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Frequency of Mutant T Lymphocytes Defective in the Expression of the T-cell Antigen Receptor Gene among Radiation-exposed People

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業　績　報　告　書

RERF TR 10－90
研究課題　3－87

原爆被爆者におけるT細胞抗原レセプター遺伝子の表現欠損を示す突然変異Tリンパ球の頻度§

Frequency of Mutant T Lymphocytes Defective in the Expression of the T-cell Antigen Receptor Gene among Radiation-exposed People

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要　約

T細胞レセプター遺伝子（αあるいはβ）発現に欠損を有する突然変異Tリンパ球の頻度を，2色フローサイトメトリー技法を用いて測定した．203名の原爆被爆者，すなわち78名の近距離被爆者（DS86線量が1.5 Gy以上）と125名の遠距離被爆者（DS86線量が0.005 Gy以下）の突然変異頻度は女性よりも男性に有意に高かった．被曝線量の影響は統計学的に有意ではなかった．これに対し，以前使われていたトリウム228を成分とする放射線診断用造影剤，トロトラストの使用により放射線に暴露した6名では有意に高い突然変異頻度が認められた．また，放射性ヨード131治療を受けた甲状腺疾患患者における変異頻度は，線量の増加と共に有意に増加した．以上のことから，このT細胞レセプターの突然変異測定法は，遺伝子毒性物質に最近被曝した人の生物学的線量の測定方法としてユニークな特徴があることが示唆される．

§本報告にはこの要約以外に訳文はない．

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Summary

The frequency of mutant T lymphocytes defective in T-cell receptor gene (α or β) expression was measured using the two-color flow cytometric technique. Results for a total of 203 atomic bomb survivors, 78 of whom were proximally exposed (DS86 doses of ≥1.5 Gy) and 125 of whom were distally exposed (DS86 doses of <0.005 Gy), showed that the mutant frequency was significantly higher in males than in females. No significant dose effects were observed. In contrast, a significant increase of mutant frequency was observed for six patients treated with Thorotrast, a contrast medium containing ^{228}Th formerly used for radiodiagnosis. In addition, thyroid disease patients treated with ^{131}I showed a dose-related increase of mutant frequency. It was suggested that the present T-cell receptor mutation assay has a unique characteristic as a biological dosimeter for the measurement of recent exposures to genotoxic agents.

Introduction

The T-cell receptor (TCR) is a heterodimer consisting of α and β chains and is associated with the CD3 components.[1] It is expressed in almost all mature T

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

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lymphocytes and plays a central role in antigen recognition and cell activation.[1] Like immunoglobulin (Ig) genes in B cells, the TCR genes undergo rearrangements in T cells. It has been reported that the spontaneous frequency of Ig gene mutations is enormously high in B-cell lines.[2–4] Therefore, it was anticipated that the TCR genes in mature T cells are also inherently unstable even after completion of the normal rearrangements that usually take place in the thymus. Furthermore, since only one of the two TCR alleles in mature T cells is believed to be actively expressed,[5] it is expected that a single mutation at the functional TCR gene will result in the phenotypic expression of TCR-defective mutants in a manner similar to that of X-chromosomal genes even though TCR genes are autosomally located.

Our previous study showed that two-color flow cytometry using anti-CD4 and anti-CD3 antibodies detects mutant T cells with altered TCR expression at a mean frequency of 2.5×10^4 among the $CD4^+$ T cells in peripheral blood from healthy donors who had not to their knowledge received sizable exposures of ionizing radiation.[6] The mutant frequency (MF) was found to increase significantly with age among these normal donors.[6] Since the mutant T-cell clones were abnormal in their expression of either one of the two chains in the TCR heterodimer, the average frequency per single TCR locus in mature T cells could be calculated as 1.3×10^4. This frequency is about 10–100 times higher than that for other loci, such as the hypoxanthine guanine phosphoribosyltransferase (HPRT) locus in T cells,[7–9] the glycophorin A (GPA) locus in erythrocytes,[10–12] and the human leukocyte antigen-A locus in T cells.[13] Thus, the TCR genes in mature T cells are quite mutable.

Since it has been well established that ionizing radiation induces somatic mutations in a dose-dependent manner both in vitro and in vivo, the frequency of TCR mutants was studied here for radiation-exposed people, i.e., atomic bomb (A-bomb) survivors, patients treated with Thorotrast and with various doses of radioactive iodine, and one person who was heavily exposed to radiation during the Chernobyl nuclear power plant accident.

Materials and Methods

Blood donors

The first group of donors consisted of a subset of A-bomb survivors who participated in the Adult Health Study at the Radiation Effects Research Foundation (RERF) in Hiroshima from May 1988 through March 1990. Among these individuals, 78 survivors had been proximally exposed (i.e., within 2,000 m of the hypocenter) and have been assigned DS86 dose estimates[14] of ≥ 1.5 Gy. Serving as controls to this group were 125 distally exposed (i.e., around 3,000 m from the hypocenter) A-bomb survivors with estimated DS86 doses of <0.005 Gy. The age and sex distributions for these individuals are given in Table 1.

The second group consisted of six Japanese men over 60 years old who had been treated with Thorotrast, a colloidal preparation of radioactive ^{228}Th used during the 1930s and 1940s as a radiological contrast medium. Its deposition as aggregates in the liver, spleen, lymph nodes, and bone marrow resulted in continuous irradiation of the surrounding tissues with alpha particles.

Table 1. Composition of A-bomb survivors sample by age, sex, and radiation dose (DS86) (n = 203)

	Kerma dose			
	<0.005 Gy		≥1.5 Gy	
Age (yr)	Males	Females	Males	Females
43–52	2	17	4	11
53–62	9	37	12	22
≥63	9	51	8	21
Total	20	105	24	54

The third group consisted of 18 thyroid cancer patients, 11 males and 7 females, who ranged from 22 to 74 years old. The time intervals between their last ^{131}I treatment and measurement of the MF were from 2 months to 5 years. Lymphocytes from one patient were tested both before and after therapy. In addition, one person who experienced acute radiation symptoms in 1986 as a result of the Chernobyl accident was examined about 3.5 years after the exposure. Moreover, lymphocytes from five unexposed healthy donors were tested two or more times during this study to evaluate the reproducibility of the assay.

Flow cytometry

Mononuclear cells were isolated from heparinized or defibrinated peripheral blood by density separation using Ficoll/Hypaque, and 2×10^5 cells were stained with fluorescein-labeled anti-Leu3a (CD4) and phycoerythrin-labeled anti-Leu4 (CD3) antibodies as specified by the supplier (Becton Dickinson Immunocytometry Systems, San Jose, Calif.). The fraction of $CD4^+$ cells among the mononuclear cells varied from 40% to 70% from individual to individual. For detecting mutant cells among $CD4^+$ T cells by flow cytometry, the lymphocyte fraction was gated by forward and right-angle light scatter, and a window for mutants was set in the region where the surface CD3 level was <1/25th of that for normal $CD4^+$ cells (see Figure 1), as described previously.[6] The MFs were calculated as the number of events in the mutant cell windows divided by the total number of $CD4^+$ T cells in the flow distribution.

Table 2 shows the results for repeated tests of lymphocytes from five reference donors during a 12-month period. The reproducibility is satisfactory and the average coefficient of variation (CV) for the mean MF was 12%.

Statistical analysis

Standard linear regression analysis was used.[15] For this purpose, natural log-transformed mutant frequency (lnMF) per 10,000 cells was used because the raw MFs of A-bomb survivors are not normally distributed. There are two cases, one in the exposed group and the other in the control group, whose MFs were zero. Because the log transformation of zero frequencies poses a problem, these two cases were omitted from the analysis. To take into account the possible effects of sex and radiation exposure, the following regression model was used:

Table 2. Reproducibility of mutant CD3-CD4$^+$ T-cell frequency ($\times 10^{-4}$) in normal donors

Donor, age (yr)	Experiment date	Mutant frequency	Mean ± SD	CV (%)
N.T. (42)	5 July 1988	2.0	1.83 ± 0.17	9.3
	30 Aug. 1988	1.8		
	4 Oct. 1988	1.6		
	20 Oct. 1988	1.9		
K.T. (53)	21 Oct. 1988	1.1	1.15	
	17 Feb. 1988	1.2	1.15	
H.M. (29)	28 June 1989	2.4	2.45 ± 0.26	10.6
	26 July 1989	2.7		
	7 Sept. 1989	2.6		
	18 Oct. 1989	2.1		
T.M. (59)	16 July 1987	4.2	4.00 ± 0.35	8.8
	18 Aug. 1987	3.6		
	1 Sept. 1988	4.2		
N.N. (43)	19 July 1988	2.7	2.25 ± 0.41	18.2
	30 Aug. 1988	1.7		
	10 Jan. 1989	2.3		
	28 Feb. 1989	2.3		

NOTE: CV = coefficient of variation.

$$\ln(\mathrm{MF}) = l + \alpha I_{\mathrm{sex}} + \beta I_{\mathrm{exp}} + e \ ,$$

where l is the average lnMF for unexposed males, I_{sex} is 1 for female and 0 for male, I_{exp} is 1 for exposed and 0 for nonexposed, and e is the residual error, which is presumed to be normally distributed with mean = 0 and variance r^2 (assumed constant for all observations). The test of $b = 0$ in this regression model is analogous to a t-test comparing the average lnMF between exposed and unexposed groups, except that it makes an adjustment for sex effects.

Results

Figure 1 shows representative results of lymphocyte flow distributions for three donors: (A) a reference donor, (B) a proximally exposed A-bomb survivor whose estimated DS86 kerma dose is 1.79 Gy, and (C) a person who was heavily exposed to radiation during the Chernobyl accident and whose chromosome aberration frequency suggested an exposure dose of approximately 3–4 Gy. For the reference donor, 20 mutant events were detected and the MF was calculated to be 2.3×10^{-4}. For the A-bomb survivor, 65 events were seen in the mutant window and the MF was 10.6×10^{-4}. The contour plot of the lymphocytes from the Chernobyl survivor showed 104 events, which corresponds to an MF of 21.1×10^{-4}.

The frequencies of TCR mutants for the 78 proximally exposed A-bomb survivors and for the 125 distally exposed controls are shown in Figure 2. Means and standard deviations of natural log-transformed MF per 10^4 cells are summarized in Table 3

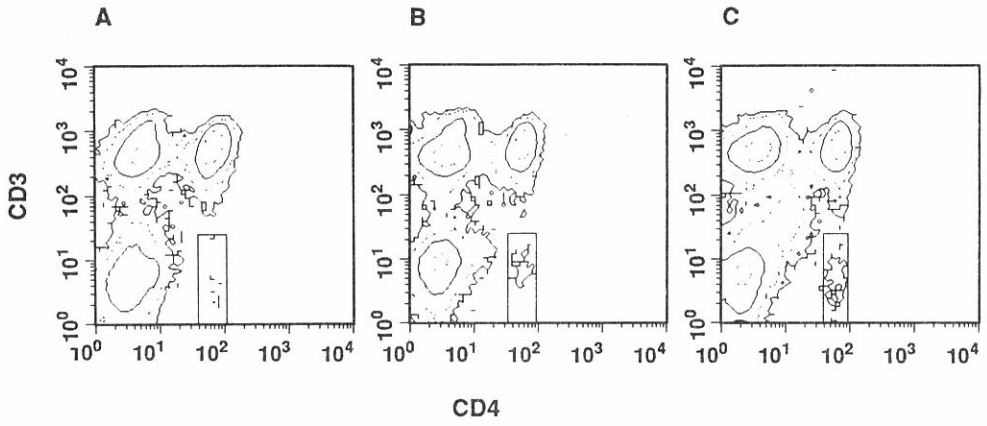

Figure 1. Flow cytograms of 2×10^5 peripheral blood lymphocytes stained with fluorescein-labeled anti-CD4 and phycoerythrin-labeled anti-CD3 antibodies. (A) a reference donor, (B) a proximally exposed A-bomb survivor, and (C) a person exposed during the Chernobyl nuclear power plant accident. Windows for mutant $CD3^-4^+$ lymphocytes are shown as squares. Contours differed in events per channel by a factor of 10 with the lowest contour representing one event per channel.

stratified by sex and exposure status. Also shown here are the analogous data for exposed males excluding three large MF values in this category. Note that when these three values are excluded, there is a dramatic decrease in the mean and standard deviation of lnMF and the standard deviations in each of the four groups become approximately equal. Thus, to satisfy the assumption of constant variance in the regression analysis, these three observations were omitted. Also note from Table 3 that the mean MF was significantly higher in males than in females.

A linear regression model was utilized to statistically compare lnMF between the two exposure groups (see Materials and Methods section). An age factor was not included in the model because there was no indication of age effects after adjustment for sex in these data (not shown). A sex factor appeared to be important since the male-to-female ratio is higher in the exposed group (Table 1), and hence without such an adjustment, differences in MF between the control and exposed

Table 3. Mean and standard deviation of natural log transformed mutant frequency per 10^4 cells of A-bomb survivors

Sex	Measure	Unexposed	Exposed	Exposed*
Male	Mean	1.20	1.39	1.21
	SD	0.35	0.63	0.41
Female	Mean	0.89	0.99	
	SD	0.38	0.39	

*Exposed group with three large values excluded from the analysis.

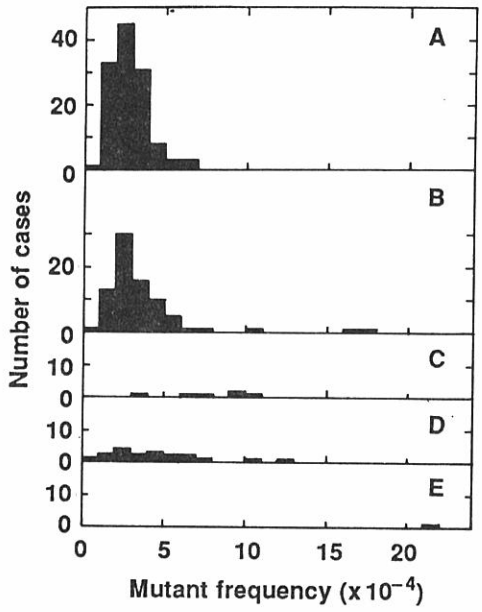

Figure 2. Mutant T-cell frequency in peripheral blood from (A) distally exposed A-bomb survivors (control group) (n = 125), (B) proximally exposed survivors (n = 78), (C) Thorotrast patients (n = 6), (D) ^{131}I-treated thyroid cancer patients (n = 18), and (E) a Chernobyl victim.

groups could be due to the different proportion of the sexes between the groups. The t-test for the significance of the exposure parameter was 1.39 on 195 df and was not statistically significant (p = .17). When the three large values were included, the test was marginally significant, t = 1.96 on 198 df (p = .051). This effect is virtually entirely due to the large values of only three individuals and further study is necessary to interpret the results (see Discussion).

As for the dose-response relationship, any model would be speculative because no information is available for low doses, viz., 0.01–1.5 Gy. Because of the lack of a difference between the MFs of exposed and control groups, any dose response would have to peak in the dose range between zero and the lowest dose value in the exposed group, viz., 1.5 Gy, although such a response does not seem very plausible. However, to test for any possible residual dose response among the exposed group, linear regression methods were utilized including dose and sex as variables. The test for a nonzero linear parameter in the dose response was not significant (p = .28). There was little difference in the results when the three large values were included. Furthermore, there is no significant curvature as tested by further addition of a squared term in dose (data not shown).

Among the six Thorotrast patients, five were found to show MFs larger than the highest MF in the control group (Figure 2). The mean MF of $7.8 \pm 2.9 \times 10^{-4}$ for the six male patients was significantly higher than that of the control male group (t-test, $p < .01$). The highest MF (21.1×10^{-4}) was observed in the person exposed during the Chernobyl accident (Figure 2).

As for the ^{131}I-treated patients, a significant linear relationship was observed between the MF and the total amount of ^{131}I radioactivity administered ($p < .0001$) (Figure 3). The fitted equation was $y = 1.86 \times 10^{-4} + 0.30 \times 10^{-4}D$, where y is MF and D is dose in gigabecquerels.

Discussion

Quantitative measurement of in vivo somatic cell mutations is undoubtedly an important area of research for the detection of human exposures to environmental genotoxic substances. At present, several methods—such as T-cell HPRT,[7–9] T-cell HLA class I,[13] and erythrocyte GPA mutation assays,[10–12]—are available for measuring in vivo MF. Practically speaking, however, the TCR mutation assay described here has some special advantages.

The T-cell colony assay for HPRT and HLA loci requires a large volume of blood (about 10–20 mL) and long-term cell culture (at least 2 weeks).[7–9] In contrast, the TCR mutation assay requires a smaller volume of blood (1 mL) because the MF is higher, and requires only 5 hr for the MF measurement. Although the erythrocyte GPA assay also requires only a small volume of blood (1 mL) and a short amount of time (2 days) for analysis, donors are restricted to MN heterozygotes and genetic analysis of the mutant cells cannot be made because they lack nuclei. In the present TCR assay, there are no restrictions on the donor genotype and mutant T-cell clones can be isolated by a cell sorter and cultivated in vitro for molecular analysis as described previously.[6] Furthermore, since the TCR mutation assay uses commercially available antibodies and a single-beam flow cytometer, this method has potential for wide use in many laboratories.

In the present study, radiation effects on the induction of TCR mutants were not unequivocally detected in the proximally exposed A-bomb survivors. Because TCR-defective mutants generally grow poorly in vitro,[6] it is suspected that such mutant T-lymphocytes have a growth disadvantage in vivo and have probably been eliminated during the more than 40 years since their exposure to radiation. If this were the case, the higher MF for the lymphocytes from Thorotrast patients and

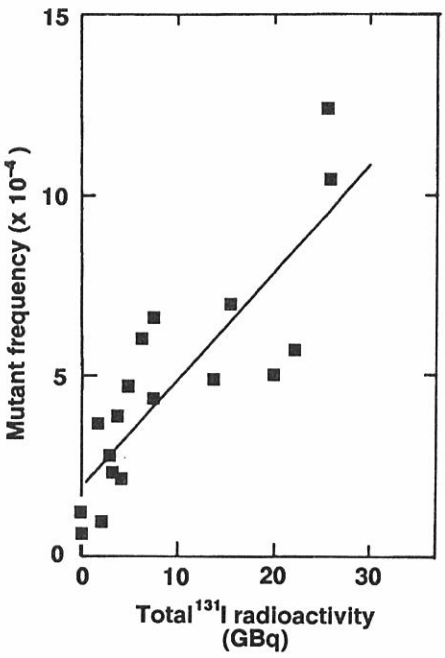

Figure 3. Correlation between mutant T-cell frequency and total radioactivity of ^{131}I received by thyroid cancer patients. The line represents a linear regression using a least squares method.

^{131}I-treated people indicates that these mutants emerged rather recently. This theory is compatible with the observation for chromosome aberrations in lymphocytes from Thorotrast patients: the majority were unstable-type aberrations.[16] This, in turn, indicates that the TCR mutants observed probably represent only a small fraction of the mutants generated continuously in vivo.

It remains to be understood why the average MF of males was higher than that of females. It has been reported that cigarette smoking increases the frequency of sister chromatid exchanges in peripheral blood lymphocytes.[17] Although negative findings are also present,[17] a higher frequency of smokers among males might partly explain the sex effect. Another factor might be medical exposures, since industrial workers have more opportunities to receive diagnostic X rays compared with housewives. As for the three male A-bomb survivors with exceptionally high MFs, viz., $>10 \times 10^{-4}$, it is not clear whether this finding is related to A-bomb radiation exposure. While it may appear interesting to note that all three males were 17 to 21 years old at the time of the bombing and that their estimated doses are 1.8 to 2.8 Gy, no trend of higher MF among males exposed before the age of 20 was observed. Therefore, it seems more likely that the similar exposure conditions of the three males are coincidental. Further studies on the mutant clones from these three individuals are in progress to clarify whether the mutants have alternative pathways that compensate for the growth disadvantage caused by defects of the TCR/CD3 complex and whether they have better opportunities to escape negative selection in vivo.

It has been reported that the peripheral blood lymphocytes from Thorotrast patients show an elevated frequency of chromosome aberrations.[16] In the present TCR assay as well, four out of six patients showed MFs greater than the highest MF observed in the control group. In contrast, the frequency of mutant erythrocytes at the GPA locus did not show a similar increase for the same patients (unpublished data). Such a discrepancy may be attributed to the difference in the sensitivity of the assay, but is more likely due to the difference in the amount of ^{228}Th deposited in the target organs. It has been reported both in rats and humans that the radioactivity of ^{228}Th is 6–10 times greater in the spleen, one of the target tissues for the T lymphocytes, than in bone, the target tissue of erythrocyte precursor cells.[18,19]

As for the patients treated with ^{131}I for thyroid malignancies, a dose-related increase of MF was observed (Figure 3). Because of the small sample size, however, no attempt was made to evaluate the effects of body weight and time interval between the last ^{131}I administration and measurement of TCR mutant frequency, factors that are supposed to affect the observed MF. Our recent results on X-ray-induced TCR mutations in a Jurkat cell line in vitro showed a maximum induction rate of approximately 10×10^{-4}/Gy (manuscript in preparation). In this context, it would be interesting to evaluate the soft tissue dose of ^{131}I-treated patients for comparison. According to Johnson,[20] 1 μCi of ^{131}I administration is calculated to give 1.3×10^{-6} Sv to the spleen, one of the major target organs of lymphocytes, for an average Caucasian woman weighing 58 kg. While the dose is inversely proportional to the total body weight, we take the value of 1.3×10^{-6}Sv/μCi here as an average dose simply because patients in the present study include both males and

females and average Japanese men weigh more and average Japanese women weigh less than the average Caucasian women. As shown in Figure 3, the regression line of MF for ^{131}I-treated patients indicates that administering about 30 GBq resulted in a net increased MF of 10×10^{-4}. This is equivalent to a committed ^{131}I dose of 1.05 Sv ($30/37 \times 10^{6}$ μCi $\times 1.3 \times 10^{-6}$ Sv/μCi) to the spleen. Therefore, in vitro data for X-ray irradiation of a Jurkat cell line and in vivo human data for ^{131}I administration appear to be in close agreement. This might, in turn, indicate that negative selection of TCR mutant lymphocytes does not take place very rapidly, viz., not within several months, since the average time interval between the final ^{131}I administration and measurement of the TCR MF is 2.2 ± 1.7 years for the nine patients who received >7 GBq of ^{131}I. Follow-up studies of these patients are in progress along with in vitro irradiation experiments using normal peripheral blood lymphocytes to substantiate this hypothesis.

It is well known that radiation exposure induces acute and chronic immunodeficiency in animals[21] and in humans.[22,23] Radiation-induced destruction of lymphocytes and bone marrow stem cells is believed to be the major factor in this immunodeficiency. Since the TCR/CD3 complex consists of the major surface molecules functioning in T-cell antigen recognition, which is the first step for a variety of other T-cell-dependent immune responses, loss or alteration of TCR expression in the surviving cells could also contribute to radiation-induced impairment of the T-cell immune response. This is compatible with our previous finding that ataxia telangiectasia patients suffering T-cell immunodeficiency show a high TCR MF.[6] Thus, determining the frequency of TCR-defective variants might be useful as a potential indicator of T-cell immunodeficiency, as well as for somatic mutagenesis.

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