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Radiosensitivity of CD4 and CD8 Positive Human T Lymphocytes by an In Vitro Colony Formation Assay

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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．

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研究課題　3-86

試験管内コロニー形成法による CD 4 及び CD 8 陽性ヒトリンパ球の放射線感受性§

Radiosensitivity of CD4 and CD8 Positive Human T Lymphocytes by an In Vitro Colony Formation Assay

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要　約

最近の試験管内リンパ球コロニー形成法の発展により，従来用いられていた皮膚線維芽細胞の代わりに，末梢血リンパ球（PBL）を用いて，ヒトの放射線感受性の多様性を検査する道が開かれた．我々の最近の結果では，コロニーの大半はCD4あるいはCD8抗原陽性のリンパ球から成ることが分かった．PBL中の$CD4^+$及び$CD8^+$の細胞の割合は，個人により異なっているので，もしも$CD4^+$と$CD8^+$の細胞の線量生存率曲線が互いに異なっていれば，サブセット頻度の違いが原因で，個人の放射線感受性に偏向がもたらされるおそれが考えられた．

今回の調査では，$CD4^+$（ヘルパー／インデューサーT）及び$CD8^+$（サプレッサー／サイトトキシックT）リンパ球をPBLから分離し，各々について線量生存率曲線を得た．その結果は，生存率を10％に減少させる線量（D_{10}）は，これらの細胞で大変よく似ていることが分かった（平均±SDは$CD4^+$では3.13±0.10 Gy，$CD8^+$では3.34±0.50 Gy，分離しなかった細胞では3.07±0.05 Gy）．このことは，放射線感受性の異なるヒトのスクリーニングにPBLをそのまま用いても構わないことを示している．

§本報告にはこの要約以外に訳文はない．

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Radiosensitivity of CD4 and CD8 Positive Human T Lymphocytes by an In Vitro Colony Formation Assay[§]

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Summary

The recent development of an in vitro lymphocyte colony assay provides a new opportunity to examine possible variations in human radiosensitivity using peripheral blood lymphocytes (PBL) in place of the hitherto used skin fibroblast assay. Our recent study showed that most of the colonies consisted of lymphocytes bearing CD4 or CD8 antigens. Since the fraction of $CD4^+$ and $CD8^+$ cells in PBL differs among individuals, it was suspected that individual radiosensitivity might be biased by the different subset frequencies if the dose-survival curves of the $CD4^+$ and $CD8^+$ cells differed.

In the present study, $CD4^+$ lymphoctyes (helper/inducer T cells) and $CD8^+$ lymphocytes (suppressor/cytotoxic T cells) were isolated from PBL and their dose-survival curves were determined. The results showed that the D_{10} (the dose required to reduce the surviving fraction to 10%) was quite similar for these two types of cells (3.13 ± 0.10 Gy [mean ±SD] for $CD4^+$, 3.34 ± 0.50 Gy for $CD8^+$ and 3.07 ± 0.05 Gy for the unsorted cells), supporting the use of a whole PBL population for screening of individuals with altered radiosensitivity.

Introduction

Due to the discovery of interleukin-2 (IL-2), it has now become possible to grow colonies of human lymphocytes from peripheral blood. Compared with skin fibroblasts, PBLs are far easier to obtain. We have therefore decided to use lymphocytes in place of skin fibroblasts for evaluating possible individual human variation in radiosensitivity.[1] Our recent studies show that it is the lymphocytes bearing CD4 markers (helper/inducer T cells) or CD8 markers (suppressor/cytotoxic T cells) that mainly form colonies under the current

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

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culture conditions.[2] Also no significant correlations were observed between each lymphocyte subset frequency in blood used for the dose-survival experiments and a parameter of the resulting survival curve, viz., the D_{10} (the dose required to reduce the cloning efficiency to one-tenth) for 22 individuals.[3] Since lymphocyte subsets of each colony were not determined in the latter experiments, we needed to clarify whether the $CD4^+$ and $CD8^+$ lymphocytes show similar radiosensitivity. If not, it would be necessary for the radiosensitivity study to use isolated lymphocytes of a specific subset so that the results would not be biased by different proportions of each lymphocyte subset among different individuals. We know of no other studies comparing the radiosensitivity of $CD4^+$ and $CD8^+$ lymphocytes using such a colony formation method.

In the present study, PBLs were sorted according to their CD4 or CD8 surface markers, and in vitro radiosensitivity was subsequently measured using the colony-formation method.

Materials and Methods

Medium and feeder cells

The culture medium consists of MEM supplemented with 1% nonessential amino acids, 24 mM HEPES, 1% L-glutamine, 1:4000 PHA-P, 9% FBS, 1% human serum, and 0.2 ng/ml recombinant IL-2.[1] As feeder cells, freshly isolated allogeneic lymphocytes from three donors and a B-cell line, OKIB, were irradiated with 50 Gy of X rays and were seeded in 96-well round bottom microplates (2×10^4 and 1.5×10^4/wells, respectively) with 0.1 ml of medium.[1]

Lymphocytes

PBLs were separated by using glass bead defibrination and Ficoll-Hypaque separation, as described previously.[4] After washing twice with Earle's balanced salt solution containing 2.5% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, the cells were stained with a phycoerythrin-labeled Leu3a (CD4) antibody and a FITC-labeled Leu2a (CD8) antibody (Becton Dickinson Immunocytometry Systems, Sunnyvale, Calif). Subsequently, the cells bearing either CD4 or CD8 surface antigens were sorted using a FACSTAR (Becton Dickinson Immunocytometry Systems, Sunnyvale, Calif). Figure 1A shows the windows used to sort $CD4^+$ or bright $CD8^+$ lymphocytes. A portion of the sorted $CD4^+$ or $CD8^+$ cells was used for the dose survival experiments, while the remaining component was tested for purity. As shown in Figure 1B and C, more than 97% of the sorted cells were found to carry the CD4 or the CD8 antigen.

The unsorted control lymphocytes (antibody treatment only) and the sorted $CD4^+$ or $CD8^+$ lymphocytes were counted after staining mononuclear cells by using Türk's solution. Before X irradiation, 2 to 70 cells were seeded with 0.1 ml of medium in each well of the 96-well microplates containing the feeder cells as described elsewhere in detail.[1]

Colony formation

The cells were incubated at 37°C in humidified 95% air/5% CO_2. After one week, 0.1 ml of the medium was removed from each well and fresh medium containing a double strength of recombinant IL-2 was added. Two weeks after the irradiation, the presence or absence of lymphocyte colonies was determined for each well using an inverted microscope. Cloning efficiency (CE) was calculated by the formula

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

To summarize the dose-response data, the surviving fraction (SF) was calculated. For this purpose, the survival data of each experiment were first fitted to a linear-quadratic equation, $-\ln CE = aD + bD^2 + c$ using an unweighted least squares method (a, b, and c are constants, D is X-ray dose in gray) to obtain fitted CE at zero dose. Since there is no reason to expect that the zero-dose CE observed was better estimated than that at any other dose, the ratio of CE observed for the irradiated cells to that fitted at zero dose for each dose-survival experiment was used as SF. D_{10}, the dose required to reduce the SF to 10%, was calculated after a and b of the fitted linear-quadratic equation.

X irradiation

Our previous studies demonstrated that the CE of 3 Gy-irradiated lymphocytes remained essentially unchanged whether X rayed from one hour before to four hours after the addition of PHA.[1] Therefore in the present study, graded doses of X rays were delivered immediately after the cells were seeded in microplates.

The X-ray machine (Shimadzu WSI-250S, Kyoto) was operated at 220 kVp, 8 mA with a 0.5-mm Al and 0.3-mm Cu filter, and the dose rate was about 0.25 Gy/min. The irradiation was performed at room temperature (approximately 15°C). Total dose was measured each time by a Victoreen 500 dosimeter (Cleveland, Ohio) which had been calibrated at the Research Institute for Nuclear Medicine and Biology of Hiroshima University.

Results

In Figure 1A are shown the windows used for sorting $CD4^+$ and bright $CD8^+$ lymphocytes. The fraction of lymphocytes falling in the two windows was 30%–50% of total lymphocytes for $CD4^+$ and 15%–20% for bright $CD8^+$ cells. As mentioned in the Materials and Methods section, more than 97% of the sorted cells were indeed $CD4^+$ or bright $CD8^+$ cells as shown in Figure 1B and C.

Summary of dose-survival data are presented in Table 1 and the dose-survival curves of the $CD4^+$, $CD8^+$, and control cells (antibody treatment only) are shown in Figure 2A, B, and C, respectively. The average CE of nonirradiated cells was 0.76 ± 0.69 (mean ±SD) for $CD4^+$ (n = 4), 0.33 ± 0.13 for $CD8^+$ (n = 4) and 0.41 ± 0.15 (n = 3) for the control cells. The fitted dose-survival curves were

expressed as $-\ln SF = 0.285\ D + 0.139\ D^2$ for $CD4^+$ cells, $-\ln SF = 0.182\ D + 0.143\ D^2$ for $CD8^+$ cells, and $-\ln SF = 0.255\ D + 0.147\ D^2$ for control cells, where D is X-ray dose in gray. The calculated D_{10} values ±SD were 3.13 ± 0.10 Gy for $CD4^+$ cells, 3.34 ± 0.50 Gy for $CD8^+$ cells, and 3.07 ± 0.05 Gy for control cells. The results clearly demonstrated that, in terms of colony-forming ability, $CD8^+$ suppressor/cytotoxic T cells in human peripheral blood were not more radiosensitive than $CD4^+$ helper/inducer T cells.

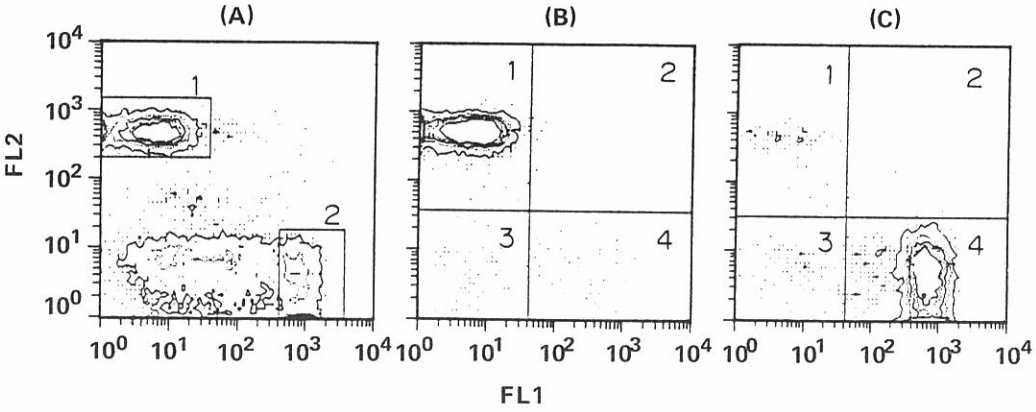

Figure 1. Two-color analysis of the lymphocytes. FL1 (abscissa) and FL2 (ordinate) represent the fluorescence intensity of anti-CD8 and anti-CD4 antibodies, respectively. (A) Peripheral blood lymphocytes before sorting. Squares 1 and 2 represent the windows used to separate $CD4^+$ and $CD8^+$ lymphocytes. (B) Rerun of lymphocytes sorted for CD4 antigen. The percentage of cells falling in square 1 was 98.7% and was less than 1% in squares 2, 3, and 4, respectively. (C) Rerun of lymphocytes sorted for CD8 antigen. The percentage of cells falling in squares 1, 2, 3, and 4 were 1.1%, 0.05%, 1.2%, and 97.7%, respectively.

Table 1. The surviving fraction at each dose point for the three types of lymphocytes tested. The values shown are mean ±SD based on three to four experiments.

Cell population	X-ray dose (Gy)					
	0	1	2	3	4	5
$CD4^+$	1.0	0.58 ± 0.20	0.34 ± 0.05	0.12 ± 0.02	0.032 ± 0.003	0.0073 ± 0.0014
$CD8^+$	1.0	0.72 ± 0.08	0.44 ± 0.19	0.15 ± 0.07	0.051 ± 0.03	0.012 ± 0.007
Unsorted	1.0	0.63 ± 0.28	0.39 ± 0.006	0.11 ± 0.03	0.035 ± 0.007	0.0072 ± 0.002

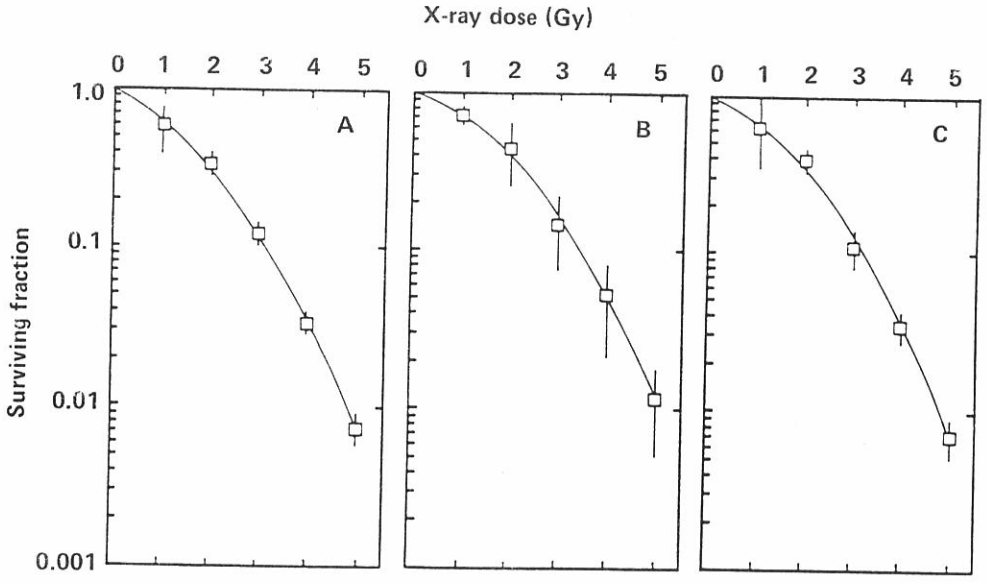

Figure 2. The dose-survival curves of $CD4^+$ (A), $CD8^+$ (B), and unsorted (C) lymphocytes. Each symbol represents the mean of four (A and B) and three (C) experiments for two donors and the bars represent the mean ±SD.

To verify that the surviving lymphocytes bore the original surface antigens on which sorting was based, colonies in Experiment 2 were randomly picked and the surface markers were examined. As summarized in Table 2, more than 95% of the colonies from sorted lymphocytes were indeed $CD4^+$ or $CD8^+$ in both the unirradiated and the 5-Gy exposed plates. On the other hand, colonies derived from unsorted lymphocytes were found to be heterogeneous, viz., more than two-thirds of them were either $CD4^+$, $CD8^+$ or $CD4^-8^-$, whereas, the others were mixtures of these three kinds of cell. The latter colonies were most probably derived from wells seeded with more than one clonogenic lymphocyte (see the Discussion section).

Table 2. Distribution of lymphocyte colonies bearing CD4 or CD8 antigens

	Sorted $CD4^+$ cells		Sorted $CD8^+$ cells		Unsorted cells	
Antigens	0 Gy	5 Gy	0 Gy	5 Gy	0 Gy	5 Gy
$CD4^+$	25	34	1	0	9	7
$CD8^+$	0	1	24	42	27	5
$CD4^-8^-$	0	0	0	1	3	1
$CD4^+ + CD8^+$	0	1	0	1	5	1
$CD4^-8^- + CD4^+$	0	0	0	0	2	0
$CD4^-8^- + CD8^+$	0	0	0	0	2	0
$CD4^-8^- + CD4^+ + CD8^+$	0	0	0	0	4	1
Total	25	36	25	44	52	15

Discussion

The present study first demonstrated that, as far as colony formation in the presence of PHA and IL-2, the radiosensitivities of $CD4^+$ (helper/inducer T cells) and $CD8^+$ (suppressor/cytotoxic T cells) lymphocytes were very similar. Since most of the lymphocyte colonies generated under these culture conditions were $CD4^+$ or $CD8^+$ lymphocytes (Table 2 and ref. 6), the present results supported the feasibility of using the lymphocyte colony assay to survey for individual variation in human radiosensitivity.

The present results were also in accord with those of a study by Stewart et al.[5] They examined the subset frequency of mass cultured lymphocytes stimulated with PHA and concluded that the radiosensitivities of $CD4^+$ or $CD8^+$ cells are very similar in terms of growth capability after radiation exposure. The similar radiosensitivity of $CD4^+$ and $CD8^+$ lymphocytes may well be understood, since they both are derived from common precursor lymphocytes in the thymus.

In Experiment 2 of the present study, some colonies derived from unsorted lymphocytes were $CD4^-8^-$. However, these colonies did not emerge for other individuals.[2] Most of the double negative cells are presumably natural killer (NK) cells of the $CD3^-16^+$ phenotype.[6] Thus, their radiosensitivity might be different from that of $CD4^+$ or $CD8^+$ cells. However, the following facts argue against this possibility. If the $CD4^-8^-$ cells were quite radioresistant, then a tail should be observed in the dose-survival curves of unsorted lymphocytes, whereas no such indication was observed up to 5 Gy (Figure 2C). On the other hand, if the $CD4^-8^-$ cells were more radiosensitive, the dose-survival curve alone may not be clearly visible unless the fraction of colonies consisting of $CD4^-8^-$ lymphocytes were sufficiently large among total colonies. (In this latter case, it would be expected that the survival curve would show a sharp decline at the low doses followed by an ordinary shouldered curve.) However, if this were the case, it would be expected that the fraction of $CD4^-8^-$ colonies among the total colonies obtained would be greatly diminished after a 5-Gy exposure. The following analysis of the results shown in Table 2 does not support that prediction.

For the unirradiated cells derived from unsorted lymphocytes, the fraction of wells without a colony is $p(0) = 0.36$. Hence, the average number of colonies per well is $\lambda = -\ln 0.36 = 1.01$, if we suppose the colony distribution follows Poisson's law. Therefore, for 96 wells, a total of $96 \times 1.01 = 97$ colonies occurred. Since 52 colonies out of 61 were examined, this indicates that the total number of colonies examined is $97 \times \frac{52}{61} = 83$. Among the colonies examined, 11 contained some $CD4^-8^-$ lymphocytes. Therefore, the fraction of $CD4^-8^-$ colonies among the total is $\frac{11}{83} = 0.13$. Likewise, for the 5 Gy-irradiated cells, $p(0) = 0.83$, thus $\lambda = 0.19$. Therefore, the fraction of $CD4^-8^-$ colonies is $2 \div (96 \times 0.19 \times \frac{15}{16}) = 0.12$ which is a value almost the same as that for nonirradiated cells.

A study is now in progress to generate colonies of $CD4^{-}8^{-}$ lymphocytes by modifying the culture conditions to directly measure the dose-survival curve of these cells.

The present results may appear incompatible with the widely accepted notion that suppressor T lymphocytes are quite radiosensitive.[7,8] However, it should be noted that this concept is derived from studies related to the T-cell-dependent immune response and not from cellular multiplication as an endpoint. Lymphocyte subset analysis of mouse spleen cells three days after total body irradiation reveals that the dose-survival curve of $Ly2^{+}$ cells (suppressor/cytotoxic T cells) is only slightly more radiosensitive (about 1.2 times) than $Ly1^{+}$ cells (helper/inducer T cells).[9] Also cancer patients who received chemotherapy or post-operative radiotherapy did not show increased $CD4^{+}/CD8^{+}$ ratios in peripheral blood lymphocytes.[10,11] These results suggest that the increased radiosensitivity of the immunosuppressor function may be caused by radiosensitive interaction process(es) among immunocytes and may not be directly related to death of the $CD8^{+}$ lymphocytes. Although it has generally been thought that suppressor T cells bear CD8 surface antigens, no suitable in vitro assay is available to identify suppressor cell function for each $CD8^{+}$ colony. Therefore, the present results simply suggested that it is quite unlikely that $CD8^{+}$ colonies largely consisted of suppressor T cells which are highly radiosensitive compared to other types of human lymphocytes which form colonies under the current experimental conditions.

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