv, etc.)	0	18 (32.1)	3 (8.3)	45 (67.2)	6 (12.2)	31 (62)
Gaps and breaks	29 (100)	33 (58.9)	32 (88.9)	17 (25.4)	36 (73.5)	8 (16)
Numerical abnormalities	0	4 (7.1)	1 (2.8)	5 (7.5)	7 (14.3)	11 (22)
Double minutes	0	1 (1.8)	0	0	0	0

t= translocations; dic= dicentrics; del= deletions; inv= inversions

10 breast cancer patients, carried out with G-banding techniques before any treatment. In the three groups of cancer patients, the frequency of cells with chromosomal abnormalities was significantly higher than in a healthy control population [12-14]. This indicates that these patients showed chromosome instability, a condition that can be considered a predisposing factor for the development of malignances, as is well established for the chromosome instability syndromes.

When the results obtained in Hodgkin's lymphoma patients and bladder or breast cancers are compared, an interesting difference observed is that in Hodgkin's the majority of alterations were gaps and breaks while in bladder or breast cancer they were structural aberrations such as translocations, deletions, inversions etc. This could indicate that chromosome instability could be due to a variety of factors, as described for the chromosome instability syndromes, which in some cases remain unknown.

Cytogenetic effects of radiotherapy

The frequency and types of chromosome aberrations induced by ionizing radiation were evaluated by G-banding techniques in 24 patients affected by several types of cancer, studied just after radiotherapy. The incidence of aberrant metaphases prior to treatment was 9.98% and increased significantly after treatment to 32.8%, as expected (Table 2). A comparison of chromosome abnormalities observed before and after radiotherapy indicated that dicentrics, rings and reciprocal translocations increased by a factor of 23, 13 and 11 respectively, after radiotherapy (Table 3). This seems to indicate that ionizing radiation produces more asymmetrical than symmetrical chromosome aberrations and more two-break than one-break anomalies, according to the results obtained by G-banding.

At the individual level, the patients affected by bladder and breast carcinoma (the two largest groups of individuals in this study) showed a clear interindividual variability in the frequency of aberrant cells, both before and after radiotherapy. This supports the hypothesis that *in vivo* sensitivity to high doses of gamma radiation varies from one individual to another.

The analysis of radiation-induced chromosome aberrations by G-banding showed that the frequency of translocations versus dicentrics was lower than the expected 1:1 ratio. This is probably due to the incapacity of G-banding techniques to detect terminal translocations between G-negative bands. Recently, using fluorescence *in situ* hybridization (FISH) with whole chromosome probes (WCP) applied simultaneously with pancentromeric probes, it has been suggested that the frequencies of translocations and dicentrics are very similar [15].

Breakpoint distribution in radiation-induced chromosome aberrations

A total of 660 breakpoints were identified, using G-banding techniques, in the chromosome aberrations detected in lymphocytes from cancer patients after radiotherapy. Taking into account the relative length of human chromosomes and chromosome arms, our results showed that chromosomes 1, 3 and 7 and chromosome arms 1p, 1q, 7q and 11p were significantly more affected than expected. The relatively higher involvement of chromosome 1 was also observed in other studies carried out with irradiations *in vitro* and using G-banding techniques [16-18]. Recent studies carried out with FISH techniques showed that chromosomes 9 and 10 were

Table 2. Cytogenetic study on lymphocytes from cancer patients before and after radiotherapy.

	Before radiotherapy	*After radiotherapy*
Metaphases analyzed	621	1192
Aberrant metaphases (%)	62 (10)	391 (32.8)
Total chromosome abnormalities	88	946
Structural abnormalities (t, dic, del, inv etc.) (%)	56 (63.6)	883 (93.3)
Gaps and breaks (%)	25 (28.4)	17 (1.8)
Numerical and other (%)	7 (8)	46 (4.9)

Table 3. Type of chromosome abnormalities observed in lymphocytes from cancer patients before and after radiotherapy.

	Before radiotherapy	*After radiotherapy*	*Increase factor*
Total alterations / cell	0.1417	0.7936	5.6
Translocations / cell	0.0128	0.1442	11
Dicentrics / cell	0.0096	0.2223	23
Deletions / cell	0.0177	0.0721	4
Inversions / cell	0.0016	0.0117	7
Acentrics / cell	0.0257	0.1426	6
Fissions / cell	0.0016	0.0067	4
Rings / cell	0.0032	0.0411	13
Marker chromosomes / cell	0.0177	0.0973	5
Gaps and breaks / cell	0.0402	0.0142	0.3
Numerical abnormalities / cell	0.0096	0.0134	1.4

more involved in translocations and dicentrics [19], but did not find an excess of involvement of chromosome 1 (the most affected in our study). Moreover, a significant excess of involvement of telomeric regions and G-negative bands has been observed. It has been suggested that the excess of involvement of G-negative bands could be due to the chromatin structure and composition of these bands [20]. In our study, a significant clustering of breakpoints of radiation-induced chromosome aberrations has been observed in bands in which fragile sites, protooncogenes, breakpoints involved in chromosomal cancer rearrangements and breakpoints involved in chromosomal evolution of the Hominoidea are located [21-24].

In conclusion, several studies seem to indicate a lack of correlation between DNA content and sensitivity to the clastogenic effect of ionizing radiation. The discrepancies appear when the chromosomes more implicated are individually identified, because they are different in each study. However, in a recent study carried out in our laboratory, in which all human chromosomes were analyzed independently by FISH painting techniques, we found in general a good correlation between DNA-content and chromosome sensitivity after blood irradiation with 5 Gy of X-rays *in vitro* [25].

Cytogenetic studies on occupationally exposed individuals

An increased frequency of chromosome aberrations has been described in populations occupationally exposed to ionizing radiation [26-31], living in areas with high levels of environmental radioactivity [32] or in those with additional exposures due to incidents such as Chernobyl [33]. However, when the doses were below 100 mSv it is difficult to establish any dose-effect relationship.

We have completed a study in 26 healthy individuals who had been occupationally exposed to ionizing radiation in radiodiagnostic, radiotherapy and nuclear medicine services in a Barcelona hospital [34]. Blood samples were cultured for 48 h in the presence of bromodeoxyuridine and the metaphases obtained were stained by the Fluorescence Plus Giemsa (FPG) technique [35]. This technique distinguishes metaphases from first, second and third mitotic division. The cytogenetic analyses were carried out exclusively in first division metaphases.

Table 4 shows the cytogenetic results obtained in exposed workers and controls. Although the frequencies of almost all chromosome aberrations were higher in the exposed group, the differences were only significant for acentric fragments ($p<0.004$). No significant influence of sex or smoking habit was observed. Because the total dose of exposure received was different in each individual, depending on the years of employment, a corrected dose was calculated from the annual dose for each individual, the mean life of lymphocytes and the exponential loss of those cells with unstable chromosome aberrations (Σ annual dose x exp (-0.693 x i /3), where i is the elapsed time in years for each annual dose (modified from [29]). When the exposed individuals were grouped into intervals of 5 mSv of total corrected dose, there were no significant differences in the frequencies of any type of chromosome aberration. Furthermore, linear regression analysis did not show any correlation between dose and the frequency of chromosome aberrations. The significant increase in chromosome aberrations that require one break (such as acentrics) but not of aberrations that require two breaks (such as dicentrics or translocations) is due to the fact that low doses and dose rates of exposure increase the number of acentric fragments more easily than that of dicentrics. Although we did not find any correlation between the dose and the frequency of chromosome aberrations, probably due to the very low doses of exposure of the individuals studied, our results suggest that acentric fragments could be considered as good qualitative

Table 4. Frequency of chromosomal aberrations in the occupationally exposed group and in controls (number/100 cells).

	Exposed group	*Control group*
Number of subjects	26	10
Cells scored	4604	2008
Dicentrics	5 (0.11)	1 (0.05)
Translocations	4 (0.09)	–
Acentric fragments	33 (0.72)	3 (0.15)
Chromosome breaks	22 (1.19)	12 (0.60)
Total structural chromosome-type aberrations	64 (1.39)	13 (0.65)
Numerical abnormalities	31 (0.67)	4 (0.20)

indicators of ionizing radiation exposure for doses below 50 mSv.

On the other hand, the number of cells with numerical abnormalities (we have considered only hyperdiploidies), was significantly higher in the exposed group ($p<0.015$). Other authors have found increases in trisomy after irradiation of lymphocyte cultures *in vitro* [36] and increases in aneuploidy in lymphocytes from victims of a radiation accident in Goiana (Brazil) [37], concluding that aneuploidy can be induced in lymphocytes by radiation and that it persists for a long time.

Biological dosimetry

Establishment of dose-effect curves for the cytogenetic effects of ionizing radiation

The main objective of a dose-effect curve is its applicability in radioprotection, and its accuracy increases with the number of cells analyzed. When an individual is exposed to an unknown dose of ionizing radiation, it is necessary to measure the dose received. The analysis of dicentric translocations in lymphocytes is considered the most sensitive biological method to quantify exposure for doses over 0.1 Gy, if sufficient metaphases are analyzed. For the estimation of a dose, each laboratory must have its own dose-effect curve [38] For X– and gamma-rays, dose-effect curves follow a linear-quadratic model $Y = C + aD + bD^2$, where Y is the frequency of dicentrics, C the basal level of dicentrics, D the dose and a and b the coefficients of the linear and quadratic terms respectively. Although a certain degree of variability in the response to radiation has been described [39], most laboratories can estimate a given dose with acceptable accuracy [40].

In our laboratory two dose-effect curves were carried out, for gamma and X-rays, by the analysis of dicentric chromosomes in uniformly stained metaphases [41,42].

Dose-effect curve for gamma rays.

Peripheral blood from a healthy individual, with no history of exposure to mutagenic agents including radiation, was irradiated using a cobalt source in the Hospital de la Santa Creu i Sant Pau (Barcelona). Doses were measured by the Unit of Radiophysics and Radioprotection of the hospital after calibration of the teletherapy unit with an electrometer (Farmer 2570). Dose rates ranged from 117.5 cGy/min to 107 cGy/min due to the decay of the cobalt source. Doses of 0, 0.1, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4 and 5 Gy were used to elaborate the curve. IAEA recommendations were followed for the irradiation [43].

To validate the curve for applicability, peripheral blood samples from six individuals were irradiated at 2 Gy in the same conditions. Three of them had been exposed occupationally.

After irradiation, blood samples were cultured for 48h in medium containing 12 μg/ml of bromodeoxyuridine in order to select only first division metaphases for the analysis.

To check whether the distribution of dicentrics within cells for each dose followed a Poisson distribution, the dispersion index $D = s^2/y$ and its normalized unit (u) were used [44]. Absolute values of u above 1.96 indicate that the distribution of dicentrics within cells does not follow a Poisson distribution, which is to be expected for this kind of analysis. Curves were fitted using the method of iteratively reweighted least squares.

Table 5 shows the summarized cytogenetic results of the dose-effect curve. The fitted values of the coefficients of the linear quadratic function $Y = C + aD + bD^2$ are: $C = (0.13 \pm 0.05) \times 10^{-2}$; $a = (2.10 \pm 0.52) \times 10^{-2}$; $b = (6.31 \pm 0.40) \times 10^{-2}$. The chi-square value of the curve was 6.6 ($p = 0.5798$), indicating a good fit.

The distribution of dicentrics for each dose follows a Poisson, as reported by other authors [45-47]. The fitted coefficients given by our results are consistent with those reported by these authors.

Table 6 shows the cytogenetic results of the six individuals whose peripheral blood was irradiated with 2 Gy. For all six individuals the 95% confidence interval of the estimated dose included the real dose, indicating an accurate estimation. However, the frequency of dicentrics per cell in the non-exposed group was higher than in the occupationally exposed group (0.31 versus 0.269), although the difference was not significant. These results were later confirmed in a more extensive study (see Adaptive response, below).

Table 5. Cytogenetic results, Papworth u and dispersion indexes of the dose effect curves for g and X-rays.

	g-rays dose effect curve				X-rays dose-effect curve			
Dose (Gy)	*Cells analyzed*	*Total dic*	*u*	*Cells s^2/y*	*Total analyzed*	*dic*	*u*	*s^2/y*
0.00	5000	8	–0.07	1.00	8811	8	–0.06	1.00
0.10	5002	14	–0.13	1.00	5048	13	–0.13	1.00
0.25	2008	22	2.61	1.08	2005	35	1.10	1.03
0.50	2002	55	–0.86	0.97	2012	67	–0.21	0.99
0.75	1832	100	0.79	1.03	1607	100	–1.30	0.95
1.00	1168	109	–0.02	1.00	1292	103	–1.20	0.95
1.50	562	100	1.08	1.06	682	101	–0.46	0.98
2.00	332	103	2.31	1.18	403	108	–0.43	0.97
3.00	193	108	–1.64	0.83	205	123	–0.80	0.92
4.00	103	103	–0.84	0.88	204	200	–0.54	0.95
5.00	59	107	0.81	1.13	200	304	–2.09	0.79

Table 6. Cytogenetic results and dose estimations after peripheral blood irradiation with 2 Gy of g-rays in six individuals.

	Cells analyzed	*dicentrics*	*dic/cell*	*Estimated dose*	*95% confidence dose interval*
Occupationally exposed					
Case 1	410	102	0.249	1.82	1.62 / 2.01
Case 2	335	103	0.307	2.04	1.82 / 2.25
Case 3	387	100	0.258	1.86	1.65 / 2.05
Non-exposed					
Case 4	380	108	0.284	1.96	1.75 / 2.15
Case 5	313	95	0.304	2.03	1.80 / 2.24
Case 6	285	100	0.351	2.19	1.95 / 2.41

Dose-effect curve for X-rays.
Blood was collected from the same individual sampled for the gamma-ray curve. Blood was irradiated using an X-ray source, with a beam quality corresponding to a half value layer of 1.43 mm Cu (180 kVp, 9 mA and 0.5 mm Cu filtration). The dose rate was 26.95 cGy / min. Blood was processed as for the gamma-ray curve.

Table 5 also shows the cytogenetic results of the dose-effect curve. The coefficients of the linear quadratic function obtained were: $C = (9.5 \pm 4.4) \times 10^{-4}$; $a = (3.43 \pm 0.68) \times 10^{-2}$; $b = (5.7 \pm 0.42) \times 10^{-2}$. Chi-square value was 15.8; df = 8 and p = 0.05.

Our results are consistent with those reported for X-ray dose-effect curves [40,48].

An example of the applicability of this dose-effect curve is described below.

Biological dosimetry in partial irradiations
Information on the absorbed dose and its distribution in the body is of great importance for an early assessment of the consequences of irradiation. Cytogenetic analysis of peripheral blood lymphocytes can provide a biological estimation of the dose received if a dose-effect curve from the same laboratory is available. Among the radiation-induced chromosome aberrations, dicentric chromosomes, sometimes combined with ring chromosomes in uniformly stained preparations, are most useful for quantitative analyses in cases of radiation accidents [48-50]. However, in the case of partial body exposure, it is difficult to quantify the fraction of the body that has been irradiated [40,50].

We studied five different partial irradiations simulated at doses of 2, 3, 4 and 5 Gy were [42].

Dose estimations, using the dose-effect curve coefficients for X-rays established previously, were made following two methods, the contaminated Poisson method [43,51] and the Qdr method [52]. Because the results of both methods were very similar, we describe only those obtained using the contaminated Poisson method. The frequency of dicentrics plus rings (dic + r) in the irradiated fraction (Y) was estimated using the following equation:

$$Y / (1 - e^{-Y}) = X / (N - n_0).$$

Y was obtained by iteration and represents the mean yield of dic+r of the irradiated fraction, e^{-Y} represents the undamaged cells from the irradiated fraction, N the number of cells scored, X the number of dic+r observed and n0 is the number of cells free of dic+r.

To calculate the standard errors (SE), the «truncated Poisson yield» $Y_1 = X / N{-}n_0$ and its variance $Var(Y_1) = X(1 + Y - Y_1) / (N - n_0)^2$, were used. The SE, calculated as the square root of $Var(Y_1)$, multiplied by 1.96, produced the 95% confidence limits for Y, that were used to calculate the estimated doses and the fraction of cells scored that were irradiated, which can be used to estimate the fraction of cells originally exposed (data not shown, see 42).

Table 7 shows the dic + r observed, the dose estimated without considering the irradiations as partial (estimated whole body dose) and the estimated dose to the irradiated fraction. The observed frequency of dic + r and the estimated whole body dose decreased with increased dilutions of irradiated blood. However, the estimated doses to the irradiated fractions were near the real dose of the irradiated fractions, and their 95% confidence intervals always included the real dose.

In conclusion, when it is not known whether irradiation affected the whole body or part of it, a first criterion to determine whether the type of exposure is partial is the Poisson distribution evaluation from «u» (see 1.5.1.1.). Our results show that overdispersion was detected when the percentages of irradiated blood were ≤75%. However, «u» for 87.5% of irradiated blood for doses of 2, 3 and 4 Gy was included in the ±1.96 interval, indicating that contamination by unirradiated cells cannot be detected. A second criterion that could help to discriminate between partial and whole body irradiation, mainly for percentages of irradiated blood >75%, is the relationship between the estimated whole-body dose and the estimated dose to the irradiation fraction. For 100% of irradiated blood at the doses of 2, 3, 4 and 5 Gy, the estimated whole-body doses were 1.93, 3.07, 4.01 and 5.07 respectively. These doses were higher than those obtained using the contaminated Poisson (1.81, 2.91, 4.00 and 4.86) or the Qdr method (1.72, 2.93, 4.01 and 4.96). On the other hand, for all dilutions, the estimated whole-body doses were always lower than the estimated doses to the irradiated fraction, irrespective of the method used. For the

Table 7. Cytogenetic results and dose estimations after *in vitro* partial irradiations.

Dose (Gy)	*% of irradiated blood*	*Cells analysed*	*Total dic+r*	*Estimated whole body dose (Gy)*	*Estimated dose (Gy) to the irradiated fraction (95% confidence limits)*
2	87.5	459	113	1.79	2.06 (1.40-2.55)
	75	508	88	1.46	2.41 (1.65-2.98)
	50	505	66	1.24	2.54 (1.63-3.18)
	25	701	29	0.59	2.03 (0.00-2.91)
	12.5	500	13	0.43	1.41 (0.00-2.56)
3	87.5	285	107	2.28	2.69 (2.01-3.21)
	75	335	106	2.07	2.93 (2.25-3.46)
	50	545	81	1.34	3.24 (2.44-3.85)
	25	521	36	0.83	2.75 (1.38-3.59)
	12.5	510	15	0.47	2.57 (0.00-3.77)
4	87.5	146	111	3.36	3.84 (3.16-4.38)
	75	185	114	3.00	3.90 (3.23-4.44)
	50	328	109	2.13	3.96 (3.28-4.52)
	25	500	61	1.19	3.33 (2.37-4.02)
	12.5	506	38	0.88	3.49 (2.19-4.35)
5	87.5	199	229	4.20	4.90 (4.42-5.31)
	75	222	116	2.74	5.23 (4.53-5.81)
	50	356	117	2.12	4.88 (4.20-5.45)
	25	506	56	1.12	5.32 (4.25-6.14)
	12.5	498	29	0.75	5.15 (3.55-6.23)

above mentioned reasons, to determine whether irradiation has affected the whole body or only part of it, it is important to consider both «u» and the relationship between the estimated whole-body dose and the estimated dose to the irradiated fraction.

To establish the proportion of the body irradiated, one must consider the fraction (f) of cells scored that were irradiated (f = X/YN). The fraction of cells originally exposed (F) and its 95% confidence intervals were calculated using the following equation, $F = (f/p) / (1 - f + f/p)$, where p is the surviving fraction of cells, taking into account the selective loss of irradiated cells due to interphase death and mitotic delay. The value of p for each dilution was estimated by the equation $p = e^{(-D/D_0)}$, where D is the estimated dose to the irradiated fraction (f) of cells scored and D_0 could be 2.7 [53] or as in our case 3.8 (the value that gives the best relationship between the estimated and the real fraction irradiated). With a D_0 of 3.8, 18 out of 20 dilutions included the real F values in their 95% confidence intervals. These results indicate that the accurate quantification of the irradiated fraction of the body is difficult, although it is possible to obtain estimated proportions close to the actual one.

Adaptive response

Adaptive response to the effect of ionizing radiation

Pretreatment of human lymphocytes with tritiated thymidine or with low doses of X-rays *in vitro*, makes these cells less susceptible to cytogenetic damage by subsequent high acute doses of X–rays [54-56]. This phenomenon has been called Adaptive Response (AR) because it is similar to the induced repair described in *E. coli* [57]. AR is dependent on the preliminary dose and on the dose rate [58]. The ability to respond to small doses of X-rays has been detected only in phytohemagglutinin-stimulated lymphocytes [56,59]. On the other hand, AR is inhibited by the presence of 3-aminobenzamide [55,60,61], which inhibits poly (ADP ribose) polymerase [62], and by cycloheximide [63]. It is now documented that ionizing radiation can induce proteins [64], the expression of certain genes, eg. the *gadd45* system [65], p53 [66-70], DNA repair mechanisms [71] and activation of transcription factors such as NF-kB [72].

Taking into account the frequencies of chromosome aberrations induced by low doses of environmental radiation, and the effect of additional radiation due to the Chernobyl accident, Pohl-Rüling *et al.* [33] assumed that repair mechanisms could be triggered by an increase of about 30-40% above the continuous environmental dose. Moreover, a significant decrease in sensitivity to bleomycin in lymphocytes of children resident in the area surronding Chernobyl with internal Cs 137 contamination, has been described [73].

Interindividual variability in the induction of AR in lymphocytes, evaluated by cytogenetic analysis, has been reported [74,75]. Other studies showed that the presence or absence of AR varies in the same donor with time, concluding that AR is not linked to the genetic constitution of the individual, but depends on unknown transient physiological parameters [74,76].

A study to determine whether an adaptive response can be induced by occupational doses of ionizing radiation was carried out in our laboratory [77]. Peripheral blood samples from 20 individuals, eight non-occupationally exposed (five males and three females with ages from 24 to 56] and twelve hospital workers occupationally exposed to X and

gamma rays (eight males and four females with ages from 30 to 49), were irradiated at 2 Gy in the same conditions. In the occupationally exposed group, the whole-body doses received were measured by film badges. The years of chronic exposure to radiation ranged from 7 to 21, and the annual doses from 0 to 28 mSv. The calculation of corrected doses was made from the annual dose received by each individual, taking into account the mean life of lymphocytes and the exponential loss of lymphocytes with unstable chromosome aberrations (Σ annual dose $\times$ exp ($-0.693 \times i/3$), where i is the elapsed time in years for each annual dose) [29]. In all cases, a control study without irradiation was made.

Chromosome analyses were carried out exclusively on first division metaphases containing 46 or more centromeres. For the 2 Gy irradiated samples, we analyzed the number of metaphases needed to include 100 dicentrics.

All metaphases with chromosome abnormalities were analyzed independently by three investigators. For comparison, the chromosome-type abnormalities considered were: dicentric chromosomes (dic), which were considered only when the acentric fragment was present, and acentrics (ace) plus chromosome breaks (csb), which were recorded together. The csb were recorded only when the broken piece was displaced with respect to the chromosome axis.

To compare the total frequencies of chromosomal abnormalities in the two groups, we used a two-tailed test of the normal approximation. To assess the effect of the doses received occupationally on the frequency of dicentrics after blood irradiation, Spearman's rank correlation test was used.

The estimation of the doses received, after blood irradiation at 2 Gy, was obtained taking into account the gamma-ray dose-effect curve of our laboratory [41].

Before irradiation, the number of dicentrics (dic) was too low to establish any comparison between populations. However, the total frequency of (ace + csb) was significantly higher in the exposed population ($p<0.001$). No differences were observed between smokers and non-smokers or males and females.

After 2 Gy irradiation of blood samples, the frequency of dicentrics was significantly lower in the exposed population than in the non-exposed ($p<0.001$) (table 8).

To assess whether the individual doses received occupationally could influence the frequency of chromosomal alterations (table 8), we used Spearman's rank correlation test. A significant negative correlation was observed between the doses received occupationally during the last year of exposure and the frequencies of dicentrics induced by the 2 Gy irradiation ($p<0.025$). The same was observed for the mean doses received during the last three years ($p<0.05$). No correlation was observed when the total corrected doses were considered.

The estimated values of the dose after 2 Gy blood irradiation and the 95% confidence intervals are also shown in table 8. The mean estimated dose was higher in the non-exposed group (2.049) than in the exposed (1.848).

Table 8. Dicentrics per cell observed after blood irradiation at 2Gy for each individual. The estimated values of the dose received and 95% confidence intervals after blood irradiation at 2Gy have been obtained taking into account the dose-effect curve of our laboratory , which follows the linear-quadratic model. For the exposed individuals the occupationally received doses are also shown.

Case	*Corrected dose (mSv)*	*Last year dose*	*Mean dose last 3 years (mSv)*	*dic/cell ± SE (mSv/year)*	*Estimated dose (Gy)*	*95% confidence dose interval (Gy)*
NE1	–	–	–	0.284±0.027	1.958	1.747 / 2.149
NE2	–	–	–	0.304±0.031	2.029	1.796 / 2.239
NE3	–	–	–	0.310±0.031	2.053	1.827 / 2.257
NE4	–	–	–	0.263±0.033	1.878	1.613 / 2.112
NE5	–	–	–	0.350±0.035	2.189	1.946 / 2.410
NE6	–	–	–	0.316±0.031	2.072	1.842 / 2.280
NE7	–	–	–	0.284±0.028	1.958	1.741 / 2.154
NE8	–	–	–	0.375±0.037	2.272	2.020 / 2.500
Total	–	–	–	0.309±0.011	2.049	1.969 / 2.125
E1	25.21	5.67	8.89	0.249±0.025	1.821	1.618 / 2.005
E2	5.29	0.44	0.85	0.307±0.030	2.043	1.819 / 2.247
E3	20.01	2.91	4.47	0.258±0.026	1.859	1.650 / 2.049
E4	18.87	4.20	5.70	0.252±0.025	1.836	1.632 / 2.021
E5	17.23	4.65	4.85	0.246±0.025	1.809	1.603 / 1.995
E6	27.43	0.54	2.18	0.261±0.026	1.869	1.661 / 2.057
E7	1.36	0.51	0.57	0.282±0.028	1.950	1.733 / 2.147
E8	18.70	0.77	0.89	0.268±0.026	1.895	1.685 / 2.085
E9	21.37	0.52	0.77	0.214±0.021	1.679	1.490 / 1.849
E10	4.42	1.09	1.13	0.222±0.022	1.711	1.519 / 1.886
E11	3.48	1.37	0.96	0.257±0.026	1.855	1.644 / 2.045
E12	1.38	0.47	0.62	0.283±0.028	1.952	1.735 / 2.148
Total				0.256±0.007	1.848	1.790 / 1.904

NE=Non-exposed, E=Exposed

These results are the first demonstration that occupational exposure to very low doses of ionizing radiation makes human lymphocytes less susceptible to subsequent irradiation *in vitro* at higher doses. This is in contrast with previous studies, in three donors, which showed the incapacity of unstimulated cells to show AR after pretreatments with low doses of radiation *in vitro* [56,59].

Interindividual variability in adaptive response has been described by several authors [74-76,78], and has also observed in our study. However, the doses received occupationally during the last three years were negatively correlated with the induction of chromosome abnormalities, indicating that, in spite of this variability, it is possible to detect a different response to subsequent high doses of radiation, which could be related to the recent doses received. Low occupational doses probably can act as an activator of the repair machinery, as described for irradiation *in vitro* [71,79].

In addition, our results could have some implications in biological dosimetry and radioprotection. The estimated dose after 2 Gy blood irradiation was, in general, lower in the occupationally exposed individuals, and in three of them the 95% confidence interval did not include the challenge 2 Gy dose. This indicates that for the estimation of doses received accidentally, it is necessary to consider the individual's occupational history of exposure to mutagens.

The results presented here do not indicate that exposure to low doses of radiation, by itself, could be beneficial, because the basal levels of chromosome abnormalities are in general higher in exposed populations [26-28, 30, 31, 34].

Adaptive response for the effect of bleomycin

Bleomycin (BLM) is an S-independent radiomimetic agent that induces double strand breaks in DNA [80,81]. Wolff et al. [82] reported that human blood lymphocytes, adapted by exposure to low doses of ionizing radiation *in vitro*, showed a decrease in the frequency of chromatid and isochromatid lesions induced by subsequent treatment with BLM. Moreover, pretreatment with low concentrations of BLM can also induce an adaptive response [83]. Recently, an adaptive response to a challenge dose of BLM in lymphocytes from children contaminated as a consequence of the Chernobyl accident [84] has been described.

In our laboratory, a study was performed to determine whether blood samples from individuals occupationally exposed to radiation are less susceptible to the clastogenic effect of BLM [85].

Peripheral blood samples from 23 individuals (11 unexposed and 12 occupationally exposed to ionizing radiation) were cultured for 48h. For each sample, two cultures were made, one as a control and another to which 0.03U/ml of BLM was added 5 h before harvesting. Cultures and culture harvesting were made as in the previous studies on the effect of ionizing radiation, and staining was by the FPG technique. The abnormalities considered were chromatid breaks (ctb) because they are the main abnormalities induced by the addition of BLM 5 h before harvesting.

Table 9 shows the cytogenetic results obtained. The frequency of ctb was significantly higher in the unexposed population than in the occupationally exposed one ($t = 2.19$; $df = 14$; one tailed $p<0.025$). Due to unequal variances, Satterthwaite's rule was applied. The rank of frequencies of ctb per 100 cells was wider in the unexposed population.

Table 9. Cytogenetic effects after bleomycin treatment.

	Unexposed	*Occupationally exposed*
Number of subjects	11	12
Cells analyzed	2138	2502
Chromatid breaks (ctb)	855 (40.5±4.93)	718 (28.5±2.35)
Population rank of ctb frequencies	21.4-66.4	18.0-42.7

In both populations, the individual frequencies of ctb, analyzed by Pearson's chi-square test, showed heterogeneity (occupationally exposed population, chi-square = 51.42, df = 11, $p<0.0005$; unexposed population, chi-square = 133.88, df = 10, $p<0.005$). Moreover, the values of Papworth's u, distributed as a standard variable were always greater than (±) 1.96 and ranged from 5.61 to 47.66 in unexposed individuals and from 2.24 to 20.75 in occupationally exposed ones. This indicates an overdispersion, which is more evident in the unexposed individuals, in whom the presence of cells with 5 or more ctb was higher than in the occupationally exposed.

After BLM treatment, no influence of sex was observed for the ctb frequencies. However, in both populations the individual frequencies of ctb were lower in smokers than in nonsmokers.

The results presented could indicate that an adaptive response induced by occupational exposure to ionizing radiation can also be detected after BLM treatment of peripheral blood cultures. This is consistent with the hypothesis that an adaptive response induced by a low dose of a mutagenic agent can be detected after a challenge dose of the same or similar DNA-damaging agent.

However, the great heterogeneity detected in both populations for the ctb frequencies differs from the results obtained with treatment of 2 Gy, where the frequencies of induced dicentrics were homogeneous in both populations. These discrepancies could be a reflection of the asynchronous cell cycle of proliferative lymphocytes. The treatment with 2 Gy of ionizing radiation was performed on G0 lymphocytes, while the treatment with BLM 5 h before harvesting resulted in an effect of BLM on cells coming from both the S and G2 stages. For this reason, we believe that at the individual level, treatment with BLM on G2 cells cannot be used to detect adaptive response.

Application of FISH techniques to the study of the cytogenetic effects of ionizing radiation

Fluorescence *in situ* hybridization (FISH) with whole chromosome probes (WCP) stains chromosomes throughout their entire length (chromosome painting). This technique, using

probes for one chromosome or different combinations of WCP allows the quantification of radiation induced chromosome aberrations [15, 86-92].

Uniform stain, which reveals dicentric chromosomes, is not appropriate for past exposures because these abnormalities disappear quickly after irradiation. However, the frequency of reciprocal translocations is relatively constant, and does not show the decrease observed for dicentrics, however, with uniform staining techniques translocations cannot be detected. Detection of reciprocal translocations (that are induced at a similar frequency as dicentrics) is easy using painting techniques, and has been used for the assessment of the level of past exposure [93-96]. Obviously, to quantify the dose of exposure, a laboratory must previously elaborate its own dose-effect curve using painting techniques.

Painting with one or several chromosome probes, combined with FISH with pancentromeric probes reveals reciprocal translocations, as well as dicentrics. For this reason, the use of chromosome painting combined with pancentromeric probes can also be used to quantify recent exposures.

Cytogenetic studies on human male germ cells

As described above, the effects of human mutagens, clastogens and aneugens have been studied almost exclusively in somatic tissues. However, currently there is a considerable discussion about the potential of ionizing radiation to induce inheritable germ cell mutations. In experimental mammals, the induction of germ cell inheritable genetic damage has been well documented. There are many studies reporting that preconceptional exposure of male and female mice to ionizing radiation induces high levels of genetic abnormalities in their offspring. In humans, however, the induction of germ-line genetic damage has been much harder to verify. Analyses of the pregnancies and offspring of Japanese atomic bomb survivors [97,98] and of cancer patients who received radiotherapy and chemotherapy [99,100] have not detected additional risk associated with exposure of either parent to these mutagens before fertilization. However, several epidemiological surveys have reported significant associations between certain occupations or environmental exposures of parents and increased risk of reproductive abnormalities [101-103].

Cytogenetic effects of cancer therapies

To supplement information obtained from epidemiological studies, germ cells from patients treated with chemotherapy and radiotherapy can be directly evaluated using biomarkers of genetic damage. This provides information on an important intermediate step in the pathway leading to transmissible germ cell mutation. Two laboratory assays have been developed to evaluate genetic damage in sperm following adverse exposures to the male: fluorescence *in situ* hybridization of sperm nuclei (sperm FISH) and the human-sperm/hamster-egg fertilization system to analyze sperm derived metaphase chromosomes (*humster* assay).

Fluorescence *in situ* hybridization (FISH) has become a popular method for estimating numerical chromosomal abnormalities in human sperm. This assay is based on the hybridization of decondensed spermatozoa with fluorescent labelled chromosome-specific DNA probes. Martin et al. [104] were the first group to use sperm FISH to address the effects of anti-cancer agents on sperm chromosomes. In their long-term effect studies, no increase in the frequency of aneuploid spermatozoa was found in patients treated with chemotherapy for Hodgkin's disease or testicular cancer. However, in a study on the short-term effect of cancer treatment, Monteil et al. [105] found an increased aneuploidy frequency in spermatozoa from a Hodgkin's disease patient after chemotherapy and radiotherapy. Similarly, Robbins et al. [106] found a transient sex chromosomal and autosomal aneuploidy increase in spermatozoa of eight Hodgkin's disease patients treated with chemotherapy. They found a fivefold increase in sperm with disomies and diploidies involving chromosomes X, Y and 8. The aneuploidy effects were transient, however, declining to pretreatment levels within approximately 100 days after the end of the therapy. These findings emphasize the advisability of using contraception during chemotherapy and also argue against recommendations that sperm banking may be performed after chemotherapy begins. The observed return to baseline at 100 days following completion of therapy suggests that conventional patient education to avoid conception during 6 months following treatment with anti-cancer agents may be advisable.

The *humster* assay allows the scoring of both structural and numerical chromosomal abnormalities in human spermatozoa. In this assay, capacitated human spermatozoa are allowed to fuse with zona-free hamster oocytes, resulting in decondensation of the human sperm chromatin material within the hamster egg cytoplasm, which after an overnight culture, reaches the first embryonic metaphase stage. Studies of abnormalities in human sperm-derived chromosomes induced by cancer treatment are both scarce and conflicting. In a study carried out by the *humster* assay, Martin et al. [107] found that radiotherapy *in vivo* induced an increase in the frequency of both structural abnormalities and hypoploidy in sperm chromosomes. Similarly, in a long-term cytogenetic study we found an increase in the frequency of structural chromosome abnormalities in the spermatozoa of six men treated several years before with chemotherapy and radiotherapy for different types of cancer [108,109]. The results obtained in our laboratory are shown in table 10. Taking into consideration only those aberrations compatible with a premeiotic origin (because it was a long-term study), we found a higher frequency of chromosomal abnormalities in cancer patients than in control donors. However, there seems to be a tendency to return to the control levels with time. It is possible that as stem-cell spermatogonia divide, those that carry chromosomal abnormalities are filtered out.

Table 10. Long term effect of CT and RT on human male germ cells (Humster assay).

		INDIVIDUALS TREATED FOR CANCER	
	CONTROL SERIES	*< 5 years after treatment*	*> 5 years after treatment*
Deletions	21	18	8
Translocations	7	5	-
Inversions	3	9	-
Marker chromosomes	8	3	-
Other	1	3	1
TOTAL STRUCTURAL	40 / 2389	38 / 429	9 / 211
	(1.7%)	(8.9%)	(4.3%)
2 x hyperploidy	80 / 2162	28 / 429	4 / 211
TOTAL NUMERICAL	(3.7%)	(6.5%)	(1.9%)

More recently, Brandriff et al. [110] analyzed sperm derived chromosomes from six Hodgkin's disease patients 3 to 20 years after treatment, which had consisted of two to six cycles of MOPP (nitrogen mustard, vincristine, procarbazine, and prednisone) with or without radiotherapy. A significant increase in the rate of hyperploidy was observed in the spermatozoa of these patients, but structural chromosome abnormalities did not increase significantly. On the other hand, no long-term cytogenetic effect was detected in spermatozoa of a man treated with MACOP-B therapy (methotrexate, doxorubycin, cyclophosphamide, vincristine and prednisone) [104].

These great variations in the results of sperm studies on cancer patients are to be expected when so many different types of cancer and treatment have been considered together. It is entirely possible that certain chemotherapeutic regimens are aneugenic and/or clastogenic whereas others are not.

While the various viewpoints remain controversial, one of the aims of germ cell cytogenetic studies must be to improve our ability to identify and estimate the actual genetic risk in humans.

Cytogenetic effects of in vitro irradiation of human spermatozoa

One way to assess the risk of transmission of genetic anomalies by human males occupationally or accidentally exposed to ionizing radiation is to determine whether there is a dose-related genetic damage in human spermatozoa irradiated *in vitro*. Cytogenetic analysis of human spermatozoa is possible after interspecific fertilization *in vitro* of zona pellucida-free hamster oocytes by human spermatozoa. Using this assay system, we have analyzed the radiation induction of structural chromosome abnormalities in sperm derived complements at the first embryo cleavage, as well as the radiation induction of micronuclei and aneuploidy in two-cell hybrid human-hamster embryos.

Induction of structural chromosome abnormalities

Using the interspecific fertilization system *in vitro*, we have established a dose-effect relationship for the cytogenetic effects of gamma-rays on human spermatozoa [111]. Semen samples from three healthy men were irradiated at doses of 0.00, 0.10, 0.25, 0.50, 1.00, 2.00 and 4.00 Gy. We observed that human spermatozoa retained a high fertilization ability even after high doses of gamma-rays. This indicates that induced DNA lesions accumulate in male germ cells, and may be transmitted to the zygote without being selected against at fertilization.

A total of 340 chromosome complements derived from non-irradiated human spermatozoa and 987 complements from irradiated spermatozoa were analyzed after sequential uniform staining-G banding. Both the frequency of spermatozoa with structural chromosome abnormalities and the incidence of such abnormalities per cell showed strong dose-effect relationships, which were best expressed by linear-quadratic equations:

$$Y = 0.06413 (\pm 0.00475) + 0.1982 (\pm 0.00833) D - 0.00763 (\pm 0.00204) D^2$$

and

$$Y = 0.07385 (\pm 0.00838) + 0.23329 (\pm 0.03124) D + 0.02317 (\pm 0.00955) D^2$$

respectively (Fig.1).

The incidence of structural abnormalities per cell showed a linear-quadratic dose-response relationship (with a positive quadratic coefficient), where the quadratic trajectory was only visible at the highest dose. The linear dose-response relationship for the induction of spermatozoa with structural abnormalities showed a saturation effect (negative quadratic

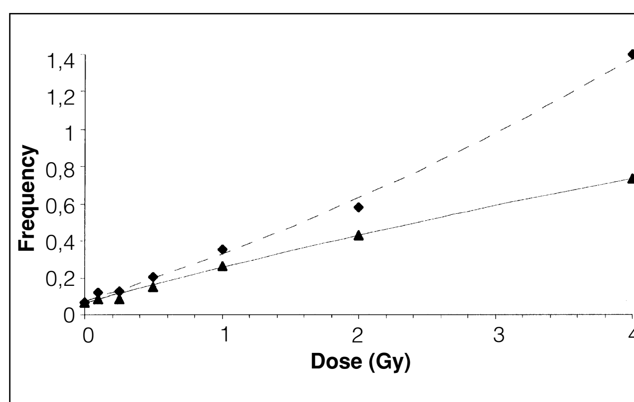

Figure 1. Cytogenetic effect of gamma radiation on human sperm. Triangles and continuous line represent the frequency of human spermatozoa with structural abnormalities. Lozenges and discontinuous line represent the frequency of structural aberrations per cell.

coefficient). This saturation effect was very small, and it could only be detected at the highest dose. This effect could probably be due to the fact that, at increasing doses, the probability that a cell will be affected by more than one chromosomal abnormality also increases, although this will be counted as a single abnormal spermatozoon. This saturation effect has also been reported in a study on the cytogenetic effects of X– and gamma-radiation on Syrian hamster spermatozoa *in vitro* [112]. In a previous study of the cytogenetic effect of gamma-radiation on human spermatozoa [113], the induction of spermatozoa with structural abnormalities was 1.5 times higher than that found in the present study, and no saturation effect was detected for the induction of spermatozoa with structural abnormalities. The reason for this apparent discrepancy could be that in the study of Mikamo et al. [113] the maximum radiation dose was 1.11 Gy. At this dose, a small saturation effect like that found in our study, can easily go unnoticed.

Chromosomal aberrations may be categorized according to the number of breaks involved and the subsequent interactions among broken ends. Thus, structural abnormalities were classified as unrejoined and rejoined. Unrejoined structural abnormalities consist of such chromosome abnormalities as breaks, terminal deletions and acentric fragments, whereas inversions, translocations, dicentrics and rings are rejoined structural abnormalities.

When analyzing these types of structural abnormality separately, we found that the incidence of unrejoined lesions was four times higher than the incidence of rejoined anomalies. The induction of unrejoined abnormalities was best fitted to the linear equation:

$$Y = 0.05198 (\pm 0.00727) + 0.24472 (\pm 0.01193) D \text{ (Fig 2)}$$

However, when analyzing the induction of rejoined chromosomal abnormalities the dose-response relationship was best fitted to the quadratic equation:

$$Y = 0.02530 (\pm 0.00531) + 0.01801 (\pm 0.00243) D^2 \text{ (Fig. 2)}$$

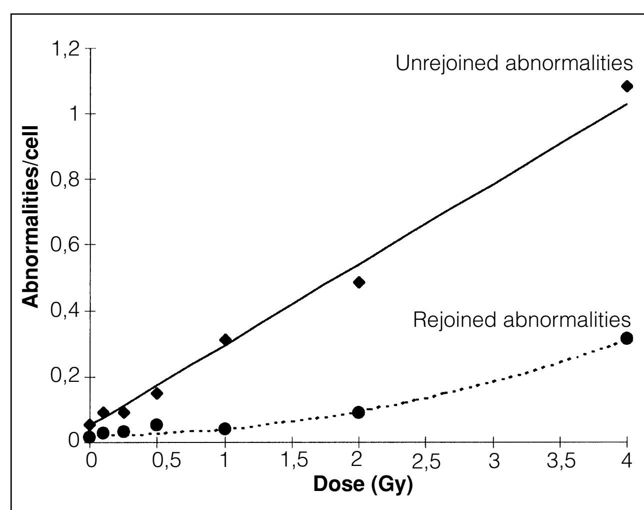

Figure 2. Induction of the different types of structural abnormalities in irradiated human sperm.

since we found that in this case the linear coefficient did not modulate the dose-response relationship (P = 0.454, Student's t test).

In human spermatozoa [111] and also in Chinese and Syrian hamster spermatozoa [112], following an acute exposure to low LET X– or gamma-radiation *in vitro*, the frequencies of unrejoined aberrations increase as a linear function of radiation dose ($Y = C + aD$). The linear curve includes a probability of breakage that is proportional to radiation dose, and a probability that the induced lesions will remain unrejoined. This probability is related to the ability of the cells to repair unrejoined abnormalities, and the linear curve assumes that this is a constant.

In contrast, the frequency of rejoined aberrations, which result from independently induced breaks and rejoining of two separate chromosome fragments increases as a linear-quadratic function of radiation dose ($Y = C + aD + bD^2$) in lymphocytes [114] as well as in human [115], and in Chinese and Syrian hamster [112] germ cells. In our study [111] the linear term was not present due to the lack of statistical meaning ($Y = C + bD^2$), showing a different kinetics in the induction of these abnormalities.

Most radiation-induced rejoined aberrations arise from the interaction of lesions present in both chromosomes participating in the exchange [116]. If rejoined aberrations are interpreted in the context of the linear quadratic dose-response model, the linear term (aD) describes the number of two-break abnormalities induced by single low-LET radiation tracks, while the quadratic term (bD^2) represents the proportion of two-break aberrations induced by two or more independent radiation tracks. Thus, from our results, it seems that in spermatozoa, rejoined aberrations were always induced by two or more independent radiation tracks. This difference could be due to the fact that nuclear volume is smaller in sperm cells than in lymphocytes. The greater nuclear diameter of the lymphocyte makes it more probable that in these cells a single gamma radiation particle will induce more than one break while going across the nucleus, thus increasing the probability of producing a rejoined aberration (aD term). Differences in the dose-response relationship for the induction of rejoined aberrations between this study and previous ones [112,115] could reflect the different systems used in detecting and scoring chromosomal abnormalities in the assessment of dose-effect curves. Tateno et al. [112] used uniform staining to score chromosome abnormalities; in these conditions, some rejoined abnormalities (e.g. translocations, inversions, marker chromosomes) can easily go unnoticed. Brandriff et al. [115] removed several kinds of abnormalities for the assessment of the dose-effect relationship in order to compare results from human sperm irradiated *in vitro* with human lymphocytes, which were analyzed using uniform stain.

Analysis of the breakpoints indicated that breakpoint distribution per chromosome was proportional to the chromosome DNA content [111]. None of the chromosomes showed a significantly higher or lower number of breaks than expected. Our results point to a random induction of breakpoints when irradiating human spermatozoa.

Induction of micronuclei

The analysis of human derived chromosomes at the first cleavage of the hybrid embryos, although useful, is time consuming. Therefore in our laboratory we have developed a new assay system, which is faster and gives an estimate of the frequency of structural and numerical chromosome aberrations. It consists of the analysis of micronuclei in two-cell human-hamster embryos. The technique used was that described by Kamiguchi et al. [117], adapted to our human-hamster interspecific fertilization system [108].

To ascertain whether the micronuclei present in the hybrid embryos were of human or of hamster origin, we hybridized them with either human or hamster genomic DNA probes. This experiment demonstrated that about 99% of micronuclei were of human origin. Furthermore, the frequency of spontaneous hamster micronuclei was determined. Only one micronucleus per 100 two-cell embryos was of hamster origin [118]. These results are consistent with the low frequency of structural chromosome abnormalities found in the hamster oocyte.

To establish a dose-effect curve for radiation-induced micronuclei, we performed the micronucleus test in two-cell human-hamster hybrid embryos after exposure of human spermatozoa to doses of 0, 0.10, 0.25, 0.50, 1.00, 2.00 and 4.00 Gy of gamma-rays. A total of 699 two-cell embryos were analyzed, 101 arising from the fertilization of hamster oocytes and non-irradiated spermatozoa, and 598 arising from irradiated spermatozoa. The induction of two-cell embryos with micronuclei increased with the dose of irradiation.

A linear relationship between the different doses of radiation and the induction of micronuclei was demonstrated (Fig. 3), although at the highest dose (4 Gy) this relationship showed a slight saturation effect.

To evaluate whether scoring micronuclei is useful for the quantification of chromosome damage induced in human spermatozoa, we compared the frequency of micronuclei per two-cell embryo (corrected for the estimated incidence of micronuclei of hamster origin) to the frequency of breaks and fragments per sperm derived complement at the different doses of irradiation. High correlations were observed between both parameters, indicating that the micronucleus test can be used to estimate chromosomal damage in human spermatozoa irradiated *in vitro* [119].

Although the micronucleus test can be used to evaluate the residual lesions left after occupational or accidental exposure to radiation, this test cannot be used to estimate the dose received at the testicular level due to the different radiosensitivities of spermatogenic cells.

Since micronuclei may contain acentric fragments [120] as well as centric fragments or whole chromosomes with damaged centromeres [121], and even groups of chromosomes that produce large micronuclei [122], we decided to carry out a FISH study with telomeric and centromeric probes for all human chromosomes to characterize the chromosomal content of micronuclei [123]. The results showed that over 75% of micronuclei were centromere-negative, indicating that they originated from acentric fragments. When double FISH was performed 100% of centromere-negative micronuclei were also telomere-positive, indicating the absence of interstitial acentric fragments. When double FISH was used to characterize centromere-positive micronuclei, two thirds of the micronuclei were also telomere-positive, indicating that they probably originated from dicentrics or from whole chromosomes. The rest were centromere-positive-telomere-negative micronuclei, derived from centric fragments or ring chromosomes.

To consider whether ionizing radiation induces aneuploidy or has mainly a clastogenic effect, results were evaluated in absolute frequencies (referred to the total number of embryos analyzed). All kinds of micronuclei increased with dose of radiation; however, centromere-negative micronuclei increased in a higher proportion than centromere-positive micronuclei (Fig. 4). Thus, this result supports the hypothesis that ionizing radiation has mainly a clastogenic effect by the induction of acentric fragments. However, although in a low proportion, ionizing radiation also induces aneuploidy through anaphase lag.

Induction of aneuploidy

An aneuploid individual usually arises at fertilization by the fusion of an abnormal gamete, which itself has resulted from a defect in meiosis. However, non-disjunction and the loss of mitotic chromosomes at the initial stages of embryogenesis

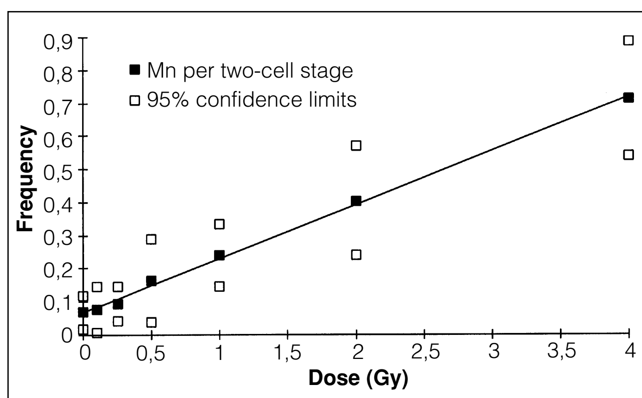

Figure 3. Dose-effect curve for the production of micronuclei (mn) by gamma-irradiation of human spermatozoa.

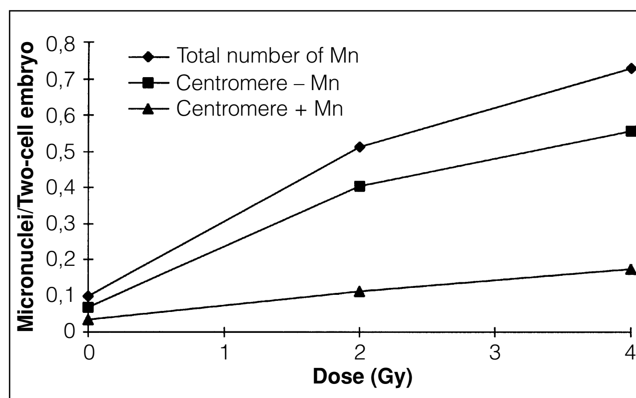

Figure 4. Induction of micronuclei (Mn) in two-cell human-hamster embryos by ^{60}Co gamma-rays.

may also result in trisomy, monosomy or chromosomal mosaicism.

A direct way to detect aneuploidy is to use FISH techniques with chromosome-specific DNA probes and to count the number of hybridization regions in interphase nuclei or metaphase spreads. In interphase cells this assay is unsuitable for detecting hypoploidy, while as much as 85% of the apparent monosomy could be due to probe overlap [124]. However, the use of chromosome-specific DNA probes in combination with the two-cell hybrid embryo stage overcomes the problem of probe overlap. An apparent case of nullisomy in one nucleus can be verified by the hybridization pattern of the sister nucleus. In the case of true nullisomy this sister nucleus must show a disomy of the same chromosome (if mitotic non-disjunction occurred) or a nullisomy of the same chromosome (if the fertilizing spermatozoon was hypoploid).

Here, we describe a new assay system which has been developed by combining two techniques, the interspecific fertilization between zona-free hamster oocytes and human spermatozoa, and the fluorescent *in situ* hybridization (FISH) technique using centromere specific DNA probes. Therefore, by tracing the marker chromosomes in two-cell hybrid embryos, reciprocal products of chromosome malsegregation can be easily traced. In this way, scoring of fluorescent spots in daughter nuclei and in micronuclei gives an estimate of the aneuploidy arising from meiosis, as well as the aneuploidy due to first mitotic division errors (both non-disjunction and anaphase lag).

To determine the baseline frequency of these numerical abnormalities we have analysed 226 two-cell embryos from one normal donor with centromeric DNA probes for chromosomes 4,7 and 18. We did not take into account those embryos showing more than one human chromosome complement because we cannot distinguish between an embryo originated by the penetration of a diploid spermatozoon and an embryo resulting from the fertilization with two normal ones. We found a two-cell embryo with mitotic non-disjunction for chromosome 18 and a micronucleus with a fluorescent signal of chromosome 7. Therefore, assuming that all chromosomes have the same probability of being involved in these processes, the frequency of non-disjunction of the first mitotic division is 3.2%. The frequency of anaphase lag is also 3.2% [125].

This test will be used to analyse the effects of physical or chemical agents on spermatozoa during the first embryonic division.

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About the authors

Since 1982, researchers at the Autonomous University of Barcelona have conducted research into the cytogenetic effects of ionizing radiations in humans, in both somatic and germinal cells. The group members belong to the departments of Biologia Cel·lular i Fisiologia (J. Egozcue, R. Álvarez, L. Barrios, A. Genescà, R. Miró, I. Ponsa and L. Tusell) and the Biologia Animal, Biologia Vegetal i Ecologia (J. F. Barquinero and M. R. Caballín).

Initially the research centred on the cytogenetic effects of ionizing radiations in lymphocytes and spermatozoa of subjects undergoing radiotherapy. This research led to the signing of two agreements with the Spanish Council of Nuclear Security in 1992, one to develop dose-effect calibration curves, which has established the reputation of the Unit of Biological Dosimetry, and the other to study the effects in human spermatozoa. Today, group members collaborate with a range of European laboratories including centres of reference in a number of countries such as the National Radiological Protection Board in the United Kingdom and the Institut für Strahlenbiologie of the GSF in Germany. In addition to the grants from the Council of Nuclear Security, the group has received other financial support from the European Commission as part of the Nuclear Fission Security Program. Josep Egozcue is a Member of the Institut d'Estudis Catalans (1992).