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Is Variation in Human Radiosensitivity Real or Artifactual? A Study by Colony Formation Method Using Peripheral Blood T-lymphocytes

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放影研 **Radiation Effects Research Foundation**
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1989年から，放射線影響研究所の業績報告書は，従来の日英両文を併記した方式では発行しない．主要な報告書については，今後も日英両文で印刷するが，それぞれ別に発行する．内容が高度に専門的であり，一般の関心が少ないと思われる報告書については英文のみとし，日本文の要約を添付する．

これにより，広島・長崎の原爆電離放射線被曝の人体に及ぼす晩発性生物学的影響に関する最近の知見を今までよりも速やかにお知らせできることと思う．

The Radiation Effects Research Foundation (formerly ABCC) was established in April 1975 as a private nonprofit Japanese Foundation, supported equally by the Government of Japan through the Ministry of Health and Welfare, and the Government of the United States through the National Academy of Sciences under contract with the Department of Energy.

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業　績　報　告　書

RERF TR 15-89
研究課題　3-86

ヒトの放射線感受性の多様性の真偽：末梢血リンパ球を用いたコロニー形成法による調査§

Is Variation in Human Radiosensitivity Real or Artifactual? A Study by Colony Formation Method Using Peripheral Blood T-lymphocytes

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要　約

試験管内のヒトTリンパ球コロニー形成に関する二つの方法，並びにその方法を用いたヒトの放射線感受性の個人差に関する調査としての線量生存率実験について記す．はじめの方法(方法1)では非照射リンパ球のコロニー形成率(CE)は10%～40%であった．実験条件の改良によりCEは30%以上となった(方法2)．試験管内でのX線照射を行った場合，90%の細胞を殺すのに必要な線量(D_{10})は，方法1では，同一対照人由来リンパ球の18回の反復実験の結果，2.87±0.28 Gy (平均±SD)であった．方法2では，D_{10}は同一対照人の28回反復実験の結果3.66±0.21 Gyと大きくなり，また異なる31人の結果は3.58±0.19 Gyであった．これらの同一人の反復調査結果と，異なる人についての1回ずつの調査結果を分散分析すると，後者で変動が有意に大きいということはなかった．この結果は少なくともG_0期リンパ球に照射してコロニー形成能力の喪失を指標として調査する限りは，ヒトの放射線感受性の多様性は，もしあるとしても大変小さなものでしかないという仮説を支持するものである．

§本報告にはこの要約以外に訳文はない．

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Is Variation in Human Radiosensitivity Real or Artifactual? A Study by Colony Formation Method Using Peripheral Blood T-lymphocytes[§]

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Summary

Two methods of producing human T-lymphocyte colonies in vitro are described, as well as dose-survival experiments using these methods for the investigation of possible differential radiosensitivity among individuals. In one method, the cloning efficiency (CE) of nonirradiated lymphocytes was between 10% and 40% (method 1), whereas subsequent improvement in assay conditions (method 2) resulted in a CE greater than 30%. In vitro X-irradiation of colonies produced using method 1 revealed that the dose required to kill 90% of the cells (D_{10}) was 2.87 ± 0.28 Gy (mean ±SD, n = 18) for repeated examinations of lymphocytes from one reference individual. Using method 2, the D_{10} values were greater, viz., 3.66 ± 0.21 Gy for 28 repeated tests of the same reference individual and 3.58 ± 0.19 Gy for 31 different individuals. Analysis of variance to compare the data from repeated examinations of one person versus data from single examinations of different persons showed that variation in the D_{10} value was not significantly greater in the latter group. These results support the hypothesis that individual variation in human radiosensitivity is quite small, if it exists at all, as far as can be determined by the loss of colony-forming ability of irradiated G_0 lymphocytes.

Introduction

The epidemiologic data derived from the atomic bomb survivors in Hiroshima and Nagasaki serve as a very important source of information for assessing the risk of late effects induced in human populations by exposure to ionizing radiation.[1] However, to fully understand the relationship between exposure to radiation and subsequent effects, it is a prerequisite that the A-bomb survivors be representative of a normal population in terms of radiosensitivity, and, if they are not, to characterize the extent to which they deviate from normality.

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

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Usually, skin fibroblasts have been used for studies of individual variation in radiosensitivity. However, ethical problems related to obtaining skin biopsies from healthy people are a major drawback of this approach. Recently, because of the discovery of the T-cell growth factor (interleukin-2), it became possible to grow peripheral T-lymphocytes in vitro.[2–6] A major advantage of using T-lymphocytes instead of skin fibroblasts is the much less invasive nature of obtaining blood lymphocytes from many healthy individuals. This report describes two methods for human T-lymphocyte colony formation and the results of dose-survival experiments using these methods which investigate the possibility of differential radiosensitivity among individuals.

Materials and Methods

Donors

In the present study, a total of 32 individuals was examined. Among these, 22 were patients who had undergone surgery for various reasons at the Second Department of Surgery, Hiroshima University School of Medicine. The other 10 were healthy laboratory volunteers. One of these, a healthy 42-year-old man, served as a reference man for repeated examinations. The age of the blood donors ranged from 20–72 years old.

Lymphocytes

Peripheral blood was defibrinated by using glass beads, and the mononuclear cells were isolated by Ficoll/Hypaque density gradient centrifugation.[7] It was confirmed that more than 90% of the cells were lymphocytes. The cells were suspended with Earle's balanced salt solution containing 2.5% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin, after which they were washed three times by centrifugation and then were used for irradiation experiments.

Colony formation

In this study, two methods were used, which differ in the type of feeder cells and serum used. In the first method (method 1), allogeneic lymphocytes and Raji cells were used as feeder cells with 10% FCS, whereas in the subsequent improved method (method 2), allogeneic lymphocytes and OKIB, another B-cell line, were used with 9% FCS plus 1% human serum. OKIB is a lymphoblastoid cell line from a leukemic patient established spontaneously in our laboratory. Both cell lines were verified to be mycoplasma-free.

Allogeneic lymphocytes obtained from three to five laboratory volunteers were separated and stored under liquid nitrogen. After thawing, the cells were washed twice and used as mixed feeder cells after exposure to 50 Gy of X rays. For B-cells, OKIB or Raji cells were employed after exposure to 50 or 100 Gy of X rays, respectively.

The results of our pilot study showed that radiosensitivity does not differ whether the lymphocytes were irradiated shortly before or soon after addition of

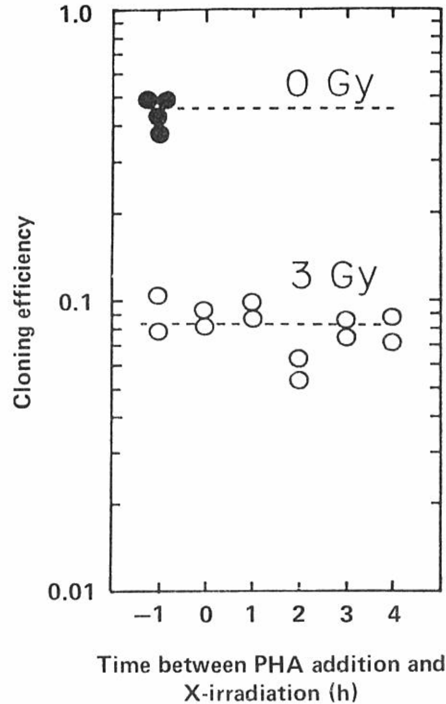

Figure 1. Using method 2, the effect on cloning efficiency (CE) of the time interval between the addition of PHA to the lymphocyte culture and X-irradiation. Closed circles = CE of nonirradiated cells, open circles = CE of 3-Gy-irradiated cells.

phytohemagglutinin A (PHA) to the culture (Figure 1). Thus, for convenience in the present study, the irradiation was performed immediately after the cells were plated in microwells with PHA (at most within one hour).

In each well of the 96-well microplates (round bottom, Costar #3596, Cambridge, Mass, USA), irradiated feeder cells (i.e., 2×10^4 allogeneic lymphocytes and 1.5×10^4 B-cells) were distributed in 0.1 ml of growth medium which consisted of MEM supplemented with 1% nonessential amino acids, 24 mM HEPES, 1% L-glutamine, 1:2000 PHA-P, and 10% FCS or 9% FCS plus 1% human serum of any ABO type.

On the next day, lymphocytes to be tested were isolated, counted after staining of mononuclear cells by Türk's solution and distributed to wells containing feeder cells in 0.1 ml of the growth medium supplemented with no PHA, but with 0.4 ng/ml of recombinant interleukin-2 (rIL-2) which was kindly provided by Takeda Pharmacy Ltd., Tokyo. Thus, the final concentrations of PHA-P and rIL-2

were 1:4000 and 0.2 ng/ml, respectively. Lymphocytes to be examined for their radiosensitivity were seeded to each well, and the average number varied from 2–2,400 in method 1 and from 2–70 in method 2, depending on the X-ray dose. One plate was used for each dose level. Immediately after the seeding, cells in the plates were X-irradiated. After incubation for one week under 5% CO_2 and 95% air at 37°C, 0.1 ml of medium was removed from each well and fresh growth medium containing a double strength of rIL-2 was added. Two weeks after irradiation, the presence or absence of lymphocyte colonies was determined for each well by means of an inverted microscope. Cloning efficiency (CE) was calculated using the formula

$$CE = \frac{-\ln\text{(fraction of wells without a colony)}}{\text{average number of lymphocytes seeded per well}} .$$

Survival curves were obtained by fitting the data to a linear quadratic equation, $-\ln(CE) = aD + bD^2 + c$, using an unweighted least squares method. Based on the a and b, a dose required to kill 90% of the cells (D_{10}) was calculated. The surviving fraction was obtained as the CE of each irradiated plate divided by that at dose zero of each fitted curve, and it was used for the summary of experiments using methods 1 and 2 and for interindividual comparisons.

X-irradiation

A Shimadzu WSI-250S machine was operated at 220 kVp, 8 mA with a 0.5-mm Al and 0.3-mm Cu filter. In the experiments using method 1 (see below), total irradiation time was calculated based on the previously measured dose rate at a distance of 70 cm to provide from 1–5 Gy (4.38 minutes for 1 Gy). However, direct dose measurements later revealed that the actual doses were about 10% in excess possibly due to scattering of X rays by the 96-well plates themselves. Accordingly, the doses delivered were estimated to be 1.10, 2.20, 3.29, 4.38, and 5.48 Gy. In experiments using method 2, the probe of a Victoreen 500 dosimeter (Victoreen Inc., Cleveland, Ohio, USA) was placed between three plates at each X-irradiation to give 1.0, 2.0, 3.0, 4.0, and 5.0 Gy. The dose rate was about 0.25 Gy/min. The dosimeter was calibrated at the Research Institute for Nuclear Medicine and Biology of Hiroshima University. Cells were irradiated at room temperature.

Statistical analysis

The data from method 2 were used to test the null hypothesis that radiation sensitivity among individuals does not differ. These data comprise D_{10} values from 28 repeated observations of one individual and single determinations of the D_{10} value of 31 individuals. The log D_{10} values are approximately normally distributed.

This null hypothesis is equivalent to assuming that all individual log D_{10} values are drawn from the same normal distribution with mean U and variance V, which is measurement error. The alternative hypothesis, i.e., that radiation

sensitivity does differ, is equivalent to assuming that the log D_{10} values are obtained from a normal distribution with variance V but with means U_i depending on the individual i. If one assumes in addition that the U_i come from a normal distribution with variance W, where $W > 0$, this is equivalent to a one-way analysis of variance with random effects. Thus, the hypothesis of interest,

$$H_0: W = 0, \text{ versus } H_1: W > 0$$

is tested using an F statistic with 31 and 27 degrees of freedom.

An upper 95% confidence limit for the CV of the individual variation ($CV = 100\% \times \sqrt{W}$) was calculated using methods similar to those of Graybill.[8]

Results

Use of Raji as feeder cells (method 1)

Results for 18 repeated tests of one individual are shown in Figure 2. The CE of nonirradiated cells was between 10% and 40% and the mean $\pm$ standard deviation (SD) of D_{10} values was 2.87 ± 0.28 Gy. As reported previously,[2] the curve shows a large shoulder and is continuously bending, thus estimates of n and D_0, based on a single-hit, multitarget model, were not made.

Improved method (method 2)

It was found that colonies are quite large and easy to score when an autologous system was included for other purposes. This system consisted of an Epstein-Barr virus-transformed (EBV-transformed) autologous B-cell line, autologous lymphocytes as feeder cells, and 5% autologous human serum in place of 10% FCS. This finding prompted us to improve the assay system. The first modification was to replace Raji with another lymphoblastoid cell line, OKIB. Raji cells remained at the center of each round-bottom microwell as a large aggregate even after two weeks' incubation, and they greatly interfered with visual observation of the growing lymphocytes. In contrast, OKIB or other EBV-transformed B-cell lines dispersed as smaller aggregates, which rendered the observation of lymphocyte growth easier. In addition, we supplemented the medium with a small amount of human serum of no special ABO type. It was found that the addition of 1% human serum not only increased the CE (Figure 3) but also the colony size considerably (data not shown). Further improvement of CE or colony size was not observed at higher concentrations of human serum, thus 9% FCS plus 1% human serum were used in the following experiments. The effect of 1% human serum appeared to be the same whether it was autologous and fresh, autologous but stored for five months at 4°C, or pooled from six individuals and stored at 4°C for five months (data not shown).

With this improved method, repeated examinations for one standard reference individual were conducted. The CE of nonirradiated lymphocytes was greatly increased (ranging from 30%–65%), and the mean $\pm$SD of D_{10} values for 28 repeats was 3.66 ± 0.21 Gy. The average survival curve is shown in Figure

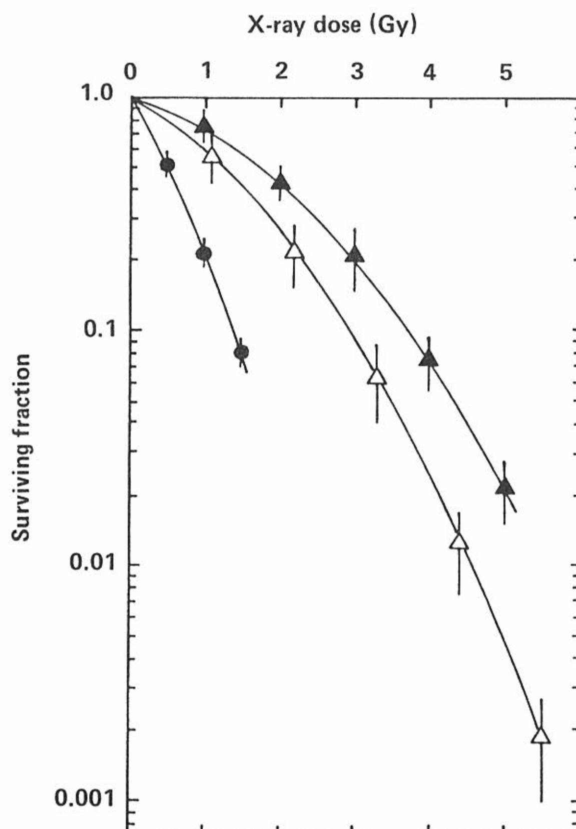

Figure 2. Summary of the dose-survival curves of G_0 lymphocytes from one donor (△ and ▲). Cells were repeatedly obtained for examination and each point represents the mean of 18 experiments using method 1 (△) and of 28 experiments using method 2 (▲). Also shown is the dose-response curve of lymphocytes from one ataxia telangiectasia patient by using method 2 (●). (Bars are the standard deviation of the mean.)

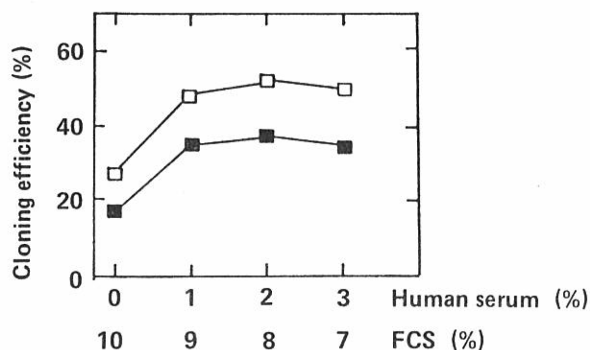

Figure 3. Effect of human serum on the cloning efficiency of nonirradiated G_0 lymphocytes. Different symbols represent different lymphocyte donors.

Compared to the results presented for method 1, D_{10} values increased by 28% and more importantly, the reproducibility became better, viz., the coefficient of variation (CV) substantially decreased from 9.8% to 5.7%. Characteristically, the dose-response curve was still continuously bending up to 5 Gy (Figure 2).

Also shown in Figure 2 is the dose-response curve of lymphocytes, obtained from a patient with ataxia telangiectasia (AT), using this improved method. As reported previously,[3,5] resting lymphocytes from AT patients are distinctively radiosensitive to the killing effect of ionizing radiation at a D_{10} value of 1.39 Gy.

Evaluating variations in individual radiosensitivity

Results of dose-survival curves for 28 repeated observations of a single donor and for 31 assays of different individuals are shown in Figure 4. The original data for this figure are presented in Table 1 for 28 repeats of one individual and in Table 2 for 31 assays of different individuals. The two sets of data are very similar. The analysis of variance table for the test of no difference in radiosensitivity, viz., in the D_{10} value, is shown in Table 3. The F test was not significant ($P > 0.25$). The 90% upper confidence limit is 4.7% for the CV for different individual D_{10} values. Given the results of present experiments, it would be very unlikely for the true CV among individuals to be greater than 4.7%.

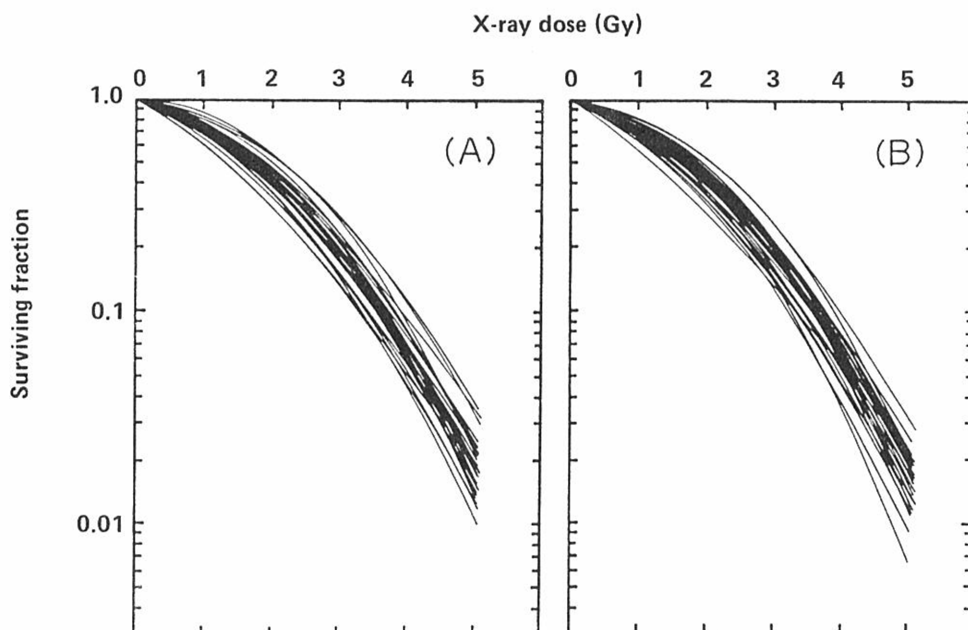

Figure 4. Dose-survival curves of lymphocytes irradiated at G_0. Results of 28 repeated tests for lymphocytes from a single donor (A) and results of a single test on each of 31 individuals (B).

Table 1. The cloning efficiency and the D_{10} value of lymphocytes irradiated at the G_0 stage, obtained from a reference donor

	X-ray dose (Gy)						
Expt.	0	1	2	3	4	5	D_{10} (Gy)
1	0.49	0.45	0.19	0.063	0.031	0.011	3.37
2	0.58	0.38	0.17	0.091	0.039	0.011	3.49
3	0.76	0.46	0.35	0.16	0.049	0.021	3.78
4	0.41	0.36	0.19	0.15	0.046	0.015	4.18
5	0.44	0.24	0.20	0.097	0.034	0.0099	3.86
6	0.35	0.29	0.18	0.066	0.032	0.0090	3.81
7	0.39	0.38	0.17	0.094	0.036	0.0096	3.84
8	0.39	0.46	0.26	0.091	0.045	0.0090	3.52
9	0.43	0.31	0.18	0.094	0.040	0.015	3.92
10	0.38	0.38	0.15	0.13	0.046	0.012	4.09
11	0.38	0.25	0.17	0.069	0.022	0.0065	3.60
12	0.29	0.24	0.12	0.063	0.023	0.0049	3.72
13	0.65	0.48	0.25	0.11	0.048	0.011	3.60
14	0.81	0.46	0.27	0.104	0.055	0.0080	3.45
15	0.53	0.41	0.27	0.084	0.031	0.0090	3.55
16	0.46	0.33	0.19	0.075	0.033	0.0096	3.61
17	0.36	0.24	0.151	0.078	0.019	0.0072	3.64
18	0.34	0.27	0.12	0.078	0.022	0.0074	3.65
19	0.37	0.41	0.16	0.087	0.027	0.0093	3.71
20	0.48	0.32	0.25	0.13	0.034	0.011	3.89
21	0.38	0.26	0.17	0.036	0.021	0.0056	3.28
22	0.63	0.46	0.19	0.16	0.031	0.0095	3.55
23	0.50	0.32	0.22	0.091	0.038	0.0077	3.69
24	0.57	0.35	0.22	0.104	0.029	0.0082	3.53
25	0.46	0.32	0.162	0.060	0.029	0.0077	3.41
26	0.48	0.34	0.26	0.084	0.031	0.0148	3.71
27	0.379	0.269	0.140	0.0575	0.0187	0.00431	3.40
28	0.505	0.357	0.224	0.0974	0.0252	0.00770	3.55

Table 2. The cloning efficiency and the D_{10} value of lymphocytes irradiated at the G_0 stage, obtained from 31 different individuals

	X-ray dose (Gy)						
Donor code	0	1	2	3	4	5	D_{10} (Gy)
Lym #56	0.34	0.33	0.15	0.078	0.017	0.0056	3.57
#57	0.43	0.32	0.19	0.055	0.020	0.0074	3.33
#58	0.39	0.35	0.16	0.078	0.029	0.0085	3.67
#59	0.49	0.28	0.22	0.084	0.030	0.0047	3.59
#60	0.60	0.57	0.28	0.12	0.043	0.0077	3.63
#61	0.57	0.36	0.23	0.097	0.036	0.0088	3.58
#62	0.58	0.39	0.21	0.115	0.036	0.0099	3.61
#63	0.41	0.35	0.17	0.052	0.014	0.0054	3.18
#64	0.29	0.24	0.11	0.044	0.017	0.0051	3.44
#65	0.53	0.45	0.23	0.11	0.043	0.014	3.76
#67	0.37	0.24	0.17	0.078	0.018	0.0060	3.61
#68	0.40	0.36	0.19	0.058	0.032	0.0074	3.58
#69	0.49	0.38	0.22	0.066	0.021	0.0070	3.33
#70	0.41	0.41	0.15	0.075	0.031	0.0085	3.58
#71	0.43	0.41	0.19	0.11	0.022	0.0096	3.64
#72	0.58	0.45	0.23	0.12	0.041	0.011	3.67
#73	0.46	0.38	0.28	0.087	0.053	0.011	3.92
#74	0.50	0.45	0.27	0.091	0.044	0.012	3.76
#75	0.35	0.35	0.11	0.055	0.014	0.0024	3.26
#76	0.37	0.25	0.17	0.081	0.023	0.0072	3.71
#77	0.44	0.28	0.16	0.072	0.015	0.0072	3.31
#78	0.49	0.43	0.26	0.104	0.032	0.012	3.72
#79	0.41	0.29	0.15	0.072	0.023	0.0088	3.41
#80	0.57	0.44	0.33	0.104	0.040	0.013	3.72
#81	0.55	0.41	0.20	0.099	0.031	0.0072	3.50
#82	0.38	0.30	0.21	0.097	0.034	0.012	3.98
#83	0.49	0.35	0.28	0.094	0.031	0.013	3.73
#84	0.53	0.46	0.20	0.104	0.025	0.011	3.47
#85	0.65	0.35	0.21	0.087	0.033	0.016	3.35
#86	0.32	0.24	0.135	0.044	0.017	0.0054	3.40
#87	0.40	0.36	0.18	0.104	0.027	0.0099	3.77

Table 3. Analysis of variance for the Log D_{10} value obtained using method 2

Source of variation	SS	df	MS
Due to individuals	0.09066	31	0.002925
Due to measurement error	0.09066	27	0.003358
Total	0.18132	58	

$F = \frac{0.002925}{0.003358} = 0.87$ Not significant ($P > 0.25$)

Discussion

The present results showed that, when using lymphocyte assay for assessing variation in human radiosensitivity, the culture conditions are quite important. In method 1, the CE was usually between 10% and 40% (mean $\pm$SD = 18 $\pm$ 10%), and the resulting average D_{10} value was 2.87 Gy, as estimated from repeated examination of one individual. However, in a subsequent improved assay, the CE was generally higher than 30% (average $\pm$SD = 47 $\pm$ 11%), and the D_{10} value increased to 3.66 Gy, based on 28 repeats of the same individual. More importantly, the reproducibility substantially increased: the CV of the mean D_{10} value decreased to nearly one-half. The increase of D_{10} values in method 2 was most probably due to increased colony size caused by the addition of 1% human serum and due to replacing feeder cells with OKIB which made colony observation easier. These modifications appear to have improved the reproducibility of the assay.

The similarity among the amount of apparent variation (Figure 4) in the lymphocyte survival curves for the repeated examination of one reference individual and for the 31 different individuals strongly suggests that any intrinsic individual variation in radiosensitivity may be quite small, as far as lymphocytes irradiated at G_0 stage are concerned. Indeed, calculation of the 95% upper confidence limit for the CV for different individual D_{10} values suggests that it is very unlikely that the true CV among individuals exceeds 3.6%. Thus, the apparent difference in the dose-survival curves among individuals (Figure 4) could be largely attributable to experimental errors.

In the studies using skin fibroblasts, the CV of the mean for D_0 (the mean lethal dose) is reportedly 10%–20% for repeated tests of some cell strains[9–11] and for a number of different strains.[9,11,12] What is the cause of such different variability in radiosensitivity between lymphocytes and fibroblasts? Perhaps expression of the repair function may differ among the cell types studied. Or maybe the fibroblast assay involves some biases introduced during in vitro culture, and alternatively the lymphocyte assay involves some factors which result in reduction of individual variation. Although it is well established that circulating lymphocytes die via interphase death in vivo after irradiation,[13] it is highly unlikely to occur now during in vitro colony assay. This is mainly because 1) the surviving fraction of G_0 lymphocytes irradiated and incubated for up to two days before PHA stimulation remained essentially the same (Nakamura, manuscript in preparation), 2) G_0 lymphocytes from AT patients are as radiosensitive as their skin fibroblasts,[3,5] and 3) the RBE for ^{252}Cf neutrons was 3 or greater for lymphocyte cell killing (Nakamura, unpublished observation)—similar to the values reported for other cultured cells[14] for which mitotic death is the common mechanism.

The possibility that the 31 individuals examined here are somehow biased and are not representative of the general population is unlikely. Currently, more than 100 individuals have been tested for lymphocyte radiosensitivity using method 2,

and the CV of the mean D_{10} value was about 7% (Nakamura et al., unpublished observation), quite similar to that of the present results based on 31 individuals.

Lastly, might the Japanese population be genetically more homogeneous than Caucasians? The fibroblast results discussed above are mainly for cells derived from Caucasians. Our recent results for paired samples of lymphocytes and fibroblasts from the same 22 individuals showed that the CV of the mean D_{10} value for the fibroblasts was 11%, about twice as large as that for the lymphocytes.[15] Therefore, it appears that the greater interindividual variability of the dose-survival curve is inherent to the fibroblast assay itself.

However, one unsolved question relates to whether the variability of lymphocyte radiosensitivity increases when lymphocytes are irradiated at the log phase. In most of the cases fibroblast data were collected for cells irradiated at the growth phase; whereas in the present study, lymphocytes were at the resting G_0 stage at the time of irradiation. A comparative dose-survival study of lymphocytes at G_0 and at a growth stage will be conducted in the near future.

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