

Research Articles

Analysis by FISH of the Spectrum of Chromosome Aberrations Induced by X-Rays in G₀ Human Lymphocytes and Their Fate Through Mitotic Divisions in Culture

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The induction, distribution, and persistence of chromosome aberrations in human lymphocytes exposed to X-rays in G₀ were analyzed in 48-, 70-, and 94-hr cultures by conventional metaphase analysis and painting of chromosomes 1, 2, and 4 by FISH. All cells that had been scored by FISH were relocated to determine by differential staining of chromatids whether they had passed through 1, 2, or ≥ 3 divisions. FISH revealed a dose-dependent induction of stable and unstable aberrations, while chromatid labeling showed mitotic lag caused by irradiation in G₀. Relative to their DNA contents, there was a small but significant overrepresentation of chromosome 4 and underrepresentation of chromosome 2 among the aberrations involving chromosomes 1, 2, and 4. FISH slightly underestimated the genomic frequency of unstable aberrations measured by conventional metaphase analysis. There was a slight excess of translocations relative to dicentrics, but the data are com-

patible with the 1:1 ratio expected from cytogenetic theory. Many of the translocations were apparently incomplete (i.e., nonreciprocal). Incomplete translocations were more frequent at higher X-ray dose and in first division, suggesting that they may be associated with complex damage and are more apt to be lost in mitosis than complete translocations. Among the incomplete translocations, t(Ab) outnumbered t(Ba) — a difference ascribable to the FISH technique. Aberration frequencies declined as the cells divided in culture. The overall decline in the frequency of aberrant cells ($\approx 29\%$ per cell generation) reflects a rapid decline in dicentrics and fragments ($\approx 60\%$ per cell generation) and the relative stability of translocations. The frequency of translocation-bearing cells underwent a modest decline in culture ($\approx 13\%$ per cell generation). *Environ. Mol. Mutagen.* 33: 94–110, 1999 © 1999 Wiley-Liss, Inc.

Key words: chromosome aberrations; chromosome painting; FISH; human lymphocytes; translocations; X-rays

INTRODUCTION

Fluorescence in situ hybridization (FISH) using whole-chromosome painting probes has become a common method for analyzing chromosome aberrations in human populations [Lucas et al., 1989, 1992; Tucker et al., 1993b, 1994; Hoffmann, 1996; Snigiryova et al., 1997; Stephan and Pressl, 1997]. FISH permits the detection not only of the asymmetrical aberrations (dicentrics, centric rings, and their associated acentric fragments) scored in conventional metaphase analysis, but also allows for the convenient detection of symmetrical aberrations, most notably translocations. FISH shows that complex aberrations derived from more than two chromosome breaks are more common than would be anticipated from classical cytogenetics, and it greatly

facilitates their analysis [Tucker et al., 1995b; Savage, 1996].

Radiation-induced asymmetrical aberrations are unstable, in that they are likely to cause cell death in mitosis and are therefore lost from populations of dividing cells [Bauchinger et al., 1986]. In contrast, symmetrical aberrations are relatively stable and can persist in a cell population. A

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longstanding assumption in radiation cytogenetics is that ionizing radiation induces dicentric chromosomes and reciprocal translocations in a 1:1 ratio in G_0 cells, because dicentrics and reciprocal translocations occur as alternative outcomes resulting from the random rejoining of two broken chromosomes [Sax, 1938; Heddle, 1965; Savage and Papworth, 1982; Tucker et al., 1993b; Natarajan et al., 1996; Stephan and Pressl, 1997]. Although dicentrics and translocations are expected to occur at equal frequency in the first cell division after their induction, the ratio is expected to change in subsequent cell divisions because dicentrics are unstable, leading to cell death when the two centromeres segregate improperly. Translocations, on the other hand, are not inherently disruptive to the mitotic process. The ease of detection of dicentrics has led to their being widely used as an indicator of clastogenic effects in classical cytogenetics. In contrast, the stability of translocations enables their detection long after induction, so long as appropriate methodology, such as FISH, is used to quantify them. Stable aberrations are therefore more suitable indicators of damage in chronic exposures or long after exposure and have been increasingly prominent in epidemiological analyses and dosimetry [Kleinerman et al., 1989; Tucker et al., 1993b; Stephan and Pressl, 1997].

Frequencies of experimentally induced chromosome aberrations are typically measured in the first mitotic division after their induction, as aberration frequencies measured in later mitotic divisions are a function both of the induction and the persistence of the aberrations through the division process. Understanding the relative stability of different classes of chromosome aberrations through mitotic divisions is therefore important to the interpretation of aberration frequencies in heterogeneous populations of dividing cells and in individuals long after radiation exposure.

In this study, we used FISH painting of chromosomes 1, 2, and 4 to characterize the chromosome aberrations in first, second, and later mitotic divisions of human lymphocytes that had been irradiated with X-rays in G_0 . After FISH analysis of cells from 48-, 70-, and 94-hr cultures, we restained the slides to distinguish numbers of divisions by differential chromatid labeling, a technique that has been extensively used to ensure that only first-division cells are scored in conventional metaphase analysis [Kleinerman et al., 1989] and to measure frequencies of sister chromatid exchange [Tucker et al., 1993a]. Through automated metaphase finding, we relocated each cell that we had analyzed by FISH (680 aberrant cells and 2,857 normal cells) and determined from the pattern of chromatid labeling the number of times that it had divided in culture. Thus, we were able to ascertain directly the frequencies of different classes of chromosome aberrations in cells that had divided a known number of times and determine the persistence of aberrations through mitotic divisions. We also compared the distribution of aberrations in first-division cells to data from

conventional scoring of unstable chromosome aberrations on Giemsa-stained slides from the same cultures.

MATERIALS AND METHODS

Chemicals and Media

5-Bromodeoxyuridine (BUdR) was purchased from Calbiochem (La Jolla, CA); Histopaque from Sigma Chemical Co. (St. Louis, MO); phytohemagglutinin (PHA) from Murex Diagnostics (Norcross, GA); and penicillin-streptomycin, RPMI Medium 1640, and fetal bovine serum (FBS) from GIBCO (Grand Island, NY). The culture medium was RPMI 1640 supplemented with 15% FBS, 1.0% PHA, penicillin (100 units/ml), streptomycin (100 μ g/ml), and 45 μ M BUdR.

Blood Collection and Irradiation

Approximately 60 ml of blood was collected into sterile vacutainers from a healthy adult female by venipuncture and maintained at ambient temperature until culturing. The whole blood was transferred to plastic centrifuge tubes for irradiation. Blood samples (15 ml) were exposed to 0, 1.5, or 3.0 Gy 220 kv X-rays from a Phillips MCN225 X-ray tube at a dose rate of 0.55 Gy/min. The irradiated blood was allowed to sit in 50 ml centrifuge tubes at ambient temperatures for 2 hr before separation of lymphocytes.

Lymphocyte Separation and Culture

The separation of lymphocytes from whole blood was performed as described by McFee et al. [1997]. Irradiated and control blood samples were subjected to density gradient centrifugation in Histopaque, and the monocyte cell layer was washed in PBS. For each culture, approximately 3×10^6 viable lymphocytes were inoculated into 10 ml of culture medium. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Colchicine was added at 0.2 μ g/ml 3 hr before harvest. Cultures were harvested at 48, 70, and 94 hr after mitogen stimulation. The harvest, as described by McFee et al. [1997], involved centrifugation, suspension of the cells in hypotonic solution (0.075 M KCl) for 15 min at 37°C, addition of 10–15 drops fixative (3 methanol: 1 acetic acid) per 15-ml tube, centrifugation, and resuspension in fixative. After another wash in fixative, the fixed cells were stored in 15 ml fixative at $-20^\circ C$ until slide making.

Slide Preparation

Cell suspensions stored at $-20^\circ C$ were centrifuged and resuspended in fresh fixative at appropriate cell density for slide making. Slides were prepared by dispensing 20 μ l aliquots of the suspensions into a film of water on a clean slide as described by McFee et al. [1997].

FISH

FISH was performed with whole chromosome painting probes, following the instructions of the supplier (Vysis, Downers Grove, IL) modified as described by McFee et al. [1997]. Direct-label probes conjugated with SpectrumOrange fluorophores were used to paint chromosomes 1, 2, and 4. The chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) prepared in a p-phenylenediamine dihydrochloride/glycerol antifade solution [Johnson and Nogueira Araujo, 1981].

Probed slides were illuminated with appropriate wavelength UV light using dual-band pass filters to permit simultaneous viewing of the probed chromosome pairs stained with SpectrumOrange and other chromosomes stained by the DAPI counterstain. The SpectrumOrange probe stains chro-

mosomes 1, 2, and 4 to slightly different intensities, and DAPI differentially binds to centromeric heterochromatin, conferring bright blue fluorescence on the centromeres. The three painted chromosome pairs (1, 2, and 4) are therefore easily distinguished from one another by differences in chromosome size, centromere position, paint intensity, and intensity of the DAPI-counterstained centromeres. The chromosomal origin of painted fragments and rearranged segments is discernible in most aberrant cells. The probe and counterstain thereby permit excellent resolution of both asymmetrical and symmetrical exchanges involving chromosomes 1, 2, or 4 with any nonpainted chromosome.

Conventional Scoring

To compare FISH data with traditional metaphase analysis, conventional scoring of unstable aberrations was performed using slides from the same cultures as were used in the FISH analysis, but stained by a modification of the fluorescence plus Giemsa technique [Littlefield et al., 1987]. This comparison was limited to first-division metaphases.

Classifying Divisions by Differential Chromatid Labeling

Differential chromatid labeling was used to distinguish cells that had undergone 1, 2, 3, or more divisions since stimulation with PHA. After culturing in the presence of BUdR, metaphase preparations were stained by a modification of the fluorescence plus Giemsa technique [Perry and Wolff, 1974; Littlefield et al., 1979] in which staining by Hoechst dye 33258 (bisbenzimid H33258) was followed by DAPI (4',6-diamidino-2-phenylindole) rather than Giemsa. When metaphases stained by the fluorescence plus DAPI technique are viewed with fluorescence microscopy, singly BUdR-substituted chromatids are bright blue, whereas doubly substituted chromatids are dull gray. Numbers of divisions that the cells have undergone are readily distinguished: 1st division (all blue); 2nd division (harlequin); 3rd division ($\approx 25\%$ blue); >3 divisions (largely gray). Proportions of cells in their first, second, and third or higher division were determined from 300 metaphases in each treatment group (48-, 70-, and 94-hr cultures treated with 0, 1.5, or 3 Gy X-rays). Proliferation indices were calculated from these proportions as follows: P.I. = $[(1 \times \text{number of 1st division metaphases}) + (2 \times \text{number of 2nd division metaphases}) + (3 \times \text{number of 3rd division metaphases}) + (4 \times \text{number of } >3\text{rd division metaphases})] / \text{number of cells scored}$.

The same differential staining procedure was used to stain chromatids after the scoring of aberrations by FISH. The presence of 45 μM BUdR in the cultures did not interfere with the FISH technique, and the chromosome painting probe did not interfere with the fluorescence plus DAPI technique. Cells could readily be assigned to 1st, 2nd, or ≥ 3 rd division in previously painted and scored slides.

Scoring Slides for Chromosome Aberrations

Probed slides were scanned by an automated metaphase finder (AKS 500 Metaphase Location System) on a Leitz Ergolux microscope to locate high-quality metaphases. Metaphases were chosen for analysis only if they were visually complete, containing all six painted chromosomes and approximately 46 total chromosomes. The positions of all metaphases scored were automatically recorded so that the identical cells could later be relocated for determining whether they had undergone 1, 2, or ≥ 3 cell divisions on the basis of differential chromatid staining.

Painted chromosomes in the 1.5- and 3.0-Gy treatment groups were scored for aberrations up to a predetermined number of aberrant cells: 60 in 48-hr cultures, 140 in 70-hr cultures, and 140 in 94-hr cultures. All aberrations involving painted chromosomes were recorded using the PAINT system of nomenclature [Tucker et al., 1995b], with the exception

that translocations were tabulated by the method recommended by Finnon et al. [1995]. Thus, a bicolored dicentric in a cell containing a bicolored acentric fragment (i.e., $\text{dic}(\text{AB}) + \text{ace}(\text{ab})$) or a pair of bicolored translocations (i.e., $\text{t}(\text{Ab}) + \text{t}(\text{Ba})$) was interpreted as a single complete (i.e., reciprocal) aberration. Rearrangements occurring singly in cells without their reciprocal counterparts (i.e., $\text{dic}(\text{AB})$, $\text{ace}(\text{ab})$, $\text{t}(\text{Ab})$, or $\text{t}(\text{Ba})$) were recorded as individual incomplete (i.e., nonreciprocal) exchanges.

Extrapolations

We adapted the method of Lucas et al. [1989, 1992] to predict frequencies of aberrations for the total genome on the basis of the frequencies measured by FISH. We assumed that the proportion of total aberrations detected by painting chromosome pairs 1, 2, and 4 reflects the probability of an exchange between the painted fraction (fp) and nonpainted fraction (1-fp) of the genome based on the relative lengths of the painted and unpainted regions. Thus, the proportion of interchanges that occur between painted and unpainted portions of the genome is given by $2(\text{fp})(1-\text{fp})$. Chromosome pairs 1, 2, and 4 represent 22.34% of the DNA content of the human genome in a female and 22.70% in a male [derived from Morton, 1991]. Thus, the labeling of chromosomes 1, 2, and 4 in cells from a female donor is expected to detect as paint–nonpaint interchanges about 34.7% of the chromosomal interchanges occurring in the complete genome, assuming that the induction and persistence of aberrations is distributed randomly among the chromosomes. Thus, one may estimate the genomic aberration frequency as approximately 2.88 times that detected in painted chromosomes 1, 2, and 4. If one includes paint–paint dicentrics among the aberrations scored, one would expect that these would represent roughly half the total paint–paint aberrations and therefore be approximated by 0.5 fp^2 . Including these dicentrics, the frequency of genomic aberrations detected by painting chromosomes 1, 2, and 4 is increased to 37.2%, and the genomic aberration frequency is given by 2.69 times that in the painted chromosomes.

RESULTS

Cell Proliferation Kinetics

Metaphases from each treatment group (0, 1.5, and 3.0 Gy in 48, 70, and 94 hr) were stained using the fluorescence plus DAPI technique to evaluate lymphocyte proliferation kinetics on the basis of the differential staining of BUdR-substituted chromatids. The data in Table I show the proliferation indices and the distribution of metaphases among divisions. In the 48-hr cultures, over 99% of the metaphases were in the first division after mitogen stimulation. By 70 hr, most cells had passed into their second division, and by 94 hr most had divided three or more times. The proliferation indices show both the progression of cells through divisions and mitotic lag caused by the X-ray exposures. Lag is indicated by a dose-dependent reduction in numbers of cell divisions that have taken place after the 1.5 and 3 Gy exposures.

Aberration Frequencies

Chromosome aberrations were tabulated by scoring FISH-painted cells in the 48-, 70-, and 94-hr cultures. After scoring by FISH, the slides were restained by the fluorescence plus DAPI technique, and every cell that had been

TABLE I. Proliferation Kinetics in Human Lymphocytes Exposed to X-Rays in G₀ and Cultured for 48, 70, or 94 Hr

X-Rays (Gy)	Proliferation index ^a and (proportion of cells having undergone 1, 2, or ≥3 divisions) ^b		
	48-hr cultures	70-hr cultures	94-hr cultures
0	1.00 (99.7—0.3—0.0)	2.34 (12.0—44.3—43.6)	3.21 (4.7—13.7—81.7)
1.5	1.00 (99.7—0.3—0.0)	2.15 (17.7—50.7—31.7)	3.12 (5.0—17.3—77.6)
3.0	1.00 (100.0—0.0—0.0)	1.89 (30.7—49.7—19.6)	2.86 (11.0—22.3—66.7)

^aProliferation index = [(1 × 1st division cells) + (2 × 2nd division cells) + (3 × 3rd division cells) + (4 × cells having undergone >3 divisions)] ÷ Σ cells.

^bDetermined from 300 metaphases on the basis of differential staining of BUdR-substituted chromatids.

TABLE II. Dose-Dependent Induction by X-Rays of Chromosome Aberrations in G₀ Human Lymphocytes Detected by FISH Painting of Chromosome Pairs 1, 2, and 4 in First Division Metaphases

A. Proportions of cells bearing chromosome aberrations			
Aberrations	% Cells affected (aberrant cells/cells scored)		
	Control	1.5 Gray	3.0 Gray
Translocations [Σt]	0.33 (2/600)	10.89 (66/606)	29.47 (94/319)
Dicentric [dic(AB) + dic(BB)]	0.00 (0/600)	9.24 (56/606)	29.78 (95/319)
Centric rings [r(B)]	0.00 (0/600)	1.49 (9/606)	4.39 (14/319)
Insertions [Aba & Bab]	0.00 (0/600)	0.33 (2/606)	3.13 (10/319)
Excess acentrics [ace + r(b)]	0.00 (0/600)	3.63 (22/606)	14.10 (45/319)
Σ Aberrations in 1, 2, & 4	0.33 (2/600)	20.30 (123/606)	50.47 (161/319)
B. Aberrations per cell			
Aberrations	Aberrations per cell (aberrations/cells scored)		
	Control	1.5 Gray	3.0 Gray
Translocations [Σt]	0.33 (2/600)	0.120 (73/606)	0.351 (112/319)
Dicentric [dic(AB) + dic(BB)]	0.00 (0/600)	0.097 (59/606)	0.329 (105/319)
Centric rings [r(B)]	0.00 (0/600)	0.015 (9/606)	0.044 (14/319)
Insertions [Aba & Bab]	0.00 (0/600)	0.003 (2/606)	0.031 (10/319)
Excess acentrics [ace + r(b)]	0.00 (0/600)	0.041 (25/606)	0.147 (47/319)
Σ Aberrations in 1, 2, & 4	0.33 (2/600)	0.277 (168/606)	0.903 (288/319)

scored was relocated to determine on the basis of differential chromatid staining whether it had undergone one, two, or three or more mitotic divisions. The aberrations tabulated for 48-, 70-, and 94-hr cultures were then retabulated on the basis of first, second, or higher divisions.

The data in Table II show a dose-dependent increase in

the frequency of both stable and unstable chromosome aberrations in first division metaphases. The increases are presented as proportions of cells bearing chromosome aberrations (Table IIA) and numbers of aberrations per cell (Table IIB). The unirradiated control showed a low frequency of aberrations compatible with historical controls. At the higher irradiation exposure (3 Gy), approximately half the cells bore detectable aberrations in chromosome pairs 1, 2, and 4, and the frequency of aberrations detected was 0.9 per cell.

Table III shows a detailed classification of the aberrations induced in chromosomes 1, 2, and 4 by 1.5 Gy (Table IIIA) or 3.0 Gy (Table IIIB) X-rays. For both doses, the aberrations are separated into groups defined by the cells having undergone 1, 2, or ≥3 divisions, as revealed by differential chromatid staining. Translocations are classified into t(Ab), in which a painted piece is translocated onto an unpainted chromosome (i.e., having an unpainted centromere), and t(Ba), in which chromosomal material identified by the DAPI counterstain is translocated onto a painted chromosome. The translocations are classified as complete when a t(Ab) translocation is accompanied by a reciprocal t(Ba) translocation in the same cell. All dicentrics involving one of the painted chromosomes were scored as to whether the other chromosome involved was unpainted (i.e., dic(AB)) or painted (i.e., dic(BB)). Dicentrics were considered to be complete when a dic(AB) was accompanied by a bicolored fragment and when a dic(BB) was accompanied by a painted fragment.

The aberrations were totaled in several ways. The sum of paint-detected events includes all aberrations involving one of the three painted chromosome pairs. Painted acentric fragments, painted rings, and BB dicentrics are included in this sum, even though they do not involve color junctions. Total numbers of color junctions are also shown. Of aberrations detected in chromosomes 1, 2, and 4 in first-division cells, 76% (523/688) involved color junctions. There was no difference in this respect between aberrations induced by 1.5 and 3.0 Gy. The proportion of aberrations involving color junctions increased to 86% (324/376) in second-division cells and 91.4% (381/417) in cells that had undergone three or more divisions. The increasing proportion of color junctions is ascribable to the instability of fragments, rings, and dicentrics relative to translocations, which is the largest class of events involving color junctions.

The total of all aberrations in chromosomes 1, 2, and 4 is calculated in a way that facilitates comparison to classical scoring. Reciprocal events (e.g., dicentric plus fragment or t(Ab) plus t(Ba) translocation) are considered a single aberration in this tabulation. The sum of aberrations is therefore given by the sum of translocations plus dicentrics, centric rings, insertions, and excess acentric fragments (i.e., those not accounted for by a dicentric or ring as its reciprocal product). Cells were

TABLE III. Classification of X-Ray-Induced Aberrations in Chromosomes 1, 2, and 4 in Cells Having Undergone 1, 2, or ≥ 3 Divisions After Irradiation in G_0

Cytogenetic events	A. Aberrations induced by 1.5 Gy X-rays					
	1st Division		2nd Division		≥ 3 rd Division	
	Cells affected (n = 606)	Number of events	Cells affected (n = 604)	Number of events	Cells affected (n = 1,351)	Number of events
Translocations						
Σ translocations	66	73	61	66	108	113
t(Ab)	59	63	53	57	96	99
t(Ba)	45	49	49	52	73	75
Complete translocations	37	39	41	43	60	61
Dicentrics						
Σ dicentrics	56	59	21	22	17	17
dic(AB)	46	49	18	19	14	14
dic(BB)	10	10	3	3	3	3
Complete dicentrics	34	34	7	8	3	3
Rings						
Centric rings: r (B)	9	9	2	2	5	5
Acentric rings: r (b)	4	4	3	4	1	1
Fragments						
Partially painted: ace (ab)	28	29	11	15	3	5
Painted: ace (b)	40	48	8	11	9	12
Excess acentrics (ab & b)	18	21	13	16	9	13
Insertions						
Aba & aba	2	2	3	3	3	3
Bab & bab	0	0	1	1	1	1
Chromosome involved						
Chromosome 1	—	56	—	31	—	47
Chromosome 2	—	42	—	29	—	38
Chromosome 4	—	53	—	34	—	50
Unidentifiable	—	1	—	4	—	7
Paint overview						
Σ paint-detected events	123	263	87	167	130	218
Σ color junctions	105	195	76	149	119	201
Total aberrations*						
Σ aberrations in 1, 2 & 4	123	168	87	114	130	153
Complex & multiple damage	24	24	16	16	17	17
B. Aberrations induced by 3.0 Gy X-rays						
Cytogenetic events	1st Division		2nd Division		≥ 3 rd Division	
	Cells affected (n = 319)	Number of events	Cells affected (n = 332)	Number of events	Cells affected (n = 325)	Number of events
Translocations						
Σ translocations	94	112	61	75	71	83
t(Ab)	78	86	51	57	69	79
t(Ba)	57	65	40	49	55	61
Complete translocations	37	37	29	31	53	57
Dicentrics						
Σ dicentrics	95	105	33	37	18	18
dic(AB)	88	98	29	32	16	16
dic(BB)	7	7	4	5	2	2
Complete dicentrics	57	60	17	20	8	8
Rings						
Centric rings: r (B)	14	14	5	5	6	6
Acentric rings: r (b)	10	10	3	4	2	2
Fragments						
Partially painted: ace (ab)	55	60	16	25	8	11
Painted: ace (b)	67	75	20	26	11	15
Excess acentrics (ab & b)	35	37	18	25	10	14

TABLE III. Continued

B. Aberrations induced by 3.0 Gy X-rays <i>Continued</i>						
Cytogenetic events	1st Division		2nd Division		≥3rd Division	
	Cells affected (n = 319)	Number of events	Cells affected (n = 332)	Number of events	Cells affected (n = 325)	Number of events
Insertions						
Aba & aba	7	7	4	4	2	4
Bab & bab	3	3	2	2	3	3
Chromosome involved						
Chromosome 1	—	99	—	45	—	35
Chromosome 2	—	61	—	43	—	41
Chromosome 4	—	71	—	29	—	34
Unidentifiable	—	1	—	4	—	3
Paint overview						
Σ paint-detected events	161	425	91	209	88	199
Σ color junctions	144	328	80	175	79	180
Total aberrations*						
Σ aberrations in 1, 2 & 4	161	288	91	152	88	130
Complex & multiple damage	65	65	26	26	26	26

*Σ aberrations = Σ translocations + Σ dicentric + Σ rings + Σ insertions + excess acentrics (ab & b); complex & multiple aberrations entail rejoining of ≥3 breaks involving ≥2 chromosomes.

considered to have complex or multiple damage if the aberrant chromosomes 1, 2, and 4 could only arise by a rejoining of ≥3 breaks involving ≥2 chromosomes. Complex and multiple damage was observed in 4.0% (24/606) of all first-division cells and in 19.5% (24/123) of aberrant first-division cells at 1.5 Gy. At 3.0 Gy, complex and multiple damage affected 20.4% (65/319) of all first-division cells and 40.4% (65/161) of aberrant first-division cells. The proportion was lower in subsequent divisions, owing to the loss of aberrant cells containing at least one unstable aberration.

Chromosome Comparisons

Table IV analyzes the distribution of aberrations among the three painted chromosome pairs. All but 21 of 861 aberrant chromosomes detected (i.e., 97.6%) could be assigned to a specific painted chromosome (i.e., chromosome 1, 2, or 4) on the basis of chromosome morphology, differences in staining intensity, and centromere staining with DAPI. The data show a slight overrepresentation of chromosome 4 and a slight underrepresentation of chromosome 2. Expected numbers of aberrations were calculated on the basis of the DNA content of the chromosomes [Morton, 1991]. Since chromosomes 1, 2, and 4 represent 8.15, 7.90, and 6.29% of the DNA in the human female karyotype, respectively, they are expected to represent 36.48, 35.36, and 28.16% of aberrations involving these chromosomes if the aberrations are randomly distributed.

The data show a significant deviation from randomness ($P < 0.01$). There is a significant overrepresentation of chromosome 4 and underrepresentation of chromosome 2

TABLE IV. Nonrandomness of X-Ray-Induced Damage Among Chromosomes 1, 2, and 4 in Human Lymphocytes Irradiated in G₀

Data & chi-square analysis	Aberrant chromosome			Total
	1	2	4	
Pooled data for 1.5 & 3 Gy X-rays				
Aberrations at 48 hr	50	44	49	143
Aberrations at 70 hr	144	109	108	361
Aberrations at 94 hr	121	101	114	336
Total aberrations induced	315	254	271	840
Chi-square test				
Observed totals	315	254	271	840
Expected totals*	306.43	297.02	236.55	840
Deviation from expected	+8.57	-43.02	+34.45	0
d ² /E	0.24	6.23	5.02	11.49
Chi-square & P-value:	$\chi^2 = 11.49$			$P < 0.01$

*Expected numbers of aberrations are calculated on the basis of the DNA content of the chromosomes [Morton, 1991].

among the aberrations induced by X-rays in these three chromosome pairs.

Genomic Equivalents

We used the data from FISH to estimate frequencies of aberrations for the total genome by adapting the method of Lucas et al. [1989, 1992] as described in the Methods section. Genomic frequencies were calculated by variations of the equation $F_p = F_g [2 (f_p) (1-f_p)]$, where F_p is the frequency of aberrations detected by FISH, F_g the frequency for the entire genome, f_p the painted fraction of the genome, and $1-f_p$ the unpainted fraction. The equation was modified to include the scoring of paint-paint dicentric

TABLE V. Prediction of Genomic Frequencies of Chromosome Aberrations Induced by X-Rays in G₀ Human Lymphocytes from Data on Painted Chromosome Pairs 1, 2, and 4^a

Aberrations	Aberrations per cell in first-division metaphases ^b					
	1.5 Gray			3.0 Gray		
	Detected by FISH	Genomic equivalent	Conventional scoring	Detected by FISH	Genomic equivalent	Conventional scoring
Unstable aberrations						
Dicentrics (AB & BB)	0.097	0.244	0.289	0.329	0.829	0.889
Centric rings	0.015		0.031	0.044		0.117
Excess acentrics	0.042		0.145	0.147		0.371
Stable aberrations						
Insertions	0.003		—	0.031		—
Translocations [Σt]	0.120	0.346	—	0.351	1.011	—
Unstable aberrations	0.154	0.388	0.465	0.520	1.310	1.377
Total aberrations	0.277	0.745	—	0.902	2.426	—

^aGenomic frequencies were calculated by an adaptation of the method of Lucas et al. [1989, 1992] using chromosomal DNA contents from Morton [1991] as described in the text.

^bFirst-division metaphases were pooled from 48-, 70-, and 94-hr cultures. The data from FISH are based on 606 metaphases at 1.5 Gy and 319 at 3.0 Gy. The data from conventional scoring are based on 325 metaphases at 1.5 Gy and 315 at 3.0 Gy.

TABLE VI. Complete and Incomplete Aberrations Among the Chromosomal Damage Induced in G₀ Human Lymphocytes by X-Rays and Detected by FISH Painting of Chromosomes 1, 2, and 4

A. Proportion of dicentric chromosomes that were part of a reciprocal exchange				
X-Ray Dose (Gy)	Divisions in culture			Σ
	1st	2nd	≥3rd	
1.5	34/59	8/22	3/17	45/98 = 0.46
3.0	60/105	20/37	8/18	88/160 = 0.55
Σ	94/164 = 0.57	28/59 = 0.47	11/35 = 0.31	133/258 = 0.52

B. Proportion of translocations that were part of a reciprocal exchange				
X-Ray Dose (Gy)	Divisions in culture			Σ
	1st	2nd	≥3rd	
1.5	39/73	43/66	61/113	143/252 = 0.57
3.0	37/112	31/75	57/83	125/270 = 0.46
Σ	76/185 = 0.41	74/141 = 0.52	118/196 = 0.60	268/522 = 0.51

C. Relative proportions of t(Ab) and t(Ba) color junctions								
Dose (Gy)	Division	Cells scored	t(Ab) translocations	t(Ba) translocations	t(Ab)/t(Ba) ratio	Deviation from 1:1		
						χ ²	P	Significance
1.5	1st	606	63	49	1.29	1.75	0.2 > P > 0.1	NS
	2nd	604	57	52	1.10	0.23	0.9 > P > 0.5	NS
	≥3rd	1351	99	75	1.32	3.31	0.1 > P > 0.05	NS
	Σ	2561	219	176	1.24	4.68	0.05 > P > 0.01	*
3.0	1st	319	86	65	1.32	2.92	0.1 > P > 0.05	NS
	2nd	332	57	49	1.16	0.60	0.5 > P > 0.3	NS
	≥3rd	325	79	61	1.30	2.31	0.2 > P > 0.1	NS
	Σ	976	222	175	1.27	5.56	0.02 > P > 0.01	*
Σ	Σ	3537	441	351	1.26	10.23	P < 0.01	**

(dicBB). We estimated genomic frequencies as Fg = 2.88Fp for translocations, 2.52Fp for dicentrics (AB + BB), and 2.69Fp for total aberrations, including both translocations and dicentrics. We compared these extrapolated values

to data obtained by conventional scoring of Giemsa-stained slides from the same cultures.

The results shown in Table V show that the FISH painting of chromosomes 1, 2, and 4 slightly underestimates

genomic frequencies of unstable aberrations measured by conventional metaphase analysis.

Aberration Comparisons

According to classical cytogenetic theory, reciprocal translocations and dicentric chromosomes result from the rejoining of two breaks involving two chromosomes. It might be anticipated on this basis that each t(Ab) translocation would be accompanied by a reciprocal t(Ba) product and that each dic(AB) dicentric would be accompanied by a bicolored fragment.

The data in Table VI show that in addition to complete (i.e., reciprocal) aberrations there are many incomplete (nonreciprocal) aberrations. Dicentrics were considered complete when a dic(AB) was accompanied by an ace(ab) fragment or a dic(BB) was accompanied by an ace(b) fragment. Translocations were considered complete when a t(Ab) color junction was accompanied by a t(Ba) color junction involving the same painted chromosome. The data show that a large fraction of the dicentrics ($\approx 48\%$) and translocations ($\approx 49\%$) detected by FISH appeared to be incomplete, even though complete aberrations are expected on the basis of cytogenetic theory.

Incomplete dicentrics (Table VIA) are more frequent in later metaphases than in first division (Fisher's exact test: $P = 0.008^{**}$). This difference undoubtedly stems from the instability of both the dicentrics and the fragment in mitotic divisions. An initially complete dicentric would be scored as incomplete in a subsequent division if the dicentric were transmitted but the fragment were lost.

There was a larger proportion of incomplete translocations (Table VIB) in first-division metaphases than after ≥ 3 divisions (Fisher's exact test: $P = 0.0002^{***}$). The difference suggests that incomplete translocations are more apt to be lost in mitotic divisions than complete translocations. A higher proportion of incomplete translocations was also observed at the higher X-ray dose (Fisher's exact test: $P = 0.018^*$), perhaps corresponding to a higher frequency of cells having multiple sites of damage.

A complete translocation detected by FISH is comprised of its t(Ab) and t(Ba) components. An assumption of reciprocity of translocation events and equal detectability of both products by FISH leads to an expectation of a 1:1 ratio of t(Ab) chromosomes to t(Ba) chromosomes. The data (Table VIC), however, reveal a significant excess of t(Ab) translocations relative to t(Ba) translocations. The disparity was observed at both 1.5 and 3 Gy and among cells that had undergone 1, 2, or ≥ 3 divisions. The excess of t(Ab) translocations was highly significant when the sample size offered sufficient statistical power.

On the basis of cytogenetic theory, one might anticipate a 1:1 ratio of translocations to dicentrics, since reciprocal translocations and dicentrics are alternate outcomes from the random rejoining of two breaks in two chromosomes.

We observed a slight excess of translocations relative to dicentrics ($t/dic = 1.06$), but the excess was not statistically significant. Pooling our data for 1.5 Gy and 3 Gy (Table III), we observed 160 first-division cells with translocations and 151 first-division cells with dicentrics among 925 cells scored (Fisher's exact test: $P = 0.62$). The differences when the two doses were considered separately or when the analysis was restricted to complete aberrations were similarly nonsignificant. The data are therefore consistent with the theoretical 1:1 ratio.

Besides comparing a genomic frequency of unstable aberrations calculated from the FISH data to frequencies measured directly by traditional scoring, we compared the ratio of centric rings to dicentric chromosomes measured by the two methods. For aberrations induced by 1.5 Gy the yield of centric rings in first-division cells was 15.2% that of dicentrics when scored by FISH (Table III) and 10.7% in classical scoring (Table V). The corresponding values for 3 Gy were 13.3% and 13.2%, respectively.

Persistence of Aberrations

Table VII shows the change in the frequency of aberration-bearing cells as they pass from first division through second and third divisions in culture. The data for cells exposed to 1.5 Gy (Table VIIA) and 3.0 Gy (Table VIIB) are shown separately, even though the stability of specific aberrations should be independent of the dose that induced them. We did not pool the dosages, as frequencies of complex aberrations and multiple damage are greater at higher doses and may influence the loss of cells bearing aberrations. The data show that dicentric chromosomes, fragments, and rings are highly unstable, being quickly lost from the dividing cell population. In contrast, translocation-bearing cells are more stable, persisting in the cell population at higher frequencies.

The decline in aberration-bearing cells is analyzed in Table VIII. There is a highly significant decline in the proportion of cells carrying dicentric chromosomes (Table VIIIA). The decline is significant at each successive division at both doses. This rapid decline reflects the instability of dicentric chromosomes, which cause lethal mitotic hindrance. Translocations also decline in frequency as the cells pass through divisions in culture, but not so sharply as dicentrics. A small but significant decrease in the frequency of translocation-bearing cells was observed at both X-ray doses (Table VIIB). The data are most compatible with a modest decline in translocation frequency over time, though translocations are certainly stable relative to dicentrics.

The data for 1.5 and 3 Gy are consistent in showing a decline in cells bearing dicentrics of almost 60% per cell generation (Table VIIC). Acentric fragments, having no regular means of mitotic segregation, are also unstable. The decline in fragment-bearing cells was also about 60% per cell generation. The decline in both dicentrics and fragments

TABLE VII. Persistence of Cells Containing Chromosome Aberrations Induced by X-Rays in G₀ Human Lymphocytes and Detected by FISH Painting of Chromosomes 1, 2, and 4 in Cell Cultures

Cytogenetic events	Percentage aberrant cells		
	First division	Second division	Three or more divisions
A. Aberrations induced by exposure to 1.5 Gy X-rays			
Translocations			
Σ translocations	10.9 (66/606)	10.1 (61/604)	8.0 (108/1351)
Complete translocations	6.1 (37/606)	6.8 (41/604)	4.4 (60/1351)
Dicentric			
Σ dicentric	9.2 (56/606)	3.5 (21/604)	1.3 (17/1351)
Complete dicentric	5.6 (34/606)	1.2 (7/604)	0.2 (3/1351)
Rings			
Centric rings: r (B)	1.5 (9/606)	0.3 (2/604)	0.4 (5/1351)
Acentric rings: r (b)	0.7 (4/606)	0.5 (3/604)	0.1 (1/1351)
Fragments			
Partially painted: ace (ab)	4.6 (28/606)	1.8 (11/604)	0.2 (3/1351)
Painted: ace (b)	6.6 (40/606)	1.3 (8/604)	0.7 (9/1351)
Insertions			
Aba & aba	0.3 (2/606)	0.5 (3/604)	0.2 (3/1351)
Bab & bab	0.0 (0/606)	0.2 (1/604)	0.1 (1/1351)
Summary of aberrant cells			
% cells with aberrant 1, 2, or 4	20.3 (123/606)	14.4 (87/604)	9.6 (130/1351)
% cells with color junctions	17.3 (105/606)	12.6 (76/604)	8.8 (119/1351)
% cells with multiple damage	4.0 (24/606)	2.6 (16/604)	1.3 (17/1351)
B. Aberrations induced by exposure to 3.0 Gy X-rays			
Translocations			
Σ translocations	29.5 (94/319)	18.4 (61/332)	21.8 (71/325)
Complete translocations	11.6 (37/319)	8.7 (29/332)	16.3 (53/325)
Dicentric			
Σ dicentric	29.8 (95/319)	9.9 (33/332)	5.5 (18/325)
Complete dicentric	17.9 (57/319)	5.1 (17/332)	2.5 (8/325)
Rings			
Centric rings: r (B)	4.4 (14/319)	1.5 (5/332)	1.8 (6/325)
Acentric rings: r (b)	3.1 (10/319)	0.9 (3/332)	0.6 (2/325)
Fragments			
Partially painted: ace (ab)	17.2 (55/319)	4.8 (16/332)	2.5 (8/325)
Painted: ace (b)	21.0 (67/319)	6.0 (20/332)	3.4 (11/325)
Insertions			
Aba & aba	2.2 (7/319)	1.2 (4/332)	0.6 (2/325)
Bab & bab	0.9 (3/319)	0.6 (2/332)	0.9 (3/325)
Summary of aberrant cells			
% cells with aberrant 1, 2, or 4	50.5 (161/319)	27.4 (91/332)	27.1 (88/325)
% cells with color junctions	45.1 (144/319)	24.1 (80/332)	24.3 (79/325)
% cells with multiple damage	20.4 (65/319)	7.8 (26/332)	8.0 (26/325)

was compatible with linearity over the short term of the experiments (Table VIII C). Translocation-bearing cells also declined in frequency through mitotic divisions, though the decline was less than that of dicentric and fragments. Despite an irregularity in the data set for 3 Gy, where the decline was observed only between first and second division, the estimate of decline for 3 Gy is identical to that from the more regular data set for 1.5 Gy: 13% per cell generation (Table VIII C). The overall decline in cells bearing detectable aberrations in chromosomes 1, 2, and/or 4 (~29% per cell generation) is intermediate between the rapid decline in cells bearing dicentric (~60%) and the lesser decline in cells bearing translocations

(~13%). The overall decline therefore reflects the great instability of dicentric through mitotic divisions and the relative stability of translocations.

DISCUSSION

Aberration Frequencies

We used the PAINT system of nomenclature [Tucker et al., 1995b] to identify and classify aberrations; however, in tabulating data we counted reciprocal aberrations as single events according to the more traditional enumeration rec-

TABLE VIII. Decline in Frequency of Aberration-Bearing Cells Through Mitotic Divisions in Human Lymphocyte Cultures Measured by FISH Painting of Chromosomes 1, 2, and 4 After Exposure to X-Rays in G₀

X-ray exposure (Gy)	Comparison of divisions		Probability (Fisher's Exact Test) & significance	
	Cells with dicentrics/ Σ cells	Cells with dicentrics/ Σ cells		
A. Decline in cells bearing dicentric chromosomes				
1.5	1st: 56/606	2nd: 21/604	<0.0001	***
1.5	2nd: 21/604	\geq 3rd: 17/1351	0.002	**
1.5	1st: 56/606	\geq 3rd: 17/1351	<0.0001	***
3.0	1st: 95/319	2nd: 33/332	<0.0001	***
3.0	2nd: 33/332	\geq 3rd: 18/325	0.041	*
3.0	1st: 95/319	\geq 3rd: 18/325	<0.0001	***
B. Decline in cells bearing translocations				
1.5	1st: 66/606	2nd: 61/604	0.71	NS
1.5	2nd: 61/604	\geq 3rd: 108/1351	0.14	NS
1.5	1st: 66/606	\geq 3rd: 108/1351	0.040	*
3.0	1st: 94/319	2nd: 61/332	0.0009	***
3.0	2nd: 61/332	\geq 3rd: 71/325	0.28	NS
3.0	1st: 94/319	\geq 3rd: 71/325	0.030	*
C. Rates of decline of aberration-bearing cells				
Aberration	X-ray exposure	Decline in frequency*	r ²	Decline per generation
Dicentrics	1.5 Gy	$\ln y = -0.98x + 3.20$	1.000	62.5%
	3.0 Gy	$\ln y = -0.84x + 4.15$	0.970	56.8%
Translocations	1.5 Gy	$\ln y = -0.15x + 2.57$	0.921	13.3%
	3.0 Gy	$\ln y = -0.15x + 3.43$	0.400	13.3%
Fragments	1.5 Gy	$\ln f = -1.14x + 3.70$	0.999	68.0%
	3.0 Gy	$\ln f = -0.83x + 4.51$	0.985	56.4%
Σ Aberrations	1.5 Gy	$\ln y = -0.37x + 3.40$	0.998	30.9%
	3.0 Gy	$\ln y = -0.31x + 4.13$	0.763	26.6%

*y: % aberration-bearing cells; x: number of cell divisions since irradiation; f: fragments/100 cells.

ommended by Finnon et al. [1995]. Though the PAINT method would give twice the number of translocations as the traditional method if all translocations were reciprocal, this difference causes little confusion, so long as the basis for scoring is clear when comparing aberration frequencies.

The frequency of aberrations involving chromosome pairs 1, 2, and 4 in untreated cultures was 2 aberrations (both translocations) in 600 painted cells. Correcting for the proportion of the genome painted, this value corresponds to about 9 aberrations per 1,000 genomes, which closely approximates the laboratory's historical control value of 7 per 1,000 genomes for blood donors of similar age. This spontaneous frequency of aberrations is consistent with other studies employing FISH [Natarajan et al., 1992; Schmid et al., 1992; Bauchinger et al., 1993; Nakano et al., 1993; Tucker et al., 1994; Boei et al., 1996]. Irradiation in G₀ with 1.5 and 3.0 Gy X-rays caused dose-dependent increases in both stable and unstable aberrations (Table II). The frequencies of different classes of aberrations detected by FISH are enriched by many stable aberrations (i.e., translocations and

insertions) that are not detectable by conventional scoring without banding.

Chromosome Comparisons

We compared aberration frequencies in the three painted chromosomes (Table IV). Though small fragments and exchanges between two painted chromosomes are not as easily distinguished as with multicolor paints [Boei et al., 1997], chromosomes 1, 2, and 4 are distinguishable on the basis of differences in chromosome morphology, intensity of labeling with SpectrumOrange, and differences in centromere staining by the DAPI counterstain. Of 861 aberrant chromosomes analyzed in this study, all but 21 (i.e., 97.6%) could be assigned to a specific painted chromosome. Other studies have similarly reported good resolution of different chromosomes painted with a single fluorochrome [Finnon et al., 1995].

Extrapolation of data from painted chromosomes to the entire genome assumes that the responses of the chromo-

somes scored are representative of the genome as a whole. Though randomness of radiation-induced damage is a long-standing assumption in cytogenetics, early studies revealed deviations from randomness in the distribution of break points and aberrations in both plants and *Drosophila* [Sax, 1938; Kaufmann, 1946; Heddle, 1965]. Chromosome banding and classical metaphase analysis of human cells irradiated in vivo and in vitro have in some instances been consistent with a random distribution of aberrations among the chromosomes [Lucas et al., 1992]. However, other studies suggested deviations from randomness [Buckton, 1983; Dutrillaux et al., 1983; Kano and Little, 1986; Knehr et al., 1994], though no consistent pattern emerged as to which chromosomes are prone to underrepresentation or overrepresentation in aberrations.

FISH has added to the controversy about the distribution of aberrations among chromosomes, in that data from chromosome painting have been consistent with a random distribution of breakage, exchange, and repair in some cases [Kovacs et al., 1994], while nonrandomness in one or more of these phenomena has been noted in others [Knehr et al., 1994; Pandita et al., 1994; Boei et al., 1997; Stephan and Pressl, 1997; Barquinero et al., 1998]. Deviations from expectations based on DNA content are sometimes small but significant [Finnon et al., 1995]. If the latter were an accurate representation of the distribution of aberrations in general, extrapolation to the whole genome from specific painted chromosomes might be justified as a practical basis for comparisons, so long as specific conclusions are drawn with cognizance of potential pitfalls owing to minor deviations from the assumption of randomness.

In our study of chromosomes 1, 2, and 4, chromosome 2 was slightly underrepresented in aberrations relative to expectations on the basis of DNA content, whereas chromosome 4 was slightly overrepresented (Table IV). We note that our analysis entails simplifications, in that we have not separated interchanges from intrachanges, and we made no special provision for the relative lengths of individual arms or for the occurrence of complex exchanges. As pointed out by Savage [1991], these factors can influence the distribution of aberrations. Nevertheless, direct comparisons to gross DNA content of the chromosomes [Morton, 1991] provide a first approximation of the relative involvement of different chromosomes in aberrations, and they permit a comparison to other studies that have used similar methods. Our data are compatible with other studies [Buckton, 1983; Knehr et al., 1994; Boei et al., 1997; Stephan and Pressl, 1997] in finding chromosome 4 most heavily represented among the three painted chromosome pairs and in showing an underrepresentation of chromosome 2 [Knehr et al., 1994; Stephan and Pressl, 1997; Barquinero et al., 1998].

Several mechanisms could account for nonrandomness of breaks and exchanges. These include differences in the density of actively transcribed genes [Natarajan et al., 1996], preferential involvement of homologous or home-

ologous sequences in chromosomal rearrangements by recombinational mechanisms [Hayata and Dutrillaux, 1985], and a higher sensitivity of interstitial telomeric sequences to breakage and rearrangement [Alvarez et al., 1993; Balajee et al., 1994; Fernández et al., 1995; Dominguez et al., 1996; Slijepcevic et al., 1996]. Although interstitial telomeres are less prevalent in primates than in other vertebrate groups [Meyne et al., 1990], there is evidence of some interstitial telomeric or quasi-telomeric sequences in humans [Hastie and Allshire, 1989], and they may be prone to recombinational events and aberrations [Hastie and Allshire, 1989; Park et al., 1992]. Chromatin conformation may also contribute to nonrandomness of damage through dependence of exchange aberrations on the proximity of sites in the interphase nucleus or differences in sensitivity of euchromatic and heterochromatic regions [Slijepcevic and Natarajan, 1994a,b; Natarajan et al., 1996].

Genomic Equivalents

Aberration frequencies measured in specific chromosomes by FISH have been extrapolated to the genome as a whole on the basis of the portion of the genome encompassed by the probe [Lucas et al., 1989, 1992]. A potential source of error in the extrapolation is that the involvement of chromosomes in aberrations may not always be proportional to target size. There is some disagreement about the conformity of FISH data to the assumption of proportionality. Knehr et al. [1994] argue that frequencies of X-ray-induced translocations and dicentrics deviate from expectations on the basis of DNA content enough to question the validity of extending FISH data to the entire genome without appropriate weighting factors. Other studies, however, have found the agreement to be generally good [Tucker et al., 1993b; Finnion et al., 1995; Boei et al., 1997]. Moreover, painted chromosomes may predict the genome as a whole reasonably even if there are modest deviations from proportionality, if an excess of aberrations in one painted chromosome is compensated by a deficit in another.

Using the method of Lucas et al. [1989, 1992] we estimated genomic aberration frequencies from our FISH data for chromosomes 1, 2, and 4 (Table V). An assumption in our use of this equation is that the deviations from randomness are small and that the overrepresentation of chromosome 4 is offset by the underrepresentation of chromosome 2.

The frequencies of unstable aberrations determined by FISH were slightly lower than those obtained by conventional scoring of Giemsa-stained metaphases from the same cultures (Table V). A tendency for FISH to underestimate dicentric frequencies relative to conventional staining has also been noted by Schmid et al. [1995], who found the frequency of γ -ray-induced dicentrics detected by FISH to be about 80% of that determined by conventional staining. Since we saw little difference between frequencies of trans-

locations and dicentrics (Table III), we doubt that we misclassified a significant number of dicentrics as translocations. We therefore suspect that FISH was equally proficient in detecting stable and unstable aberrations and that total genomic aberration frequencies may be slightly underestimated by FISH. Our finding that t(Ab) translocations are significantly more frequent than t(Ba) translocations (Table VIC) supports the same interpretation, because this difference is most readily explained by small pieces of unpainted chromosomal material being less readily detected than small pieces of painted material. Differential chromosome involvement in aberrations may also contribute to a slight underestimation of genomic frequencies if chromosomes 1, 2, and 4 are collectively involved in aberrations less often than expected on the basis of their proportion of the human genome.

In contrast to our findings, Ellard et al. [1995] reported that FISH gave a higher estimate of the genomic frequency of chromosome aberrations induced by bleomycin in human lymphocytes than that obtained by conventional scoring of Giemsa-stained chromosomes. They attributed the difference to the detection by FISH of many complex nonreciprocal exchanges. Chromosomal damage induced by bleomycin tends to be strongly overdispersed [Dresp et al., 1978; Kligerman et al., 1992; Hoffmann et al., 1993, 1994], whereas that induced by X-rays tends to be Poisson-distributed [Littlefield et al., 1989; Hoffmann et al., 1993]. Cells with complex aberrations are therefore more common after bleomycin treatment than after low LET ionizing radiation. Consequently, classical scoring is more apt to underestimate the overdispersed damage induced by bleomycin than aberrations induced by X-rays, except at high doses where multiple exchanges are prevalent.

Thus, two factors in scoring offset one another: slight underestimation of single damage by FISH owing to technical factors in staining, and underestimation of multiple damage by classical scoring because of the inability to detect complex exchanges that do not appreciably alter chromosome morphology. The former is apt to be more prominent in the spectrum of aberrations induced by X-rays and the latter in that induced by bleomycin.

Aberration Comparisons: Complete vs. Incomplete Aberrations

As in other FISH studies, we observed that a significant fraction of the translocations and dicentrics were incomplete, in the sense that a reciprocal product could not be identified in the same cell (Table VI). There was no difference between translocations and dicentrics with respect to proportions of incomplete aberrations (49% and 48%, respectively). Since these proportions are so similar, the completeness of the aberrations is unlikely to be a significant factor in the relative frequency of translocations and dicentrics in our study. Our value of 51% complete translocations

is lower than that of Stephan and Pressl [1997], who reported that 79% of translocations in chromosomes 2, 4, and 8 of irradiated lymphocytes were complete. Our results are in closer agreement with those of Finnon et al. [1995] who reported 59% complete aberrations for both dicentrics and translocations in painted chromosomes 2, 3, and 5.

Complete translocations are comprised of a t(Ab) chromosome and the reciprocal t(Ba) chromosome, whereas incomplete translocations are represented by either t(Ab) or t(Ba), but not both. In our study, t(Ab) translocations consistently outnumbered t(Ba) translocations (Table VIC), and the difference between t(Ab) and t(Ba) for the data set as a whole was highly significant ($P < 0.01$). This finding is consistent with other FISH studies that have reported higher frequencies of t(Ab) than t(Ba) chromosomes [Boei et al., 1997; Matsumoto et al., 1998]. Even in studies in which the difference between the two classes was nonsignificant [Finnon et al., 1995], t(Ab) was the majority class. The ratio of t(Ab) to t(Ba) in our study of chromosomes 1, 2, and 4 was 1.26:1. In comparison, Finnon et al. [1995] reported a t(Ab)/t(Ba) ratio 1.14 for chromosomes 2, 3, and 5, while Tucker et al. [1995a] reported a ratio of 1.21 for chromosomes 1, 2, and 4. In the extensive data set of Matsumoto et al. [1998] the median t(Ab)/t(Ba) ratio among eight cultures exposed to γ -rays was 1.29, and the results were consistent for two fluorochromes — one covering chromosomes 1, 2, and 4 (t(Ab) vs. t(Ba)) and another covering chromosomes 3, 5, and 6 (t(Ac) vs. t(Ca)). Therefore, several independent data sets show an excess of translocated chromosomes with unpainted centromeres.

Since the difference between t(Ab) and t(Ba) applies to different chromosomes and probes, it is probably ascribable to the greater visual ability to see a painted segment on an unpainted chromosome than an unpainted segment on a painted chromosome. This difference would be most important when the translocated segment is small because of the nearness of the break to a telomere. This view of the discrepancy between t(Ab) and t(Ba) is compatible with an estimate that the smallest exchange detectable by chromosome painting is about 14.6 megabases for unpainted chromosomal material, but only 11.1 megabases for painted material [Kodama et al., 1997]. The fact that the excess of t(Ab) is independent of dosage and numbers of cell divisions (Table VIC) is also consistent with its being ascribable to technical factors in FISH. If so, some of the translocations and dicentrics scored as incomplete are undoubtedly complete aberrations for which insufficient resolution by FISH prevented the detection of the reciprocal product. This can contribute to an underestimation of aberration frequencies quantified by the PAINT method. We minimized this effect by counting either a sole t(Ab), a sole t(Ba), or a paired t(Ab) plus t(Ba) as a single translocation.

Aberration Comparisons: Translocations vs. Dicentrics

According to classical cytogenetic theory, the frequency of induction of stable chromosome aberrations (translocations) and unstable aberrations (dicentrics) by ionizing radiation should be equal because both arise by random rejoining of two breaks [Sax, 1938; Heddle, 1965; Savage and Papworth, 1982; Tucker et al., 1993b; Stephan and Pressl, 1997]. Several studies using traditional metaphase analysis have confirmed this expectation [Heddle, 1965; Buckton, 1976; Tucker et al., 1993b]. The relative frequencies of dicentrics and translocations change rapidly after the first division, because unstable aberrations cause anaphase bridges that lead to death in mitotic division, whereas stable aberrations (translocations) do not cause mitotic death [Sax, 1941; Bauchinger et al., 1986; Kleinerman et al., 1989]. Thus, the equality of radiation-induced dicentrics and translocations cannot be observed in cell populations that have undergone mitotic divisions. Radiation-induced translocations, but not dicentrics, persist in human peripheral lymphocytes long after irradiation *in vivo* [Kleinerman et al., 1989; Tucker et al., 1993b; Stephan and Pressl, 1997].

FISH analysis is well suited to testing whether the frequencies of translocations and dicentrics actually fit the hypothesized 1:1 ratio. Such comparisons require that scoring be strictly limited to first-division metaphases, since later divisions will be enriched for translocations relative to dicentrics owing to their relative stabilities. In some FISH studies, frequencies of translocations and dicentrics in first division cells are equal, as expected on the basis of classical theory. For example, Kovacs et al. [1994] found no difference in their frequencies in chromosome 4 painted by FISH in human fibroblasts. However, translocation/dicentric (*t/dic*) ratios greater than 1.00 have been reported in many other studies using FISH: 1.6–2.4 [Lucas et al., 1989], 1.5–2.0 [Natarajan et al., 1992], 1.8 [Schmid et al., 1992], 1.5 [Nakano et al., 1993], 1.4–1.8 [Tucker et al., 1993b], 1.48 [Knehr et al., 1994], 1.15 [Ellard et al., 1995], and 1.13 [Stephan and Pressl, 1997]. Some studies report X-ray-induced translocation:dicentric ratios just marginally greater than the theoretical 1:1. Finnon et al. [1995] reported a ratio of 1.17 from studies of painted chromosomes 2, 3, and 5; this difference, which is statistically significant, dropped to 1.12 when corrected for the higher spontaneous frequency of translocations than dicentrics. The latter value does not differ significantly from the theoretical 1:1.

Besides the possibility of an actual difference in the frequencies at which these two classes of aberrations are formed, experimental factors may contribute to a *t/dic* ratio greater than 1.00 [Natarajan et al., 1994; Ellard et al., 1995]. Translocations may be overestimated by misclassification of some dicentrics as translocations in FISH scoring, inclusion of second-division cells among the cells scored, and inclu-

sion of spontaneous translocations (which outnumber spontaneous dicentrics) at low dosages.

Studies using centromere probes [Straume and Lucas, 1993] or conventional staining [Nakano et al., 1993] have confirmed that dicentrics have been misclassified as translocations in some cases. Nakano et al. [1993] reported first-division cells to have a ratio of X-ray-induced translocations to dicentrics of 1.58 in FISH analysis of chromosomes 1, 2, and 4, but 1.06 in conventional analysis of the same metaphases. Straume and Lucas [1993] reported that an excess of translocations relative to dicentrics in chromosomes 1, 2, and 4 was eliminated by the use of centromere probes to avoid the misclassification of dicentrics as translocations. In other cases, higher frequencies of translocations cannot be so explained [Bauchinger et al., 1993; Tucker et al., 1993b]. Taking precautions to observe centromeres, Tucker et al. [1993b] found that translocations consistently outnumbered dicentrics in lymphocytes treated with γ -rays and analyzed by FISH painting of chromosomes 4 or 1, 3, and 4.

The question of whether the frequency of translocations equals or exceeds that of dicentrics is complicated by observed differences in the *t/dic* ratio among chromosomes in some studies [Boei et al., 1997; Griffin et al., 1995; Granath et al., 1996]. Differences in *t/dic* ratios have also been noted among species, and it has been proposed that species in which the karyotype includes metacentric, submetacentric, and acrocentric chromosomes (e.g., humans and Chinese hamsters) tend to show more translocations than dicentrics, whereas those in which all chromosomes are acrocentric (e.g., mouse) show a 1:1 ratio [Dominguez et al., 1996; Natarajan et al., 1996].

We tried to avoid bias in the *t/dic* ratio by systematically eliminating second-division metaphases on the basis of differential chromatid labeling and by analyzing all aberrant cells both with the probe and with the counterstain alone to minimize the chance of misclassifying dicentrics as translocations. Our data for first-division cells (Table III) show a small excess of cells bearing translocations relative to those with dicentrics (1.06 ratio); the difference is nonsignificant and the data are therefore consistent with the 1:1 ratio predicted from cytogenetic theory. Our data for the ratio of translocations to dicentrics induced by X-rays in chromosomes 1, 2, and 4 are in accordance with those of Finnon et al. [1995] for chromosomes 2, 3, and 5.

Though the difference in frequency between translocations and dicentrics in our study was nonsignificant, we cannot argue strongly against the small excess of translocations being real. Other studies that have similarly avoided misclassification of dicentrics as translocations and excluded second-division cells have measured *t/dic* ratios >1; for example, an analysis of chromosomes 1, 4, and 12 in 46-hr cultures revealed an excess of translocations over dicentrics (ratio \approx 1.2–1.3) even when spontaneous translocations were subtracted from the totals [Bauchinger et al.,

1993]. In light of the evidence from many FISH studies [Natarajan et al., 1994], we are inclined to believe that a ratio of translocations to dicentrics slightly higher than the theoretical 1:1 is not a technical artifact and describes the actual spectrum of aberrations induced by X-rays.

An excess of translocations relative to dicentrics can be explained on several grounds. First, the difference may be a reflection of the fact that “dicentric” is a more tightly defined category (i.e., two centromeres) than “translocation.” In theory, one should expect equality between dicentrics plus their associated fragments and reciprocal translocations. However, FISH reveals a high incidence of complex aberrations [Savage, 1996], and translocations observed by FISH include incomplete translocations (sometimes called terminal translocations), interstitial translocations (i.e., insertions), and complex translocations [Natarajan et al., 1992, 1996], in addition to reciprocal translocations. Thus, translocations may be more heterogeneous than dicentrics, and that heterogeneity contributes to their excess [Natarajan et al., 1996].

The fact that agents that inhibit DNA repair can have differential effects on dicentrics and translocations has led Natarajan et al. [1996] to propose a possible mechanistic basis for differences in frequency between the two classes of aberrations. Specifically, potentiation of the induction of dicentrics but not translocations by specific inhibitors has been taken as evidence that the two kinds of aberrations may arise from different lesions or different processing (e.g., misrepair) of lesions [Natarajan et al., 1994, 1996].

Aberration Comparisons: Dicentrics vs. Centric Rings

Ratios of interchromosomal exchanges to intrachromosomal exchanges, called F values, have varied widely among laboratories, ranging from 5 to 20 [Nakamura et al., 1998]. We found agreement between FISH and classical scoring (Table V), in that the yield of centric rings was 13–15% that of dicentrics for FISH ($F = 6.5\text{--}7.5$) and 11–13% for conventional metaphase analysis ($F = 7.6\text{--}9.3$). Finnon et al. [1995] reported the frequency of X-ray-induced centric rings to be 5–10% that of dicentrics ($F = 10\text{--}20$) using FISH painting of chromosomes 2, 3, and 5, a value in good agreement with their results from classical scoring. Stephan and Pressl [1997] similarly found that centric rings equalled about 8% of the dicentric yield for painted chromosomes 2, 4, and 8 in irradiated lymphocytes. Thus, F values from FISH seem to agree with those from traditional cytogenetic analysis.

Persistence of Aberrations

Early cytogeneticists noted that aberrations tend to be eliminated from populations of dividing cells [Sax, 1941], and classical studies explored the details of the process by which aberrations are lost [Sax, 1941; Conger, 1965; Sasaki

and Norman, 1967; Carrano and Heddle, 1973]. The decline in aberrations is nonuniform, in that unstable aberrations (i.e., dicentrics, rings, and fragments) are lost quickly, whereas stable aberrations (i.e., inversions and translocations) persist in populations of dividing cells [Sax, 1941]. These studies [Conger, 1965; Sasaki and Norman, 1967; Carrano and Heddle, 1973] support the view that dicentric chromosomes are lost at about 50% per cell division, a rate that is consistent with expectations based on multiple centromeres behaving independently. If centromeres behave independently, a dicentric chromosome should have a 50% chance of forming a bridge and a 50% chance of falling free in mitosis. The former pattern would be mitotic lethal, while the latter would produce daughter cells containing the dicentric.

Several studies have shown the loss of unstable aberrations to approximate the theoretical 50% per division. For example, Bauchinger et al. [1986] found the loss of dicentrics and rings to be about 55% in the first division, and the data of Boei et al. [1996] show frequencies of dicentrics and fragments to decline by 57% and 52% per division, respectively. In early studies, the decline in fragment-bearing cells generated debate about the effects of fragments vis-à-vis the division kinetics of aberrant and normal cells [Sasaki and Norman, 1967; Carrano and Heddle, 1973]. More recent studies [Bauchinger et al., 1986] show a frequency of fragments in the second generation less than half that in the preceding division (about 40%). The decline reflects the loss of fragments in the first division (about 20%), the proportion of daughter cells receiving a fragment (about 50%), and the unhindered replication of normal cells in the population [Bauchinger et al., 1986].

Studies using FISH have lent support to the classical interpretation of aberration stability. Kovacs et al. [1994] found dicentrics involving chromosome 4 in human fibroblasts to be rapidly eliminated from the cell population, while frequencies of translocations remain essentially constant. Boei et al. [1996] showed that the decline in frequencies of both dicentrics and fragments involving chromosomes 2 and 8 was about 57% per cell division. They found a somewhat more rapid decline (63.5%) for dicentrics involving painted chromosomes 1 and 4 [Boei et al., 1997].

Matsumoto et al. [1998] have recently reported a rapid decline in frequencies of γ -ray-induced dicentric chromosomes, ring chromosomes, and acentric chromosomes from lymphocyte cultures after irradiation in G_0 . Translocations showed greater stability than these demonstrably unstable aberrations, but nevertheless declined in frequency in culture [Matsumoto et al., 1998]. Although the decline was related to time in culture rather than to specific numbers of divisions, it suggests that translocation frequencies decline more quickly in early divisions and then stabilize at a frequency substantially above the spontaneous frequency. On the basis of their observation that cells bearing translocations had a higher frequency of dicentrics than cells

without translocations, Matsumoto et al. [1998] suggested that the presence of the unstable dicentrics may contribute to the early decline in translocations. There was, however, a decline in translocations even in cells not carrying dicentrics, so the decline cannot be ascribed wholly to the presence of a dicentric in the same cell [Matsumoto et al., 1998].

We measured the persistence of all classes of aberrations detected by FISH in chromosomes 1, 2, and 4 through the first few divisions in culture (Table VII). The 29% decline in the overall frequency of aberrant cells per division (Table VIII) is a function of a rapid decline in cells bearing dicentrics (~60%) and the much more modest decline in cells bearing translocations (~13%). The data are consistent in showing a decline in cells bearing dicentrics (Table VIII) slightly greater than 50% per cell generation (62% at 1.5 Gy; 57% at 3 Gy). The measured rate may exceed 50% for several reasons: 1) chance; 2) some cells having undergone >3 divisions; and 3) longer than average distances between centromeres in dicentrics of chromosomes 1, 2, and 4, increasing the chance of a twist between centromeres on the metaphase plate. Acentric fragments, having no regular means of mitotic segregation, are highly unstable (Table VIII). We estimate the decline in the frequency of cells bearing fragments (68% for 1.5 Gy; 56% for 3 Gy) to be similar to the 60% in the data of Bauchinger et al. [1986] using conventional metaphase analysis and somewhat lower than the 70% in the classical study of Sasaki and Norman [1967].

There are several possible reasons for a decline in translocations. If, as suggested by Matsumoto et al. [1998], the frequencies of unstable aberrations are higher in translocation-bearing cells than in cells without translocations, other aberrations could drive some translocations out of the population. This mechanism would not apply, however, if translocations and dicentrics were randomly distributed in the cell population with respect to one another, and most observations from classical cytogenetics are compatible with randomness. The assumption that translocations are stable in a strict sense implies selective neutrality, and some translocations may, in fact, be deleterious. For example, translocations whose break points disrupt a critical gene or are not precisely balanced at the molecular level may confer a selective disadvantage, even though they do not cause mitotic hindrance, as do dicentrics.

The loss of aberrations over time is reflected in a declining frequency of dicentric chromosomes after *in vivo* exposure of humans [Ramalho et al., 1996] or laboratory animals [Kligerman et al., 1990; Hande et al., 1996; Spruill et al., 1996; Tucker et al., 1997] to ionizing radiation. Though unstable through divisions, a few dicentric chromosomes may persist *in vivo* long after irradiation because some lymphocytes are long-lived. Translocations, however, are much more persistent than dicentrics because they do not mechanically hinder cell division and may be replenished from translocation-bearing stem cells. Nevertheless, fre-

quencies of translocations induced by ionizing radiation in mice [Hande et al., 1996; Natarajan et al., 1996; Spruill et al., 1996] and rats [Tucker et al., 1997] have also been shown to decrease over time, though much more slowly than dicentrics. Indirect evidence comparing present translocation frequencies with dicentric frequencies measured some years ago suggests that frequencies of translocations also decline slowly in humans *in vivo* [Natarajan et al., 1996]. Some evidence suggests that nonreciprocal translocations are less stable than reciprocal translocations [Spruill et al., 1996; Tucker et al., 1998], but other studies do not support the same interpretation [Boei et al., 1996; Matsumoto et al., 1998].

Taken together, the available data are sufficient to conclude that translocations are not completely stable, either in cultured cells or in animals *in vivo*. Exposure estimates based on cytogenetic data obtained long after exposure could presumably be increased to correct for aberration instability, but current information is insufficient to provide a reliable means of doing so. Further analyses of aberration stabilities *in vivo* can contribute to a better ability to correct for the loss of aberrations. While corrections for aberration loss offer the prospect of improved precision in using chromosome aberrations in somatic cells as dosimeters of exposure at varying intervals after irradiation, they will require a better understanding of variation among individuals. Such variation encompasses not only the behavior of aberrations through cell divisions, but also a complex of variables associated with the genetic constitution, age, other exposures, immunologic history, lymphocyte division rates, and variation in spontaneous translocation frequencies.

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