Technical Report Series

Flow-cytometric Measurements of Somatic Cell Mutations in Thorotrast Patients

Shigeko Umeki, M.D.; Seishi Kyoizumi, Ph.D.; Yoichiro Kusunoki, Ph.D.; Nori Nakamura, Ph.D.; Masao Sasaki, Ph.D.; Takesaburo Mori, M.D.; Yuichi Ishikawa, M.D.; John B. Cologne, Ph.D.; Mitoshi Akiyama, M.D.

放影研 RERF **Radiation Effects Research Foundation**

A Cooperative Japan–United States Research Organization

RERF Technical Report Series

Technical reports are the basic medium for reporting of original research carried out at the Radiation Effects Research Foundation. Reports in this series receive both internal and external peer review and may serve, in part or in toto, as the basis for publication in the open scientific literature. Although they may be quoted and cited, these reports are considered to be internal publications of the Foundation. Copies are available upon request from Publication and Documentation Center, RERF, 5-2 Hijiyama Park, Minami-ku, Hiroshima, 732 Japan.

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.

業績報告書シリーズ

トロトラスト投与患者における体細胞突然変異のフローサイトメトリー法による解析§

Flow-cytometric Measurements of Somatic Cell Mutations in Thorotrast Patients

梅木繁子[1]　京泉誠之[1]　楠 洋一郎[1]　中村 典[1]　佐々木正夫[2]　森 武三郎[3]
石川雄一[4]　John B. Cologne[5]　秋山實利[1]

要　約

電離放射線被曝は，発癌リスク因子として以前からよく知られている。電離放射線により突然変異が誘発され得るので，体細胞突然変異頻度を正確に測定する方法があれば，癌リスク評価のための有用な手段となる。今回の研究では，体内に沈着している二酸化トリウムから放出されるアルファ粒子に継続的に被曝しているため，発癌リスクが増加しているトロトラスト投与患者18人について，赤血球グリコフォリンAおよびT細胞レセプター遺伝子座における生体内体細胞突然変異頻度を測定した。対照者と比較すると，トロトラスト投与患者では，T細胞レセプター遺伝子座位に突然変異頻度の有意な増加が認められたが，赤血球グリコフォリンA座位には認められなかった。

§本業績報告書は研究計画書RP3-87と7-88に基づく。訳文は要約のみ。承認1991年10月30日。印刷1992年10月。

放影研 [1]放射線生物学部および[5]統計部　[2]京都大学放射線生物研究センター　[3]放射線医学総合研究所 生理病理研究部　[4](財)癌研究会病理部。

Technical Report Series

Flow-cytometric Measurements of Somatic Cell Mutations in Thorotrast Patients§

Shigeko Umeki, M.D.[1]; Seishi Kyoizumi, Ph.D.[1]; Yoichiro Kusunoki, Ph.D.[1]; Nori Nakamura, Ph.D.[1]; Masao Sasaki, Ph.D.[2]; Takesaburo Mori, M.D.[3]; Yuichi Ishikawa, M.D.[4]; John B. Cologne, Ph.D.[5]; Mitoshi Akiyama, M.D.[1]

Summary

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

Introduction

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; 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

§This technical report is based on Research Protocols 3-87 and 7-88; approved 30 October 1991; printed October 1992. The complete text of this report will not be available in Japanese.

Departments of [1]Radiobiology and [5]Statistics, RERF; [2]Radiation Biology Center, Kyoto University; [3]Division of Physiology and Pathology, National Institute of Radiological Sciences, Chiba; [4]Department of Pathology, Cancer Institute, Tokyo.

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]

The discovery of cancer-suppressor genes such as the retinoblastoma susceptibility gene[5] and the p53[6] and DCC[7] genes has 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 being used in our laboratory to measure in vivo somatic mutation frequencies. Studies on atomic bomb (A-bomb) survivors and persons with recent radiation exposure using the GPA[9,10,12] and TCR mutation assays,[13] respectively, have revealed dose-related increases in 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 undertaken to measure the frequencies of in vivo GPA and TCR mutants in Thorotrast patients, who are at increased risk of developing malignant diseases.

Materials and Methods

Subjects

Peripheral blood samples were obtained during April 1987 to April 1990 from 18 Japanese men aged 67 to 83 years (mean $\pm$ *SD* = 74 $\pm$ 4) 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 were below 0.005 Gy (from the distally exposed group) and who were between 67 and 83 years old at the time of examination. Twenty-three such males served as subjects for the erythrocyte GPA mutation assay and 19 males for the lymphocyte TCR assay (the mean age of both groups was 74±4 years).

Glycophorin A gene mutation assay

The erythrocyte GPA mutation assay, as described previously in detail,[8–10] uses four monoclonal antibodies (MoAbs) 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 M and N allele products. These MoAbs were labeled with appropriate fluorescent dyes such as fluorescein (the "-F" suffix on the antibody name) or biotin-streptavidin-phycoerythrin ("-B-AvPE"). By combining 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 NØ and homozygous NN erythrocytes and the hemizygous MØ and homozygous MM

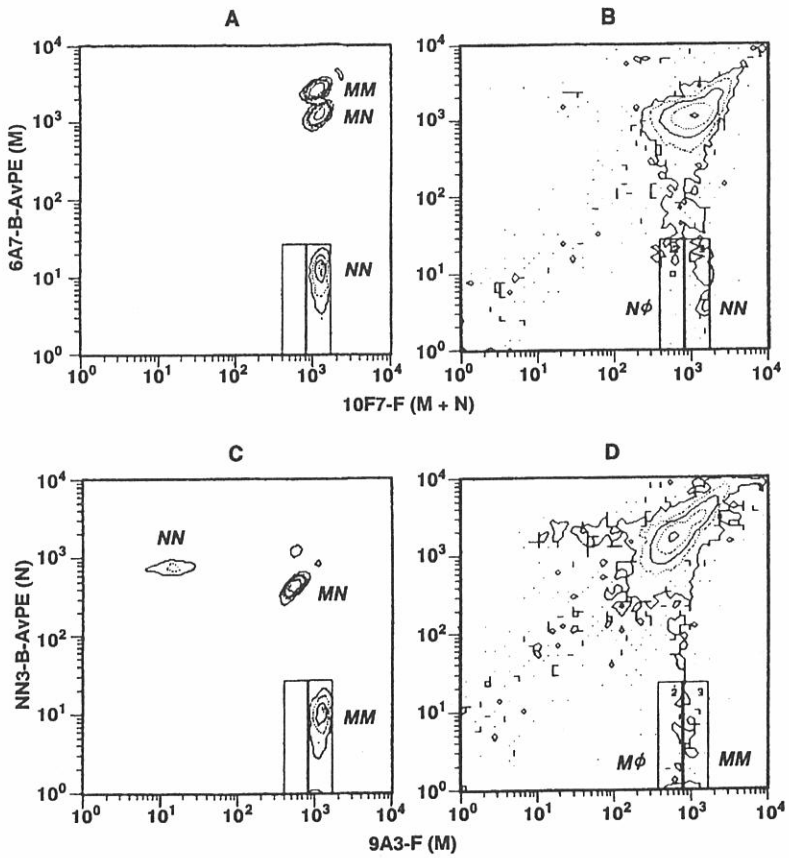

Figure 1. Fluorescence distributions of assay 1 (top) and assay 2 (bottom) on a standard mixture of MM-, MN-, and NN-type erythrocytes (A, C) and of 2×10^5 erythrocytes from one Thorotrast patient (#3) (B and D). The contour plots in B and D differ from those in A and C by a factor of 10 in events per channel, with the lowest contour representing 1 event per channel. The windows labeled NØ and NN, MØ 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]

erythrocytes, can be detected from heterozygous donors of MN blood type by assay 1 and assay 2, respectively (Figure 1).

T-cell antigen receptor gene mutation assay

As for the lymphocyte TCR mutation assay, the T-cell antigen receptor 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 the 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"); 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 that for X-chromosomal genes. For the TCR to be expressed on the T-cell surface, the complete CD3/TCR 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 surface and cytoplasmic accumulation of these molecules instead. Therefore, the expression of CD3 antigen on the cell surface can be used as a marker for TCR mutations (Figure 2).

Our previous studies showed a significant age-dependent increase of T cells with altered TCR expression in normal donors[11] and a highly elevated frequency in patients with ataxia telangiectasia[11] and with Bloom's syndrome (unpublished data). In addition, the isolation and cloning of these T cells from peripheral blood indicate that their abnormalities can be accounted for by alterations in TCR expression as defects of protein expression and partial protein deletion.[11] These

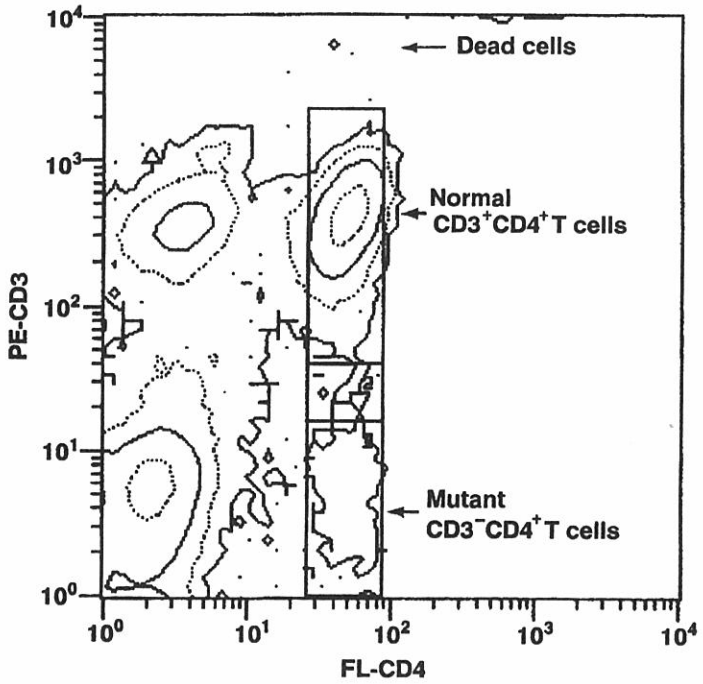

Figure 2. Flow cytogram of 2×10^5 peripheral blood lymphocytes from a Thorotrast patient (#16), stained with fluorescein-labeled anti-CD4 (FL-CD4) and phycoerythrin-labeled anti-CD3 (PE-CD3) antibodies. CD4 is a T-cell differentiation antigen expressed mainly on the surface of helper/inducer T lymphocytes.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

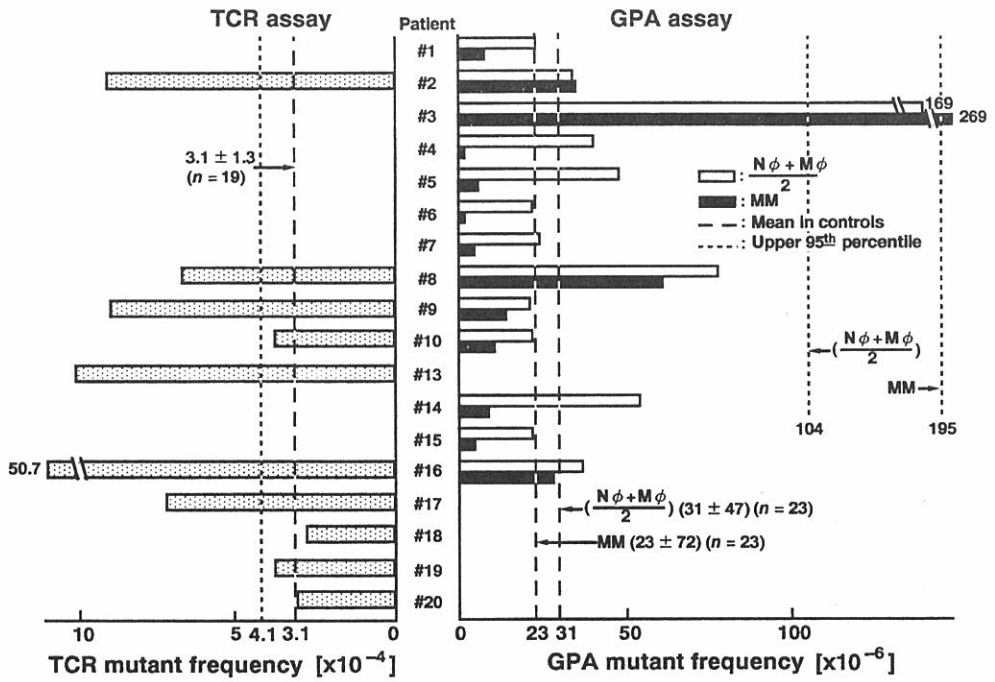

Figure 3. Observed T-cell receptor (TCR) and glycophorin A (GPA) mutant frequencies (Mfs) in Thorotrast patients. The dashed lines represent the mean Mf of the control group. The dotted lines represent the upper 95th percentile of the Mf distribution in controls.

observations strongly indicate that such alterations in TCR expression are induced by somatic mutagenesis of TCR genes. Hereafter, these T cells with altered TCR expression are called "TCR mutant T cells."

Statistical analysis

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

Results

As shown in Figure 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, NØ and MØ, and the

frequency of MM homozygous-type mutants are also shown. (NN is not shown because it is not reproducible.[9,10])

The geometric mean Mf of 37 ± 23 (*SD*) × 10^{-6} for hemizygous-type mutants was not significantly different from that of the age-matched male controls (n = 23, mean = 31 ± 47 × 10^{-6}, t test: $p > .5$). In addition, no significant increase of the Mf for MM homozygous-type mutants was observed ($p > .1$). Only one patient (#3) had an unusually high GPA Mf, as compared with the upper 95th percentile of the control Mf distribution ([NØ + MØ]/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 found to have developed hepatic cancer. It remains to be known, however, whether the increased Mf has a causal relationship to the development of the diseases in these patients.

As for the TCR mutation assay, 10 of the 18 Thorotrast patients could be examined (Figure 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 ± 5.9 × 10^{-4} for the 10 male patients was significantly higher than that of the male controls (n = 19, mean = 3.1 ± 1.3 × 10^{-4}, t test: $p < .02$).

Discussion

What causes this difference in the results between TCR and GPA mutations? Our previous studies of the A-bomb survivors reveal that the effects 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-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-bombings. In fact, studies on female patients treated with radiotherapy for uterine cervical 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 that 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 mutations are biased toward lower doses of Thorotrast. Further study should 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] that is, the radiation effects detected in T lymphocytes are due to recent exposures. Long-term follow-up studies should provide information on whether 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 doses.

Acknowledgments

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.

References

1. Mori T, Kato Y, Ishihara T, Sugiyama H, Mori W, Irie H, Hatakeyama S, Kamiyama R, Iwato S, Nakashima T, Kojiro M, Ikezaki H, Akita K: The effects of thorium on the human being. In *Research on Thorium Fuel (SPEY 9)*. Ed by Shibata T, Kanda K, Kobayashi K. Tokyo, Ministry of Education, Science, and Culture, 1984. pp 229–50
2. Horta J, Horta ME, Motta C, Tavares MH: Malignancy in Portuguese Thorotrast patients. Health Phys 35:137–51, 1978
3. van Kaick G, Lieberman D, Lorenz D, Lorenz WJ, Hührs H, Scheer KE, Wesch H, Muth H, Kaul A, Immich H, Wagner G, Wegener K: Recent results of the German Thorotrast study—epidemiological results and dose effect relationships in Thorotrast patients. Health Phys 44(Suppl):299–306, 1983
4. Mori T, Kato Y, Kumatori T, Maruyama T, Hatakeyama S: Epidemiological follow-up study of Japanese Thorotrast cases—1980. Health Phys 44(Suppl):261–72, 1983
5. Klein G: The approaching era of the tumor suppressor genes. Science 238:1539–45, 1987
6. Sager R: Tumor suppressor genes: The puzzle and the promise. Science 246:1406–12, 1989
7. Fearon ER, Cho KR, Nigro JM, Preinsinger AC, Thomas G, Kinzler KW, Vogelstein B: Identification of a chromosome 18q gene that is altered in colorectal cancers. Science 247:49–56, 1990
8. Langlois RG, Bigbee WL, Jensen RH: Measurements of the frequency of human erythrocytes with gene expression loss phenotypes at the glycophorin A locus. Hum Genet 74:353–62, 1986
9. Langlois RG, Bigbee WL, Kyoizumi S, Nakamura N, Bean MA, Akiyama M, Jensen RH: Evidence for increased somatic cell mutations at the glycophorin A locus in atomic bomb survivors. Science 236:445–8, 1987 (RERF TR 1-87)
10. Kyoizumi S, Nakamura N, Hakoda M, Awa AA, Bean MA, Jensen RH, Akiyama M: Detection of somatic mutations at the glycophorin A locus in erythrocytes of atomic bomb survivors using a single beam flow sorter. Cancer Res 49:581–8, 1989 (RERF TR 9-88)
11. Kyoizumi S, Akiyama M, Hirai Y, Kusunoki Y, Tanabe K, Umeki S: Spontaneous loss and alteration of antigen receptor expression in mature $CD4^+$ T cells. J Exp Med 171:1981–99, 1990 (RERF TR 22-89)
12. Akiyama M, Kyoizumi S, Hirai Y, Hakoda M, Nakamura N, Awa AA: Studies on chromosome aberrations and HPRT mutations in lymphocytes and GPA mutations in erythrocytes of atomic bomb survivors. In *Mutation and the Environment, Part C:*

Somatic and Heritable Mutation, Adduction and Epidemiology. Ed by Mendelsohn, ML, Albertini RJ. New York, Wiley-Liss, 1990. pp 69–80

13. Kyoizumi S, Umeki S, Akiyama M, Hirai Y, Kusunoki Y, Nakamura N, Endoh K, Konishi J, Sasaki M, Mori T, Fujita S, Cologne JB: Frequency of mutant T lymphocytes defective in the expression of the T-cell antigen receptor gene among radiation-exposed people. Mutat Res 265:173–80, 1992 (RERF TR 10-90)
14. Sasaki MS, Takatsuji T, Ejima Y, Kodama S, Kido C: Chromosome aberration frequency and radiation dose to lymphocytes by alpha-particles from internal deposit of Thorotrast. Radiat Environ Biophys 26:227–38, 1987
15. Clevers H, Alarcon B, Wileman T, Terhost C: The T-cell receptor/CD3 complex: A dynamic protein ensemble. Annu Rev Immunol 6:629–62, 1988
16. Kronenberg M, Sin G, Hood LE, Shastri N: The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Annu Rev Immunol 4:529–91, 1986
17. Meyer J, Jack HM, Ellis N, Wabl M: High rate of somatic point mutation in vitro and near the variable-region segment of an immunoglobulin heavy chain gene. Proc Natl Acad Sci USA 83:6950–3, 1986
18. Jack HM, Wabl M: High rate of deletions in the constant region segment of the immunoglobulin μ gene. Proc Natl Acad Sci USA 84:4934–8, 1987
19. Snedecor GW, Cochran WG: *Statistical Methods* (6th ed). Ames, Iowa State University Press, 1967. pp 114–6
20. Kaul A, Noffz W: Tissue dose in Thorotrast patients. Health Phys 35:113–21, 1978
21. Norimura T, Tsuchiya T, Hatakeyama S, Yamamoto H, Okajima S: Long-term tissue distribution and steady state activity ratios of ^{232}Th and its daughters in rats after intravascular injection of Thorotrast. J Univ Occupat Environ Health (Kitakyushu) 11:287–97, 1989