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業績報告書シリーズ

免疫ペルオキシダーゼ染色によって分類されたヒトリンパ球亜集団細胞中におけるX線誘発小核§

X-ray Induction of Micronuclei in Human Lymphocyte Subpopulations Differentiated by Immunoperoxidase Staining

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要　約

本研究では，小核アッセイ法を用いて，ヒト末梢血リンパ球亜集団細胞の放射線感受性を確認することを目的とした。ヒト末梢血から分離された単核細胞にX線を照射した。3日間培養後に細胞を固定し，免疫ペルオキシダーゼ染色法で染色した。リンパ球亜集団細胞は単クローン抗体 Leu4(CD3)，Leu2a(CD8)と Leu19(CD56)を用いて特定した。

特異的抗体と反応させた後に染色された二核細胞中の小核を数えて，線量-反応曲線を求めた。$CD8^+$(サプレッサー／細胞毒性)細胞の線量-反応曲線は，$CD3^+$(汎T)細胞のそれと全く同様であった。ところが $CD56^+$(ナチュラルキラー)細胞は感受性が有意に低かった。しかし，測定できた $CD56^+$ の二核細胞は全二核細胞の4％以下しかなかった。

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放影研[1]遺伝学部および[2]統計部。

Technical Report Series

X-ray Induction of Micronuclei in Human Lymphocyte Subpopulations Differentiated by Immunoperoxidase Staining§

Sadayuki Ban, D.M.Sc.[1]; Mimako Nakano, Ph.D.[1]; John B. Cologne, Ph.D.[2]

Summary

In this report we sought to confirm the radiosensitivity of human peripheral blood lymphocyte subpopulations using a micronucleus assay. Mononucleated cells isolated from peripheral blood were irradiated with X rays. After being cultured for 3 days, cells were fixed and stained using the immunoperoxidase staining technique. Lymphocyte subpopulations were characterized by means of the monoclonal antibodies Leu4 (CD3), Leu2a (CD8), and Leu19 (CD56).

Dose-response curves were obtained by scoring the number of micronuclei in binucleated cells that reacted with a specific antibody and were then stained. The dose response of $CD8^+$ (suppressor/cytotoxic) cells was quite similar to that of $CD3^+$ (pan T) cells. In comparison, $CD56^+$ (natural killer) cells were significantly less sensitive, although scorable binucleated $CD56^+$ cells made up less than 4% of the total number of binucleated cells.

Introduction

It is well known that radiation efficiently induces micronuclei in living cells. The documented dose-response relationships for ionizing irradiations suggest that the micronucleus assay is one of the easiest and most reliable tests for the determination of radiation sensitivity. Human peripheral blood lymphocytes have been used for quantitative comparisons of frequencies of radiation-induced micronuclei because of the ease of sample preparation.[1–5] However, if an extreme difference in radiosensitivity exists between lymphocyte subpopulations, individual radiosensitivity as determined in whole blood could be affected by immunological changes caused by the disturbance of interactions between lymphocyte subpopulations.

Stewart et al.[6] irradiated human peripheral blood mononuclear cells with X rays and analyzed the cell survival by flow cytometry 6 days after irradiation.

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They showed that the unstimulated $CD8^+$ (suppressor/cytotoxic) cells were twice as radiosensitive as $CD4^+$ (helper/inducer) cells, whereas Leu19 (natural killer) cells were about 5 times more radioresistant than $CD4^+$ cells. We were concerned by the unusual radiosensitivities of the unstimulated $CD8^+$ and Leu19 cells and sought to confirm them with the micronucleus assay.

In the present study, human peripheral mononuclear lymphocytes were exposed to X rays before phytohemagglutinin (PHA) treatment. The human lymphocyte subpopulations fixed on glass slides were differentiated with the immunoperoxidase staining technique reported by Slavutsky and Knuutila.[7] The frequencies of micronuclei in cells, which were characterized with Leu4 (CD3), Leu2a (CD8), and Leu19 (CD56), were then compared.

Materials and Methods

Micronucleus assay

Peripheral blood was obtained from three healthy males—a 41-year-old nonsmoker (donor A), a 22-year-old smoker (10 cigarettes/day, donor B), and a 40-year-old smoker (20 cigarettes/day, donor C)—and one healthy 24-year-old nonsmoking female (donor D). Mononucleated cells were separated from 10 mL of heparinized blood from each donor by Ficoll-Paque density-gradient centrifugation. Immediately after suspension of the cells in 9 mL of RPMI medium (GIBCO, Grand Island, N.Y.) supplemented with 20% fetal calf serum (FCS) and *L*-glutamine, the cells were put into plastic tubes (1.5 mL per tube) and exposed to X rays. Irradiated cells were transferred into plastic flasks containing 8.5 mL of fresh medium with PHA (Wellcome, Dartford, U.K.; final concentration = 1.7%), and cultured at 37°C in a 95% air – 5% CO_2 incubator for 48 hr. Cultures were continued for another 24 hr in the presence of cytochalasin B (Sigma Chemical Co., St. Louis, Mo.; final concentration = 3 μg/mL). The number of micronuclei in binucleated cells was scored under a microscope (×1000). Criteria for scoring the micronuclei were similar to those presented by Fenech and Morley.[4] The trinucleated and tetranucleated cells observed occasionally were excluded. Some multinucleated cells were found in $CD56^+$ cells, and these were also excluded. The diameters of the micronuclei were one-third or less than those of the main nuclei.

Immunoperoxidase staining

The immunoperoxidase staining technique reported by Slavutsky and Knuutila[7] was followed. After being cultured, cells were hypotonically treated at 37°C in a solution made up of one part of culture medium (RPMI 1640 plus 10% FCS) and one part of buffer (50 mM glycerol, 5 mM KCl, 10 mM NaCl, 0.8 mM $MgCl_2$, 10 mM sucrose, and 1 mM $CaCl_2$). Cells were attached to glass slides by means of a cytocentrifuge (400 g for 8 min), air dried for 3–4 hr, and then fixed with acetone containing 0.024% formaldehyde for 1 min. PBS(–) buffer with 0.08% FCS was used to wash the cells in the following treatments. Cells were treated with primary antibody (Beckton Dickinson Immunocytometry Systems, Mountain View, Calif.) for 15 min and with secondary biotinylated antibody (Beckton Dickinson) for 15 min at room temperature. The concentration of primary antibodies was determined before assay, because a high concentration

of primary antibodies caused dark staining that concealed the micronuclei. Cells that reacted with primary antibody were stained with the Vectastatin ABC kit (avidin/biotinylated horseradish peroxidase; Beckton Dickinson) for 15 min, and with 3-aminoethyl-9-ethylcarbazole (AEC) solution in the presence of hydrogen peroxidase for 10 min. The AEC solution was a mixture of 0.5 mL of 0.8% AEC in dimethylformamide and 9.5 mL of 0.1 M acetic acid buffer. Nuclei and micronuclei were stained with 10% Giemsa. Lymphocyte subpopulations were identified with the following monoclonal antibodies: Leu4 (CD3), Leu2a (CD8), and Leu19 (CD56); $CD3^+$ is a pan T-cell marker, $CD8^+$ is a marker of suppressor/cytotoxic T cells, and $CD56^+$ is a marker of natural killer cells.

X-ray irradiation

Cells were irradiated with X rays at room temperature. The X-ray generator was operated at 220 kVp, 8 mA, 0.3 mm Cu plus 0.5 mm Al external filtration. The dose rate measured with the Victoreen condenser chamber was 1.1 Gy/min. Cells received doses of 0, 0.5, 1.0, 1.5, 2.0, and 3.0 Gy.

Statistical analysis

The outcome (number of micronuclei per cell; see Table 2) can be thought of as a Poisson variable with mean depending on dose and cell type, as well as, perhaps, other factors distinguishing individual donors. Because the experimental design incorporated repeated observations on some individuals and different doses applied to separate aliquots of a single blood sample from each donor, we employed statistical regression methods suitable for correlated Poisson data.[8]

Because of the small number of individuals tested, it was necessary to make some assumptions concerning the correlations among observations. Using methods for estimating components of variance in random effects models,[9,10] we concluded that only one random effect involving donors—donor by dose level interaction—contributed significantly to correlations among multiple measurements within individual donors. Measured frequencies were therefore assumed to be correlated within each separately irradiated aliquot of donor blood, but pairs of observations between dose levels or between donors were assumed to be uncorrelated. We assumed the response to be linear-quadratic in X-ray dose d:

$$\lambda_{ij}(d) = \alpha_{ij} + \beta_{ij}d + \gamma_{ij}d^2 \quad , \tag{1}$$

where i {1,2,3} represents cell population ($CD3^+$, $CD8^+$, and $CD56^+$, respectively) and j {1,2,3,4} represents individual donor (A, B, C, and D, respectively).

For each dose/cell type/donor combination, a large number of cells, $N_{ij}(d)$ (typically 1,000 or as many as could be found) were scored for micronuclei. Taking these as independent and using the fact that a sum of independent Poisson variables is Poisson with mean equal to the sum of their individual means, we then have

$$\mu_{ij}(d) = N_{ij}(d)\lambda_{ij}(d) = N_{ij}(d)\alpha_{ij} + N_{ij}(d)\beta_{ij}d + N_{ij}(d)\gamma_{ij}d^2 \tag{2}$$

as the mean number of micronuclei in $N_{ij}(d)$ scored cells at dose d.

The regression model (2) may be fit using covariates (N_{ij}, $N_{ij}\,d$, and $N_{ij}\,d^2$) with no intercept term. Separate parameters based on the indices i and j are estimated

by incorporating appropriate indicator variables and their cross-products with d and d^2 in the regression model. Tests concerning the regression parameters are based on normal approximation theory as well as on deviance tests for generalized linear models with dependent data.[11] Note that it is not necessary to specify correctly the correlations among all observations within a single donor; the method guarantees that approximately statistically consistent estimates of the regression parameters will be obtained even if an incorrect working hypothesis concerning the correlation structure is used.

Results

Immunoperoxidase staining was carried out on the unirradiated cells of donor A before and after cell cultivation (Table 1).

In this case, 57% of the cells were stained with Leu4, 27% with Leu2a, and 22% with Leu19. For cells cultured with PHA for 48 hr and cytochalasin B for 24 hr, 90% of binucleated cells were stained with Leu4, 40% with Leu2a, and only 3.6% with Leu19.

Table 2 shows the raw micronucleus data for the three types of cells and four donors.

For unknown reasons, the micronucleus frequency in $CD56^+$ cells showed a downturn in response at 3 Gy (Figure 1). Attempts to fit this behavior using other simple dose-response models were unsuccessful, and we therefore excluded these points from further analysis (see the Discussion for further comments on this point).

Through a process of testing various parameters in the regression model, we determined that the linear-quadratic model that best fit the individual cell-type data was

$$\lambda_i(d) = \alpha + \delta_\alpha I_{(i=3)} + (\beta + \delta_\beta I_{(i=3)})\, d + \gamma d^2 \quad , \tag{3}$$

where $I_{(i=3)}$ is an indicator that is 1 when $i = 3$ ($CD56^+$ cells) and 0 otherwise. The fitted dose-response curves are displayed in Figure 1. This model is interpreted as fitting a single linear-quadratic dose response jointly to $CD3^+$ and $CD8^+$ cells

Table 1. Fraction of unirradiated lymphocyte subpopulations as determined by immunoperoxidase staining before and after cultivation in the presence of phytohemagglutinin

Time after cultivation	Antibody (antigen)	No. of mononucleated cells checked	No. of binucleated cells checked	No. of stained cells (%)
0 hr	Leu4 (CD3)	5000		2871 (57.4)
	Leu2a (CD8)	5000		1347 (26.9)
	Leu19 (CD56)	5000		1096 (21.9)
72 hr	Leu4 (CD3)		5000	4475 (89.5)
	Leu2a (CD8)		5000	1991 (39.8)
	Leu19 (CD56)		5000	178 (3.6)

Table 2. Frequency of micronuclei in $CD3^+$ cells, $CD8^+$ cells, and $CD56^+$ cells of four donors.

Donor	Dose (Gy)	No. of cells scored	Micronucleus distribution per cell						Avg. no. of micronuclei per cell
			1	2	3	4	5	6	
			$CD3^+$ cells						
A (41-yr-old male)	0	3000	58	4	1				0.023
	0.5	3000	174	23	3				0.076
	1.0	3000	354	47	4	1			0.155
	1.5	3000	500	97	12				0.243
	2.0	3000	588	157	21	3			0.326
	3.0	3000	735	318	51	14	3		0.532
B (22-yr-old male)	0	1000	18	1					0.020
	0.5	1000	47	6					0.059
	1.0	1000	112	13	1				0.141
	1.5	1000	143	24	2				0.197
	2.0	1000	214	42	9	1			0.329
	3.0	1000	311	129	17	3	1		0.637
C (40-yr-old male)	0	1000	44	6	1				0.059
	0.5	1000	88	11	1				0.113
	1.0	1000	111	31	1	1			0.180
	1.5	1000	149	35	3			1	0.234
	2.0	1000	174	41	6	2	1		0.287
	3.0	1000	205	81	24	8	1		0.476
D (24-yr-old female)	0	1000	18	1					0.020
	0.5	1000	58	2					0.062
	1.0	1000	98	13					0.124
	1.5	1000	154	30	3				0.223
	2.0	1000	228	33	6	1			0.316
	3.0	1000	265	93	11	6	1		0.513
			$CD8^+$ cells						
A (41-yr-old male)	0	3000	63	8	1				0.027
	0.5	3000	190	16	2		1		0.078
	1.0	3000	347	48	4				0.152
	1.5	3000	524	92	12	1			0.249
	2.0	3000	654	158	23	2	1		0.351
	3.0	3000	749	314	54	15	2		0.536

Continued

Table 2. Continued

Donor	Dose (Gy)	No. of cells scored	Micronucleus distribution per cell 1	2	3	4	5	6	Avg. no. of micronuclei per cell
			$CD8^+$ cells						
B (22-yr-old male)	0	1000	24						0.024
	0.5	1000	57	5					0.067
	1.0	1000	96	11	1				0.121
	1.5	1000	126	30	4				0.198
	2.0	1000	209	44	3	2			0.314
	3.0	1000	301	104	22	3	1		0.592
C (40-yr-old male)	0	1000	42	4					0.055
	0.5	1000	55	15	2				0.091
	1.0	1000	133	18	3	1			0.182
	1.5	1000	186	34	3	1			0.267
	2.0	1000	173	51	10	3	1		0.322
	3.0	1000	241	92	18	4	2		0.505
D (24-yr-old female)	0	1000	15	1					0.017
	0.5	1000	56	5					0.066
	1.0	1000	103	12					0.127
	1.5	1000	150	31	6				0.230
	2.0	1000	227	39	7	1			0.330
	3.0	1000	287	97	12	3	1		0.530
			$CD56^+$ cells						
A (41-yr-old male)	0	343	14						0.041
	0.5	265	17	1					0.072
	1.0	289	27	1					0.100
	1.5	273	39	3					0.165
	2.0	347	60	11		1			0.248
	3.0	186	40	8	3				0.349
B (22-yr-old male)	0	236	15	2					0.081
	0.5	197	18	3					0.122
	1.0	180	18		1				0.117
	1.5	128	20	3					0.203
	2.0	199	37	5	1				0.251
	3.0	158	34	7	1	1			0.348

Continued

Table 2. Continued

Donor	Dose (Gy)	No. of cells scored	Micronucleus distribution per cell 1	2	3	4	5	6	Avg. no. of micronuclei per cell
			$CD56^+$ cells						
C (40-yr-old male)	0	491	29	2					0.067
	0.5	445	40	1					0.094
	1.0	373	42	3					0.129
	1.5	386	42	6					0.140
	2.0	352	55	9		1			0.219
	3.0	356	53	11	1				0.219
D (24-yr-old female)	0	165	4						0.024
	0.5	155	10						0.065
	1.0	174	14						0.080
	1.5	180	23	5					0.183
	2.0	107	19	3					0.234
	3.0	104	21	4	1				0.308

NOTE: The percentages of binucleated $CD56^+$ cells that were scored in 2,000 binucleated cells on control (0 Gy) slides were 1.9%, 2.85%, and 3.7% for donor A; 2.35% for donor B; 3.45% and 3.9% for donor C; and 1.9% for donor D.

and a separate linear-quadratic dose response to $CD56^+$ cells, except that the quadratic term is common to all three cell types. The parameters δ_α and δ_β are the differences in intercept and slope, respectively, between the joint $CD3^+/CD8^+$ and $CD56^+$ responses. Estimated values of the regression parameters are shown in Table 3 along with their standard error estimates obtained under the assumed correlation model defined in the previous section (see "Statistical analysis"; the estimated correlation parameter was .28).

It is of particular interest to note from the fitted model that there was no statistically significant evidence of any difference between $CD3^+$ and $CD8^+$ dose responses (this may be confirmed by examining Figure 1), but the $CD56^+$ cells exhibited a smaller linear term (0.0935 – 0.0615 = 0.032 versus 0.093 micronuclei per cell per gray; $p < .001$), suggesting that the $CD56^+$ cells are more radioresistant than the other two cell types. In addition, the background frequency of micronuclei in $CD56^+$ cells (0.0292 + 0.0264 = 0.0556 micronuclei per cell) was about twice that of the other two cell types (0.0292 micronuclei per cell; $p < .001$).

Further refinement of the model to include separate dose responses for individual donors resulted in a substantially better fit to the observed data (the deviance decreased from 268.13 to 100.82 with a concomitant decline of 15 in degrees of freedom; fitted model not shown). Although there was a significant improvement in the fit of the model when allowing for differences among donors, these differences were similar to what we observed when separate fits were made

to replicated studies on donor A alone. Thus we cannot conclude anything regarding individual heterogeneity in this study (see the Discussion).

Discussion

Fujiwara et al.[12] determined the proportion of Leu1 (pan T), Leu2a (suppressor/cytotoxic T), Leu3a (helper/inducer T), and HLA-DR (B and monocyte) cells in 104 Adult Health Study participants in Hiroshima, including 75 atomic bomb (A-bomb) survivors and 29 distally exposed controls. They found no effect of radiation exposure on the proportions of subpopulations. Their findings are very important because they show that immunological balance has been maintained in the A-bomb survivors by the interactions between lymphocyte subpopulations. In addition, their findings suggest that there is no difference in radiation sensitivity between lymphocyte subpopulations examined or that a difference, if any exists, may no longer be detectable some 40 years after the exposures.

Stewart et al.[6] demonstrated unusual radiosensitivities of human $CD8^+$ and Leu19 cells. When $CD4^+$ and $CD8^+$ cells were stimulated with PHA, both types of cells exhibited similar radiosensitivity. Nakamura et al.[13] also showed similar X-ray sensitivities of PHA-stimulated $CD4^+$ and $CD8^+$ cells as determined by an in vitro lymphocyte colony assay.

In the present study, dose responses of micronucleus induction were compared in $CD3^+$, $CD8^+$, and $CD56^+$ cells that were exposed to X rays before PHA stimulation. Micronuclei were detected in interphase cells that had undergone one cell division. To discriminate between dividing and nondividing cells, Fenech and Morley advocated blocking cytokinesis with cytochalasin B. The micronuclei can then be scored in binucleated cells after the first mitosis.

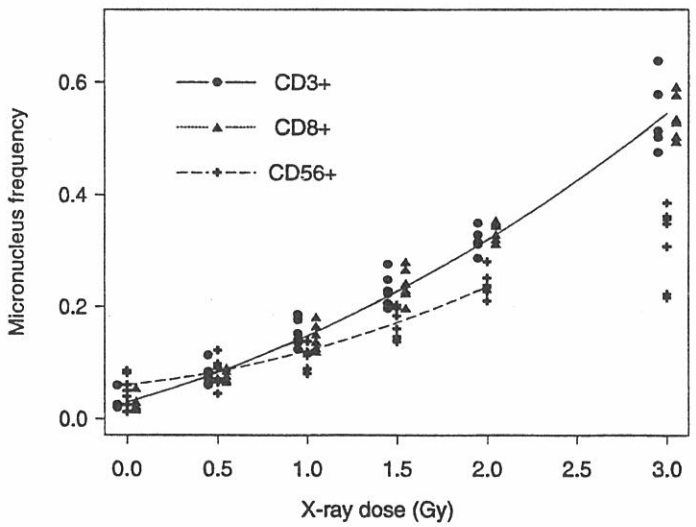

Figure 1. Observed micronucleus frequencies and estimated dose responses from model (3). Data are from all donors combined; $CD56^+$ points at 3 Gy were not used in fitting the curves. Some of the symbols have been shifted slightly to the right or left of their dose to enhance their legibility.

Table 3. Estimated regression parameters for individual cell-type dose-response model (3)

Term	Parameter*	Estimate	*SE*
Intercept ($CD3^+$, $CD8^+$)	α	.0291	.0020
Difference in intercept ($CD56^+$)	δ_α	.0308	.0065
Slope ($CD3^+$, $CD8^+$)	β	.0917	.0047
Difference in slope ($CD56^+$)	δ_β	−.0575	.0057
Quadratic coefficient (all cell types)	λ	.0267	.0018
Indicator ($CD56^+$, 3.0 Gy)	ζ	−.1136	.0275

*See Equation (3) and the subsequent discussion for a definition and interpretation of these parameters.

Comparison of micronucleus frequencies after exposure to X rays shows that $CD3^+$ and $CD8^+$ cells exhibited quite similar radiosensitivities. In comparison, $CD56^+$ cells were significantly less sensitive than $CD3^+$ and $CD8^+$ cells. This could result from differences in the spatial arrangements of chromosomes during repair processes. Multinucleated cells having more than five nuclei were occasionally observed in $CD56^+$ subpopulations but not in $CD3^+$ and $CD8^+$ subpopulations. Many nuclei in binucleated $CD56^+$ cells were not circular. This suggests that the nucleoid structure in $CD56^+$ cells might be different from those in $CD3^+$ and $CD8^+$ cells. Different forms of chromosomal arrangements could cause a significant difference in micronucleus induction between the $CD56^+$ cells and other types of lymphocytes, such as through a smaller interaction between breaks produced by independent tracks in $CD56^+$ cells than in $CD3^+$ or $CD8^+$ cells. Our hypothesis could be similar to that reported by Gordon et al.[14] They suggested that the specific structural differences between the nucleoids derived from either Chinese hamster–V79-monolayer or spheroid cultures could correlate with the different radiosensitivities observed between the two types of cells.

Another possible explanation for the low sensitivity of $CD56^+$ cells is an in vitro selection by which a radioresistant fraction of the $CD56^+$ cell population would enter into interphase through the first mitosis. Only the small fraction of $CD56^+$ cells that underwent one division within 72 hr was tested (Tables 1 and 2). For this reason, the lower sensitivity of $CD56^+$ cells was not reflected in the radiosensitivity of $CD3^+$ cells.

We are puzzled by the lack of fit of data generated by the linear-quadratic model to the $CD56^+$ micronucleus frequency at 3 Gy. The downturn at 3 Gy with $CD56^+$ cells could be due to damaged cells not surviving to express micronuclei. We thought it possible that the dose response for $CD56^+$ might be linear, but a strictly linear model, including the 3.0-Gy points, fit significantly worse than the linear-quadratic model (with increase of deviance of 6.23 on 1 degree of freedom) and had a nearly twofold higher linear coefficient, both evidence that a strictly linear dose response does not adequately fit the $CD56^+$ data. Although such downturns in dose response are often suggestive of cell killing in chemical mutagenesis assays, we have no clue as to its cause in the present study.

Individuals vary widely in the radiation sensitivity of their body tissues. An in vitro radiosensitivity assay using numerous skin fibroblast strains demonstrated this wide variation.[15,16] It is very important to know if persons with a high sensitivity to radiation damage also have a high risk of cancer or other diseases due to occupational or accidental exposure to radiation. In a previous study, we did not find any evidence that A-bomb radiation preferentially induced breast cancer in women whose skin fibroblast cells in vitro were more sensitive to cell killing by radiation, although the sample was not large.[17] Although it has become important to assess individual susceptibility to ionizing radiation in the general population, assessing the radiosensitivity of skin fibroblasts with the colony-formation assay is at present too laborious and time-consuming a task to use with large populations. On the other hand, the micronucleus assay using human lymphocytes is a simpler, less expensive, and also more sensitive method of biological dosimetry than the chromosome-aberration assay[18] and the colony-formation assay.[19]

We did not determine the radiosensitivity of $CD4^+$ cells. However, the lack of a difference in radiosensitivity between $CD3^+$ and $CD8^+$ cells suggests that $CD4^+$ cells should not have an unusual radiosensitivity. Although $CD56^+$ cells exhibited reduced sensitivity under the current culture circumstances, they constituted a small fraction of lymphocytes. This report therefore confirms that sorting lymphocyte subpopulations should not be necessary for the determination of an individual's radiosensitivity using the micronucleus assay.

It is difficult to draw any conclusions regarding heterogeneity in radiosensitivity using the present data, because there are few repeated determinations of the dose response among donors. Differences among donors' dose-response curves were of similar magnitude to differences between replicated experiments within a single donor. Thus, the heterogeneity we observed among the donors in this study might be due to random variation. A careful study of heterogeneity in individual susceptibility to radiation would require a much larger number of individuals with concurrent measurement of factors possibly related to radiation sensitivity. Such a study is currently underway.

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