RAPID COMMUNICATION Jpn. J. Cancer Res. 82, 1349–1353, December 1991

Flow Cytometric Measurements of Somatic Cell Mutations in Thorotrast Patients

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Exposure to ionizing radiation has long been well-recognized as a risk factor for cancer development. Since ionizing radiation can induce mutations, an accurate way of measuring somatic mutation frequencies could be a useful tool for evaluating cancer risks. In the present study, we have examined *in vivo* somatic mutation frequencies at the erythrocyte glycophorin A (GPA) and T-cell receptor (TCR) loci in 18 Thorotrast patients who have been continuously irradiated with alpha-particles emitted from the internal deposition of thorium dioxide and who thus have increased risks of certain malignant tumors. When compared with controls, the results showed a significantly higher frequency of mutants at the lymphocyte TCR loci but not at the erythrocyte GPA loci in the Thorotrast patients. The discrepancy between the results of the two assays is discussed.

Key words: Thorotrast patient — Somatic mutation — GPA gene — TCR gene

Thorotrast, a colloidal solution of thorium dioxide, was used in many countries during the 1930s to 1950s for the radiographic visualization of body cavities, the cerebral arteries, liver and spleen.[1-4] Only a small percentage of Thorotrast is eliminated from the body and the rest is deposited mainly as aggregates in the reticuloendothelial system, liver, spleen, lymph nodes and bone marrow. These aggregates continuously irradiate the tissues with alpha particles emitted by the decay of ^{232}Th and its daughter nuclei.[1] Epidemiological, pathological and clinical studies have demonstrated that intravascularly administered Thorotrast causes a marked increase in the incidence of malignant hepatic tumors and leukemia.[1-4]

Discoveries of cancer suppressor genes such as the retinoblastoma susceptibility gene,[5] p53[6] and DCC[7] genes have strengthened the somatic mutation theory of carcinogenesis. Furthermore, it is well documented that ionizing radiation is both carcinogenic and mutagenic. Therefore, measurement of the somatic mutation frequency in people exposed to ionizing radiation may serve as a biological indicator of cancer risk.

The recent development of monoclonal antibodies and of the flow cytometric technique has opened a new era for the rapid enumeration and isolation of rare mutant blood cells, an approach that holds great promise for identifying biological markers for risk evaluation. Currently, erythrocyte glycophorin A (GPA) mutation[8-10] and lymphocyte T-cell antigen receptor (TCR) gene mutation[11] assays are used in our laboratory for the measurement of *in vivo* somatic mutation frequency. Results from studies on atomic bomb (A-bomb) survivors and cases of recent radiation exposure using the GPA[9,10,12] and TCR mutation assay,[13] respectively, revealed dose-related increases of mutant frequencies. It has also been reported that peripheral blood lymphocytes from Thorotrast patients show an elevated frequency of chromosome aberrations.[14] The present study was therefore undertaken to measure the frequencies of *in vivo* GPA and TCR mutations in Thorotrast patients, who are at increased risk of developing malignant diseases.

Peripheral blood samples were obtained during April 1987 to April 1990 from 18 Japanese men aged 67 to 83 (mean$\pm$SD$=74\pm4$) who had been treated with Thorotrast. Unfortunately, except for a few cases, the Thorotrast doses were not available. The control group consisted of male A-bomb survivors whose estimated radiation doses are below 0.005 Gy (distally exposed group) and who were between 67 to 83 years old at the time of examination. A total of 23 such males served for erythrocyte GPA assay (mean age of 74 ± 4) and 19 males for lymphocyte TCR assay (mean age of 74 ± 4).

The erythrocyte GPA mutation assay, as described previously in detail,[8-10] uses 4 monoclonal antibodies (MoAb) to GPA: the GPA (M)-specific MoAbs 6A7 and 9A3; the GPA (N)-specific MoAb NN3; and the GPA-specific MoAb 10F7 that binds equally to both the

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[7] Abbreviations used in this paper: GPA, glycophorin A; TCR, T-cell antigen receptor; Mf, mutant frequency.

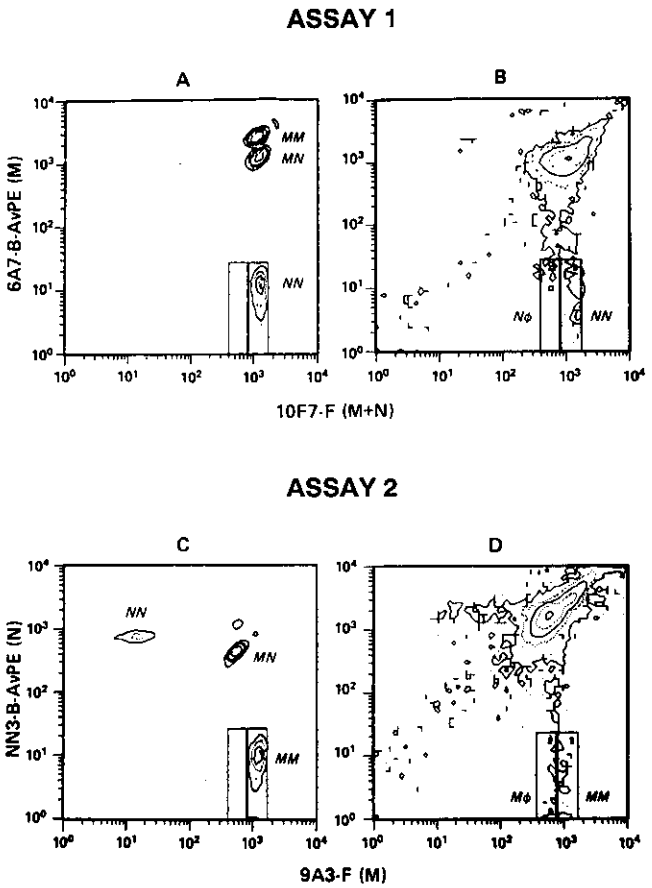

Fig. 1. Fluorescence distributions of assay 1 and assay 2 on a standard mixture of MM, MN and NN type erythrocytes (A, C), and of 2×10^5 erythrocytes from one Thorotrast patient (#3) (B and D). Contour plots in B and D differ from those in A and C by a factor of 10 in events per channel, with the lowest contour representing 1 event per channel. The windows labeled Nϕ, Mϕ and NN and MM (B and D) correspond to the hemizygous and homozygous mutants and were defined using a standard erythrocyte mixture (A or C), as described previously.[10]

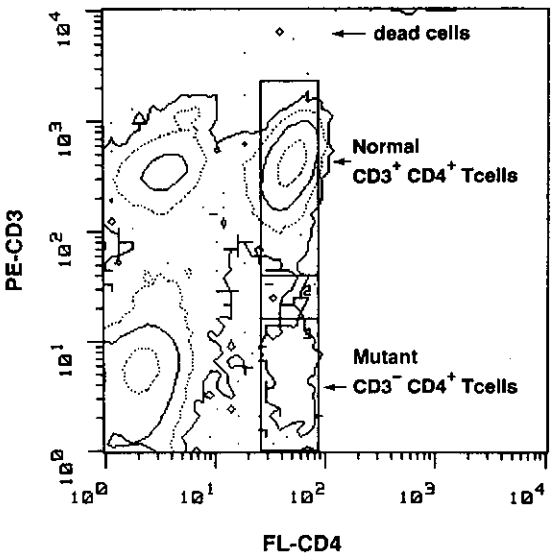

Fig. 2. Flow cytogram of 2×10^5 peripheral blood lymphocytes from a Thorotrast patient (#16) stained with fluorescein-labeled anti-CD4 (FL-CD4) and phycoerythrin-labeled anti-CD3 (PE-CD3) antibodies. CD4 is a T cell differentiation antigen expressed mainly on the surface of helper/inducer T lymphocytes. 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 (indicated as $CD3^-4^+$) where the surface CD3 level was $\leq$1/25th of that of normal $CD4^+$ cells, as described previously.[11] The Mfs were calculated as the number of events in the mutant window divided by the total number of $CD4^+$ T cells in the flow distribution.

M and N allele products. These MoAbs were labeled with appropriate fluorescent dyes such as fluorescein (-F suffix on the antibody name) or biotin-streptavidin-phycoerythrin (-B-AvPE). By using combinations of MoAbs 6A7-B-AvPE and 10F7-F (assay 1), and MoAbs 9A3-F and NN3-B-AvPE (assay 2), two sets of mutant erythrocyte types, that is, the hemizygous Nϕ and homozygous NN erythrocytes and the hemizygous Mϕ and homozygous MM erythrocytes can be detected from heterozygous donors of MN blood type by assay 1 and assay 2, respectively (Fig. 1).

As for the lymphocyte TCR mutation assay, the T-cell antigen receptor (TCR) is a heterodimer consisting of α and β or γ and δ chains and is associated with a molecular complex referred to as CD3 antigen,[15] a differentiation antigen expressed on the surface of mature T lymphocytes. TCR genes undergo DNA rearrangements during the normal maturation of T lymphocytes in the thymus,[16] similar to immunoglobulin (Ig) gene rearrangements that occur during B-cell maturation.[17,18] As in Ig genes, only one of the two alleles of the TCR gene is expressed in T cells (a phenomenon called "allelic exclusion"), and thus TCR genes are functionally hemizygous although they are autosomally located. This means that a single mutation in the functional TCR genes results in the phenotypic expression of TCR-defective mutants in a way similar to X-chromosomal genes. For the TCR to be expressed on the T-cell surface, the complete TCR/CD3 complex is required. Thus, any defect in one of the two molecules that make up the TCR heterodimer results in a loss of the expression of CD3 molecules on the T-cell

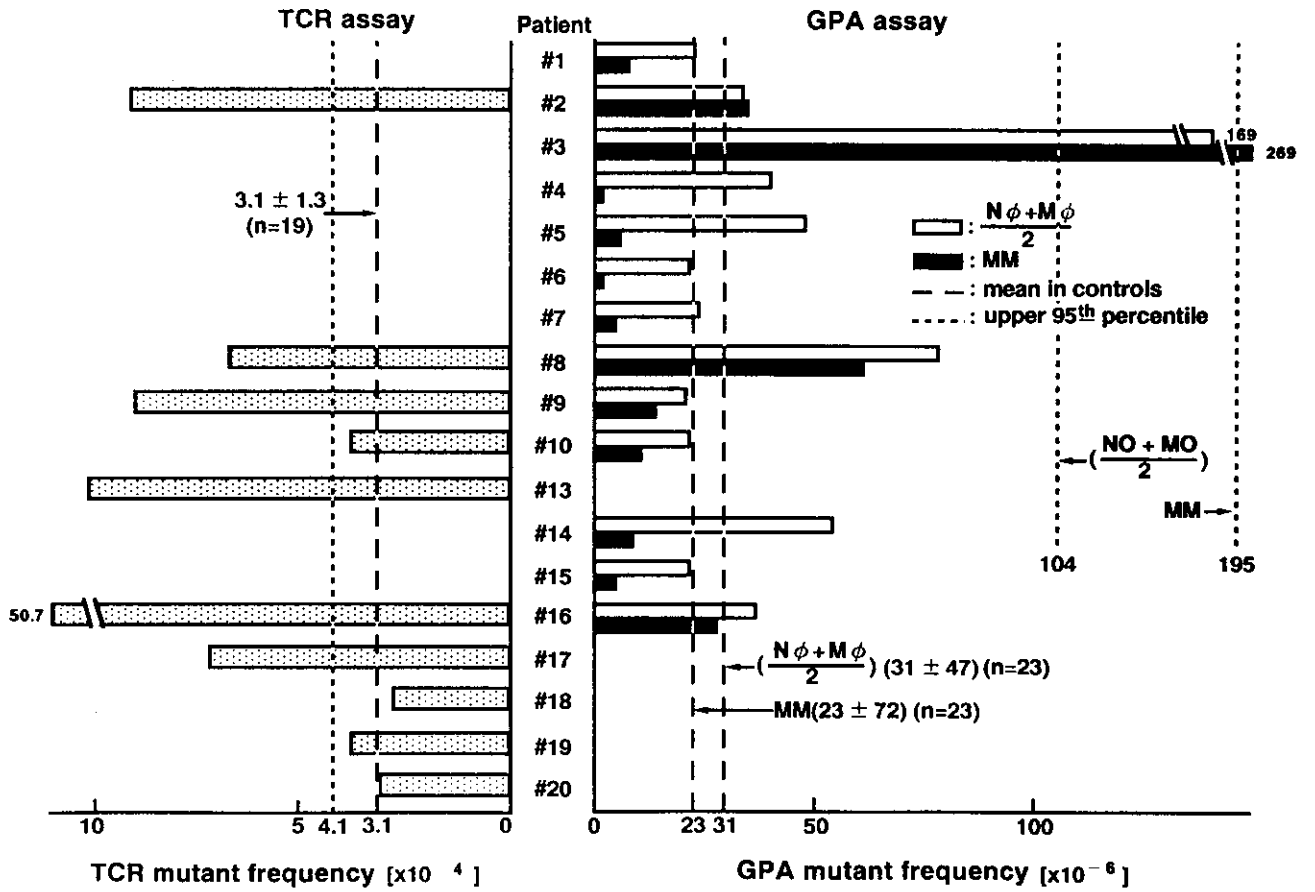

Fig. 3. Observed TCR and GPA mutation frequencies in Thorotrast patients. The broken lines represent the mean Mf of the control group. The dotted lines represent the upper 95th percentile of Mf distribution in controls.

surface and cytoplasmic accumulation of these molecules instead. Therefore, expression of CD3 antigen on the cell surface can be used as a marker for TCR mutations (Fig. 2).

The results for the 18 Thorotrast patients are summarized in Fig. 3.

We tested for a possible significant increase of mutant frequency (Mf) in TCR and GPA assays by comparing the Thorotrast patients to a suitable group of controls. The t test for unequal variances was employed with approximate degrees of freedom calculated by Welch's method.[19] Natural log-transformed Mf was analyzed bacause we have determined through previous experience that the log-transformed Mf is approximately normally distributed. Geometric means and standard deviations of Mf were obtained by exponentiating the mean of *ln*Mf and by applying the delta method, respectively. The upper 95th percentile of the distribution of Mf was calculated by exponentiating the quantity (mean + 1.645 × standard deviation) where the "mean" and "standard deviation" are of the *ln*Mf in the controls.

As shown in Fig. 3, 13 of the 18 Thorotrast patients were MN heterozygotes, and so they compose the group tested using the erythrocyte GPA mutation assay. The average frequencies of hemizygous type mutants, Nϕ and Mϕ, and the frequency of MM homozygous type mutants, are also shown. (NN is not shown because of its poor reproducibility.[9, 10])

The geometric mean Mf of 37 ± 23 (SD) $\times 10^{-6}$ for hemizygous type mutants was not significantly different from that of the age-matched male controls (n=23, mean $31 \pm 27 \times 10^{-6}$, t test: $P > 0.5$). In addition, no significant increase of Mf for MM homozygous type mutants was observed ($P > 0.1$). Only one patient (#3) had an unusually high GPA Mf, as compared to the upper 95th percentile of the control Mf distribution

(Nϕ+Mϕ)/2: 169 vs. 104; MM: 269 vs. 195). In the follow-up studies, this patient was found to have developed leukemia. However, blood sampling was done before the diagnosis and thus the high Mf cannot be attributed to the therapy for the disease. Another patient (#5) was also found to have developed hepatic cancer. It remains to be known whether the increased Mf had a causal relationship to the development of these diseases.

As for the TCR mutation assay, 10 of the 18 Thorotrast patients could be examined (Fig. 3). In contrast to GPA, the TCR Mf was greater than the 95th percentile of the control population in 6 of the 10 patients. The geometric mean Mf of $6.9 \pm 5.9 \times 10^{-4}$ for the 10 male patients was significantly higher than that of the male controls (n=19, mean $3.1 \pm 1.3 \times 10^{-4}$, *t* test: $P < 0.02$).

What causes this difference in the results between TCR and GPA mutations? Our previous studies of the A-bomb survivors revealed that the effect of A-bomb radiation could still be detected by the GPA assay[9, 10, 12] but not by the TCR assay.[13] Because *in vitro* X-ray irradiation of peripheral blood lymphocytes resulted in a sharp increase of TCR Mf (Umeki *et al.*, manuscript in preparation), it was suspected that the TCR mutants may have been eliminated *in vivo* during the period since the A-bomb radiation exposure. In fact, studies on female patients treated with radiotherapy for uterine cancers revealed that the TCR Mf decays with a half-life of about 2 years (Umeki *et al.*, manuscript in preparation). Thus, the TCR assay detects recent exposures to radiation and is not always a more sensitive test than the GPA assay.

On the other hand, it has been reported, both in rats and in human beings, that the radioactivity of ^{232}Th and its daughters is 6 to 10 times higher in the liver and spleen — the spleen is one of the target tissues for T lymphocytes — than in bone marrow — the target tissue for erythrocyte precursor cells.[20, 21] Thus, it is conceivable that the deposition of Thorotrast may not be uniform and lymphocytes would have received higher doses than erythrocyte precursor cells in bone marrow. Alternatively, the present results may simply be fortuitous in that patients measured for erythrocyte GPA mutation are biased toward lower doses of Thorotrast. Further study will clarify this point.

This finding is compatible with the observation that the majority of chromosome aberrations in lymphocytes from Thorotrast patients were unstable types,[14] namely, the radiation effects detected in T lymphocytes are due to recent exposures. Long-term follow-up studies should provide information on whether or not these two mutation assays may serve as biological risk indicators among radiation-exposed people. In this regard, it should be noted that the dose distributions of internal exposures may not be homogeneous and hence an appropriate assay must be used for proper evaluation of internal exposure doses.

We wish to thank M. Yamaoka and K. Tanabe for their excellent technical assistance and M. Takagi for manuscript preparation. We also thank M. Edington for his editorial assistance.

(Received July 4, 1991/Accepted October 1, 1991)

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