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業　績　報　告　書

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PHA刺激及び未刺激ヒトリンパ球細胞におけるガンマ線及び核分裂中性子誘発小核§

Gamma-ray- and Fission-neutron-induced Micronuclei in PHA-stimulated and Unstimulated Human Lymphocytes

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要　約

人造放射性同位体^{252}Cfは，癌の中性子療法に有効であり，他方，原爆放射線から放出される核分裂中性子の影響を評価する上でも有用な情報を提供するモデルと考えられている．本研究の目的は，健康ヒト末梢血リンパ球細胞中の小核誘発を指標として，^{252}Cfから放出される低線量率核分裂中性子と^{60}Coから放出される低及び高線量率ガンマ線の生物学的影響を比較・評価することである．

3名の正常健常人（女性1人，男性2人）から得られた末梢血細胞に，採血後直ちに，又はPHA存在下で24時間培養後に放射線を照射した．照射線量は，^{60}Coガンマ線で0，0.5，1.0，2.0，3.0Gy，^{252}Cf放射線で0，0.25，0.5，1.0，1.5Gyであった．なお，^{252}Cf放射線の2/3が中性子で，1/3はガンマ線であった．培養開始48時間後にcytochalasin Bを添加し，その24時間後に標本を作成した．各標本当たり1,000個の二核細胞中の小核を数え，線量−効果関係（$E=\mu+\alpha D+\beta D^2$）を求めた．^{60}Coガンマ線の線量−効果関係ではβ値が有意であったが，^{252}Cf放射線のβ値は有意ではなかった．G_0，G_1-Sのいずれの時期においても，低線量率ガンマ線よりも高線量率ガンマ線の小核誘発効果は高かった．低線量率^{60}Coガンマ線及び^{252}Cf放射線照射の場合，G_0期及びG_1-S期間における感受性に有意な差は認められなかった．高線量率^{60}Coガンマ線に対してはG_0期よりもG_1-S期が有意に高い放射線感受性を示した．^{252}Cf中性子の小核誘発効果は高く，その生物学的効果比（RBE）は，

§本報告にはこの要約以外に訳文はない．
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種々の培養細胞を用いて出された線量−生存率関係を指標として報告されているRBE値とよく一致していた.

高線量率^{60}Coガンマ線に対する細胞周期上の感受性差を説明することはできないが，G_0期及びG_1-S期における染色体の三次元構造の違いが損傷間の異なる相互作用を引き起こし，染色体損傷修復に影響を及ぼすのではないだろうか．あるいは，潜在的致死損傷の回復(PLDR)—この機構については広く研究されているけれども，ほとんどわかっていない—が関与しているのかも知れない．^{252}Cf放射線のRBE値が高く，G_0期及びG_1-S期間に感受性差が認められないことは，不均一な増殖形態をもつ癌の治療に有用であろう．ヒト集団に及ぼす原爆放射線，あるいは事故による放射線の影響を考える上で，個々人の放射線感受性差が被爆後の種々の医学・生物学的影響にどのように反映するのかを検討するのは重要である．本報告で用いた小核試験法は，電離放射線及び中性子の生物学的線量測定法としては非常に簡便であり，検査コストが安く，再現性の高い線量−効果関係が得られることから，大集団のヒト放射線感受性を評価する上での有用な一方法である.

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Technical Report Series

Gamma-ray- and Fission-neutron-induced Micronuclei in PHA-stimulated and Unstimulated Human Lymphocytes[§]

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Summary

Two groups of normal human blood cells, one stimulated with phyto-haemaglutinin (PHA) for 24 hr (G_1-S phase of the cell cycle) and one unstimulated (G_0 phase), were irradiated with ^{60}Co gamma rays or ^{252}Cf radiation. A comparison of radiation-induced micronucleus frequencies showed that the high-dose-rate gamma rays were more effective in inducing micronuclei than were low-dose-rate gamma rays. In the cells exposed to low-dose-rate irradiation, there was little difference between the frequency of micronuclei in the G_0 phase and the G_1-S phase. However, cells in the G_1-S phase were more sensitive than G_0-phase cells to high-dose-rate gamma rays. The relative biological effectiveness of ^{252}Cf neutron irradiation measured in micronucleus assays was consistent with the value obtained for the lethal effect of ^{252}Cf on cultured cells.

Introduction

When chromosomes or chromosome fragments fail to be incorporated into the daughter nuclei during mitosis they remain in the cytoplasm as micronuclei.[1,2] Radiation efficiently induces micronuclei, and the dose-response relationships have been well documented for blood cells exposed in vitro to ionizing radiation. Countryman et al.[3,4] investigated the X-ray induction of micronuclei in human lymphocytes and analyzed the relationship between the number of micronuclei and

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§The complete text of this report will not be available in Japanese.

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the structural abnormalities of their chromosomes. They found that the number of micronuclei per cell increases linearly with X-ray doses up to 400 R,* and demonstrated that part of the chromosomal damage was repaired during split irradiation with two doses of 200 R.

Micronuclei are also detected in interphase cells that have undergone one cell division. To discriminate between dividing and nondividing cells, Fenech and Morley[5,6] advocated blocking cytokinesis with cytochalasin B. The micronuclei can then be counted in the binucleated cells after the first mitosis.

It is important to assess the biological effects of mixed gamma-ray and neutron radiation, both for medical applications and in order to estimate the effects of exposure to atomic bomb (A-bomb) radiation. For this purpose, we felt that ^{252}Cf, a man-made, spontaneously fissioning isotope, which is believed to be effective in administering neutron therapy for cancer,[7] might provide relevant information for evaluations of the effect of neutrons released in A-bomb radiation. In the present study, the frequencies of micronuclei induced in normal human lymphocytes were investigated after exposures to low-dose-rate (LDR) and high-dose-rate (HDR) gamma-ray radiation and LDR ^{252}Cf radiation.

Our results confirmed (1) that some chromosomal aberrations are repaired during prolonged LDR irradiation, and therefore a nonlinear dose-response curve is expected for acute high-dose irradiation; (2) that chromosomal radiosensitivity is higher in the G_1-S phase than in the G_0 phase; and (3) that the relative biological effectiveness (RBE) of ^{252}Cf neutrons, using micronucleus induction as an index, is consistent with the RBE values found in other studies of its lethal effect on cells.

Materials and Methods

Cells and culture conditions

Peripheral blood was obtained from 3 healthy individuals (a 23-year-old female, a 23-year-old male, and a 38-year-old male). To compare the radiosensitivities at different phases of the cell cycle, the heparinized blood was immediately suspended in a fivefold volume of RPMI medium (GIBCO, Grand Island, N.Y.) supplemented with 20% fetal calf serum and phytohaemaglutinin (PHA [Wellcome, Dartford, England], final concentration = 1.7%), and divided into two groups. One milliliter of the cell suspension was put into each test tube (Pyrex glass, internal diameter = 15 mm, length = 125 mm). After irradiation, 1 mL of the culture medium was added to each test tube, and the cells were incubated in 95% air + 5% CO_2 at 37 °C. Forty-eight hours after blood collection, 2 mL of the culture solution containing 6 μg/mL of cytochalasin B (Sigma Chemical Co., St Louis, Mo.) was added to each test tube.

Irradiation

Irradiation was commenced either within 1 hr (G_0) or 24 hr (G_1-S) after collection of the blood. All irradiations were performed at room temperature in the Research Institute for Nuclear Medicine and Biology of Hiroshima University. Gamma

*Traditional radiation units are retained if they appear in the work being cited. In all current RERF reports, the International System of Units is employed.

irradiations were carried out at distances of 302.5 cm or 50 cm from the ^{60}Co gamma source (Shimazu Seisakusho, Tokyo). The dose rates measured with a Victoreen condenser chamber were 15.09–15.32 mGy/min (the LDR) and 921.4–937.0 mGy/min (the HDR). Neutron irradiations were performed 8 cm from the encapsulated ^{252}Cf source (Type X-35, Amersham International, Buckinghamshire, England).[8] To maintain a uniform irradiation field, the table supporting the tubes was rotated slowly around the source. The dose rate, as determined using Three-Terminal Ionization Chambers (Types IC-17 and IC-17G, Far-West Technology, Inc., Goleta, Calif.) and a Flicke dosimeter, was 10.90–11.25 mGy/min in the culture solution. The ratio of neutrons to gamma rays in the doses was 2.0. The doses employed for the gamma irradiations were 0, 0.5, 1.0, 2.0, and 3.0 Gy, and for the ^{252}Cf irradiations they were 0, 0.25, 0.5, 1.0, and 1.5 Gy. Thus at the highest gamma-ray doses it took 3.26 hr to expose the cells at the LDR but only 3.2 min at the HDR.

Micronucleus specimens

At 72 hr after blood collection, the cells were washed twice with phosphate-buffered saline (PBS[–]), and 5 mL of PBS(–) was added to each test tube. Five milliliters of Carnoy's fixative (methanol 3: glacial acetic acid 1, v/v) was added to the buffer saline and left standing for 15 min. The cells were fixed twice with Carnoy's fixative to remove red blood cells. Drops of the cell suspension were then placed on glass slides and stained with 2% Giemsa. The frequency of micronuclei in 1,000 binucleated cells for each dose point of each donor was scored under a microscope (×1000). Criteria for scoring the micronuclei were similar to those presented by Fenech and Morley.[5,6] The trinucleated and tetranucleated cells observed occasionally were excluded. The micronuclei were the same color or lighter than the main nuclei. The diameters of the micronuclei were one-third or less than those of the main nuclei.

Curve fitting

The dose responses of induced micronucleus frequency were analyzed using standard linear regression methodology.[9] Computations were performed using the GLIM 3.77 statistical program.[10] Expected frequencies were fitted using the general model:

$$E(Y_{ijk}) = \mu_{jk} + \alpha_{ijk}D + \beta_{ijk}D^2 \ ,$$

where D is dose in gray and Y_{ijk} is micronucleus frequency (per cell) for source i (1,2,3), phase j (1,2), and donor k (1,2,3). The model was constructed by stepwise addition of factors representing source, phase, and donor. Statistical tests of the significance of various parameters in the model were based on the regression F test or the t test (estimated coefficient divided by its standard error).

Calculation of RBE

$RBE_{n+\gamma}$ values were obtained from the regression curves that were fitted to the gamma-ray (quadratic) and ^{252}Cf (linear) radiation dose responses. Since it is assumed that the gamma rays and the neutron particles in the ^{252}Cf radiations act

independently, the RBE of neutrons (RBE_n) can be calculated as follows:

$$RBE_n = (RBE_{n+\gamma} - f_\gamma) / f_n \ ,$$

where f_n (0.67) and f_γ (0.33) represent the fractions of dose due to neutrons and gamma rays, respectively.

Results

Table 1 shows the micronucleus frequencies in cells exposed to LDR and HDR ^{60}Co gamma rays and to LDR ^{252}Cf radiations at the G_0 and G_1-S phases. Data were obtained by scoring the number of micronuclei in 1,000 cells from each dose point of the three donors.

There was no significant effect of cell phase on the intercept of the dose response ($F_{1,75}$ = 0.96, $p > .5$ [the subscript on F represents numerator and denominator degrees of freedom, respectively]); therefore, a single intercept term, μ, was used in the dose-response model. Linear terms in the dose response differed significantly ($F_{2,74}$ = 521.9, $p < .001$) among the three sources. However, only HDR gamma irradiation showed a significant difference in the slopes of the dose responses between the G_0 and G_1-S phases (t_{71} = 5.0, $p < .001$ [the subscript on t represents degrees of freedom]). Quadratic terms were significant for both gamma sources (LDR, t_{70} = 2.5, $p < .02$; HDR, t_{70} = 3.0, $p < .005$) but not for the LDR ^{252}Cf source (t_{70} = 0.3, $p > 5$).

There was evidence of variability among the separate donor dose-response curves when separate parameters were estimated for each donor ($F_{14,57}$ = 2.51, $p < .02$). However, all of this variability was due to a single observation (615) at 1.5 Gy in the ^{252}Cf dose response; removing this point negated the significance of the interdonor variability ($p > .2$).

Coefficients of the final dose-response model are given in Table 2. This model reflects a single overall intercept (μ), a linear-quadratic dose response for LDR ^{60}Co gamma radiation, separate linear-quadratic dose responses for HDR ^{60}Co gamma radiation within each phase (but sharing a common quadratic coefficient), and a strictly linear dose response for ^{252}Cf. The final model may be written as follows:

$$\begin{array}{ll}
\mu + \alpha_1 D + \beta_1 D^2 & \text{LDR } ^{60}\text{Co} \\
\mu + \alpha_{2,1} D + \beta_2 D^2 & \text{HDR } ^{60}\text{Co, } G_0 \\
\mu + \alpha_{2,2} D + \beta_2 D^2 & \text{HDR } ^{60}\text{Co, } G_1\text{-S} \\
\mu + \alpha_3 D & \text{LDR } ^{252}\text{Cf} \ .
\end{array}$$

Table 3 shows the RBEn+g and RBEn values at specific micronucleus freies. Since a linear-quadratic dose response best fitted the gamma irradiation data, whereas the neutron irradiation dose response was strictly linear, the $RBE_{n+\gamma}$ values decreased with increasing dose. The calculated values of RBE_n range from 2.7 (HDR ^{60}Co [G_1-S] at a micronucleus frequency [micronuclei/cell] of 0.4) to 5.7 (LDR ^{60}Co at a micronucleus frequency of 0.05).

Table 1. Number of micronuclei observed in 1,000 peripheral blood cells from each of 3 donors after low-dose-rate (LDR) or high-dose-rate (HDR) ^{60}Co gamma-ray irradiation or LDR ^{252}Cf irradiation at the G_0 or the G_1-S phase of the cell cycle

Radiation source	Dose (Gy)	Donor	Irradiated at:	
			G_0	G_1-S
No radiation	0	1	20	22
		2	25	32
		3	24	22
LDR ^{60}Co γ	0.5	1	51	77
		2	63	63
		3	46	60
	1.0	1	99	116
		2	102	118
		3	99	106
	2.0	1	219	216
		2	216	267
		3	233	231
	3.0	1	348	336
		2	382	370
		3	353	388
HDR ^{60}Co γ	0.5	1	75	71
		2	72	94
		3	59	62
	1.0	1	139	139
		2	129	185
		3	120	152
	2.0	1	299	335
		2	283	363
		3	262	350
	3.0	1	454	555
		2	420	550
		3	396	554
LDR ^{252}Cf	0.25	1	93	100
		2	93	95
		3	89	102
	0.5	1	193	182
		2	189	184
		3	171	211
	1.0	1	398	375
		2	364	350
		3	347	412
	1.5	1	615	563
		2	506	572
		3	487	546

Table 2. Coefficients of the final dose-response model

Radiation source	Phase	Parameter	Est. value	*SE*
No radiation	G_0 and G_1-S	μ	0.0126	0.00504
LDR ^{60}Co γ	G_0 and G_1-S	α_1	0.0879	0.0107
		β_1	0.00971	0.00362
HDR ^{60}Co γ	G_0	$\alpha_{2,1}$	0.104	0.0109
	G_1-S	$\alpha_{2,2}$	0.143	0.0109
	G_1-S	β_2	0.0119	0.00362
LDR ^{252}Cf	G_0 and G_1-S	α_3	0.358	0.00628

Standard errors (*SE*) were calculated according to standard linear regression theory. HDR: high dose rate; LDR: low dose rate.

Table 3. Radiation doses necessary to cause specific micronucleus frequencies (A), and the relative biological effectiveness (RBE) of ^{252}Cf against low- and high-dose-rate (LDR, HDR) ^{60}Co radiation (B)

A

Micronucleus frequency	Dose (Gy) necessary to cause the specific micronucleus frequency			
	^{252}Cf	LDR ^{60}Co	HDR ^{60}Co (G_0)	HDR ^{60}Co (G_1-S)
0.05	0.10	0.41	0.35	0.26
0.10	0.24	0.90	0.77	0.58
0.15	0.38	1.36	1.17	0.89
0.20	0.52	1.78	1.53	1.19
0.30	0.80	2.55	2.21	1.75
0.40	1.08	3.24	2.82	2.28

B

Micronucleus frequency	RBE of ^{252}Cf against:					
	LDR ^{60}Co		HDR ^{60}Co (G_0)		HDR ^{60}Co (G_1-S)	
	$RBE_{n+\gamma}$	RBE_n	$RBE_{n+\gamma}$	RBE_n	$RBE_{n+\gamma}$	RBE_n
0.05	4.1	5.7	3.5	4.8	2.6	3.4
0.10	3.8	5.2	3.2	4.3	2.4	3.1
0.15	3.6	4.9	3.1	4.2	2.3	3.0
0.20	3.4	4.6	2.9	3.9	2.3	3.0
0.30	3.2	4.3	2.8	3.7	2.2	2.8
0.40	3.0	4.0	2.6	3.4	2.1	2.7

The doses for each type of radiation were calculated from regression curves using the coefficients in Table 2. The gamma rays (γ) and the neutron particles (n) in the ^{252}Cf radiation were assumed to act independently.

Discussion

A comparison was made of the frequency of induced micronuclei in lymphocytes exposed to radiations of different qualities and dose rates at different stages of the cell cycle. Earlier studies demonstrated that there is a wide variation in cell cycle times in PHA-stimulated lymphocytes.[11] Bender and Prescott[12] reported that ^{3}H-TdR is not incorporated into DNA in the first 24 hr of a lymphocyte culture. Kikuchi and Sandberg[13] reported that S-phase begins at 16–20 hr before metaphase in leukocytes. Using the sister chromatid differential staining method, Crossen and Morgan[14] found that 47–98% of lymphocytes from 20 healthy individuals underwent at least one mitotic division in a 48-hr culture. The shortest cell cycle time was 12 hr. Using a similar technique, Craig-Holmes and Shaw[15] found lymphocytes with cell cycle times of 12–48 hr. Based on these reports, the major fraction of PHA-stimulated lymphocytes could be expected to have entered into the G_1-S phase by 24 hr after the beginning of the culture.

The regression methodology used to fit the dose-response curves should be viewed as only approximate. The observed micronucleus frequencies per 1,000 cells are the sums of correlated binary observations, and the scores at different dose points for an individual donor are also correlated. Therefore, basic assumptions implied in the use of the F and t tests are not strictly met. However, we evaluated the fitted model using plots of residuals and found that the variance was reasonably constant and the distribution was approximately normal. It should be noted, though, as may be seen from Figure 1, that the model did not fit the zero-dose observations well. We attribute this to the inability of the linear-quadratic model to adequately fit curvature in the low-dose region. Unfortunately, we did not obtain additional low-dose observations in order to better model that portion of the curve.

The micronucleus frequency induced with LDR gamma rays was lower than that induced with HDR gamma rays at the same total dose. The simplest and most often cited explanation for this reduction might be that some fraction of the potential chromosomal aberrations that would have resulted from the interaction of separate ionization tracks is repaired during the more prolonged period of LDR irradiation. Fenech and Morley[6] compared the induced micronucleus frequencies following gamma irradiation at dose rates of 0.5 Gy/min and 4 Gy/min, but observed no dose rate effect. The low-dose rates we used were 15.09–15.32 mGy/min, about 30 times lower than their dose rates.

With HDR gamma irradiation, the frequency of induced micronuclei was lower in the G_0-phase cells than in the G_1-S phase cells, most notably at doses > 1 Gy (Figure 1). Wolff[16] determined the X-ray-induced frequencies of dicentrics and rings of unstimulated (G_0) and PHA-stimulated (G_1) human lymphocytes. He showed that the yield of two-break aberrations per cell is higher in cells irradiated at the G_1 phase than in those irradiated at the G_0 phase. Our micronucleus induction results are thus consistent with his. In addition, dose-fractionation studies by Wolff[16] showed that chromosome breaks are repaired in PHA-stimulated and unstimulated lymphocytes. The repair time after a dose of 1 Gy is from 4 to 5 hr in both types of cells. Countryman et al.[3,4] showed a linear response for the frequency of X-ray-induced micronuclei over a dose range of 100–400 R, but

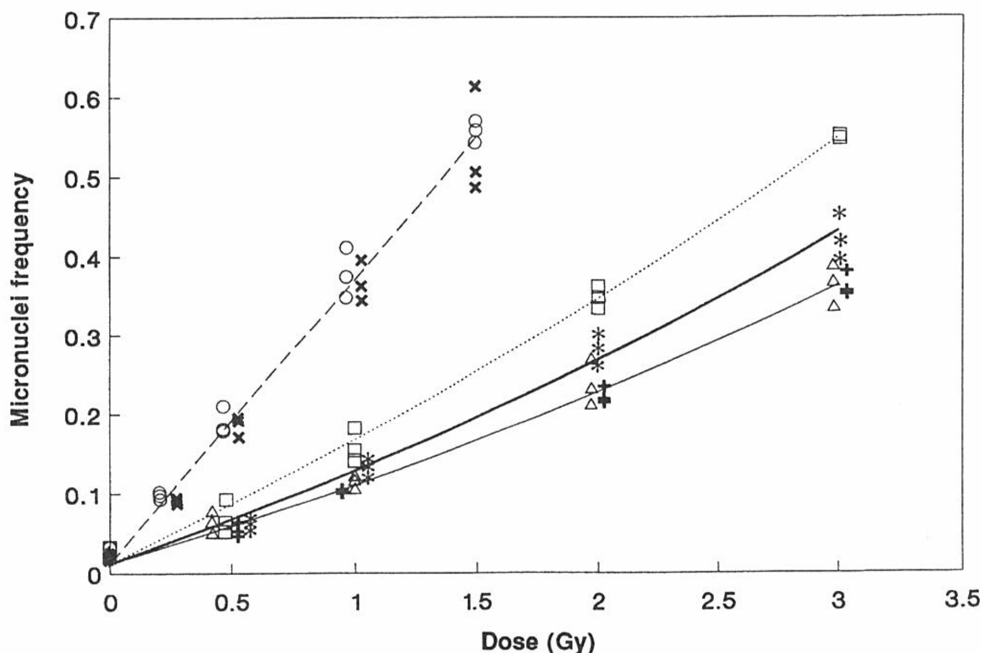

Figure 1. Dose responses of micronuclei frequencies in PHA-unstimulated cells (G_0 phase: x, *, +) and cells treated with PHA for 24 hr (G_1-S phase: O, □, △) exposed to ^{60}Co gamma rays at the low dose rate (+, △) and high dose rate (*, □), and to low-dose-rate ^{252}Cf radiation (x, O). Data were fitted by the linear model for ^{252}Cf radiation and the linear-quadratic model for ^{60}Co gamma rays using the coefficients in Table 2. Some of the symbols have been shifted slightly to the right or left of their dose to enhance their legibility.

significant dose fractionation effect, which indicates two-track interactions. A comparison of our data after exposures to the LDR gamma-ray and the LDR ^{252}Cf radiations shows no difference in micronucleus induction between the G_0 and G_1-S phases. This indicates that there was no difference in unrepairable damage in both types of cells. Therefore, the fact that the HDR gamma exposures showed a significant difference between the G_0 and G_1-S phases indicates a different postirradiation response, i.e., greater interaction between breaks produced by independent gamma tracks in the G_1-S phase than in the G_0 phase. This could result from differences in the spatial arrangement of chromosomes during repair processes. The important contribution of repair of potentially lethal damage (PLDR) could be another possible reason for the different radiosensitivities found in stimulated and unstimulated lymphocytes, although the process of PLDR is not clear. PLDR after irradiation has been investigated in various phases of the cell cycle.[17] Iliakis and Nuesse[18] observed PLDR only in cells that had not entered the S phase. PLDR could be due to the cell progression, which would result in either viable types of chromosome aberrations or cell death.

The frequency of micronuclei per dose is high when ^{252}Cf irradiation occurs in the G_0 phase or in the G_1-S phase, with no significant difference in frequency between the two phases. Dose-response curves after ^{252}Cf irradiation were well

fitted by the linear model (Table 2). Because ^{252}Cf radiation effectively induces the same amount of damage in both the G_0 and G_1-S phases, it could potentially be useful in treating cancers consisting of cells that proliferate heterogeneously.

Prosser et al.[19] analyzed X-ray dose-response curves for the frequency of micronuclei and chromosome aberrations, using the quadratic model $Y = C + \alpha D + \beta D^2$. The value of the α coefficient for their micronucleus study was approximately 2 times greater than that for chromosome aberrations, but the β term was 10 times lower. The linear and quadratic models they obtained from the HDR X irradiations of 5 individuals' blood cells were $Y = (0.0092 \pm 0.0032) + (0.146 \pm 0.005)D$ with 8 df, and $Y = (0.0129 \pm 0.0018) + (0.117 \pm 0.006)D + (0.0087 \pm 0.0016)D^2$ with 7 df. The coefficients are in good agreement with those obtained from the HDR ^{60}Co gamma irradiations at G_0 (Table 2). Our dose-response curves after gamma-ray irradiation were also fitted to the quadratic model, and the β values were not so high. Prosser et al.[19] noted that the background frequency of micronuclei is significantly higher than that of acentrics. Our micronucleus background values were consistent with theirs. From a theoretical analysis, Savage[20,21] concluded that acentric fragments and micronuclei are quite different parameters and that no simple relationship exists between them.

So far, a few reports have examined neutron dose responses employing the micronucleus assay method. Roberts et al.[22] irradiated BHK21 cells with ^{60}Co gamma rays (48 Gy/min) and 14.8-MeV neutrons (58.3 mGy/min). Although they obtained a similar RBE of 2 at a micronucleus frequency (the number of micronuclei per cell in their experiment) of 0.3 or less, a direct comparison of RBEs is questionable since their gamma-ray dose rate was very high. Goud et al.[23] irradiated Balb/c-strain mice with ^{252}Cf radiation or ^{60}Co gamma rays and counted the number of micronuclei in polychromatic erythrocytes. They obtained values of $RBE_{n+\gamma} = 2.1$ and $RBE_n = 2.7$.

A number of RBE values at D_{10} doses (the dose required to kill 90% of the cells) have been reported, using as an index the lethal effect on culture cells irradiated in the presence of oxygen. These values are in the range of 1.9–4.3.[7,24–27] Ban et al.[8] reported that with LDR ^{252}Cf irradiation and HDR irradiation of HeLa-derivative MR cells, $RBE_{n+\gamma} = 2.05$ and $RBE_n = 2.6$ at the 50% survival rate and $RBE_{n+\gamma} = 2.1$ and $RBE_n = 2.7$ at a mutation rate of 5×10^{-5}. The RBE values of ^{252}Cf neutrons obtained using micronucleus induction as a measure are consistent with those obtained using the cell survival assay.

Parallel experiments with blood samples obtained from a large number of individuals appear to show a marked range of radiation response distributions (unpublished data). Thus this type of study could potentially be utilized to assess whether individuals are differentially radiosensitive, and if so, what the underlying causes for the variation might be (e.g., donor age, sex, and genetic and/or other physiological differences).

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