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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.

RERF TR 3-91
研究課題 11-89

業績報告書シリーズ

フローサイトメトリーを用いたヒト末梢血リンパ球におけるHLA-A 遺伝子座突然変異検出法[§]

Development of a Flow-cytometric HLA-A Locus Mutation Assay for Human Peripheral Blood Lymphocytes

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要　約

フローサイトメトリー法を用いて, HLA-A2 または A24 ヘテロの血液提供者のリンパ球中に存在する HLA-A2 または A24 対立遺伝子の発現喪失突然変異細胞頻度の測定法を開発した.

末梢血リンパ球中の突然変異頻度は10^{-4}のレベルであり, 血液提供者の年齢と共に上昇した. 突然変異クローンの分子レベルでの解析の結果, 約3分の1は体細胞組換えに起因することが判明した. 残りの3分の2についてはサザンブロット法でいかなる変化も検出されなかった.

原爆被爆者についての小規模な調査では, 有意な線量効果は認められなかった.

試験管内突然変異誘発実験の結果は, HLA-A24 遺伝子に関する突然変異頻度は 1 Gy 当たり約2×10^{-4}の割合で増加することが認められた. この値はリンパ球のX染色体に位置しているヒポキサンチン　フォスフォリボシルトランスフェラーゼ遺伝子座について報告されている値よりも約10倍高い. また, これらの突然変異の大半は大きな染色体レベルでの欠失に起因していることも明らかになった.

[§]本報告にはこの要約以外に訳文はない.　この報告は研究課題RP11-89に基づく.　承認1991年6月10日,　印刷1992年2月.

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Technical Report Series

Development of a Flow-cytometric HLA-A Locus Mutation Assay for Human Peripheral Blood Lymphocytes§

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Summary

A flow-cytometric technique was developed to measure the frequency of mutant lymphocytes lacking expression of HLA-A2 or A24 allele products among donors heterozygous for HLA-A2 or A24.

It was found that the mutant frequency of lymphocytes in peripheral blood was on the order of 10^{-4} and increased with donor age. Molecular analyses of mutant clones revealed that about one-third were derived from somatic recombinations and that the remaining two-thirds did not show any alterations after Southern blot analysis. A small-scale study on atomic bomb survivors did not show a significant dose effect.

An in vitro mutagenesis study showed that the mutant frequency at the HLA-A24 locus increased at a rate of roughly 2×10^{-4}/Gy, about 10 times greater than that reported at the X-chromosomal hypoxanthine phosphoribosyltransferase locus in lymphocytes. These mutants were found to be mostly derived from large chromosomal deletions.

Introduction

Recent discoveries of cancer suppressor genes such as the retinoblastoma susceptibility gene,[1] and the p53[2] and DCC[3] genes have strengthened the somatic mutation theory of carcinogenesis. It has been well documented that ionizing radiations are both carcinogenic and mutagenic, and hence measurement of the

§*This technical report is based on Research Project 11-89. The complete text of this report will not be available in Japanese; approved 10 June 1991; printed February 1992.*

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somatic mutation frequency would serve as an indicator of human exposure to radiation. In contrast to the variety of in vitro mutagenesis assays available, the number of techniques to measure in vivo mutant frequency in humans is quite limited.

Historically, the discovery of mitogens provided the first opportunity to culture peripheral blood lymphocytes (PBLs); it had previously been considered that PBLs were terminally differentiated and did not divide. Observations of lymphocyte metaphase chromosomes provided a definitive answer to questions about the total chromosome number in the human genome. In addition, a series of human aneuploid syndromes, such as Down's syndrome, Kleinfelter's syndrome, Turner's syndrome, and so on, had been recognized.[4] Subsequently, an increased frequency of lymphocytes bearing chromosomal abnormalities was found among atomic bomb (A-bomb) survivors.[5] Although the lymphocyte chromosome analysis can be performed in standard cytogenetic laboratories, it requires well-trained personnel and a considerable amount of time and hence is not suitable for rapid screening of a large number of people.

The discovery of the T-cell growth factor interleukin-2 provided the second tool for the long-term culture and cloning of PBLs. Techniques for the quantitative detection of 6-thioguanine-resistant mutants defective in the hypoxanthine phosphoribosyltransferase (HPRT) gene—which are widely used for in vitro mutagenesis assays—could be applied to PBLs.[6,7] In addition, Southern blot analysis using DNA extracted from clonally grown mutants made it possible to dissect the mutants at the molecular level. This epochal development made the human in vivo mutant frequency (Mf) easily measurable. Unfortunately, however, it was also found that HPRT-defective mutant lymphocytes appeared to be prone to negative selection in vivo. Results of studies on A-bomb survivors[8] show that the slope of the dose-response curve is quite shallow, roughly 1/10th of that obtained after in vitro irradiation, and hence the assay was found to be unsuitable for use as a lifetime biological dosimeter.

The third tool was elaborated by Jensen's group at Lawrence Livermore National Laboratory. Using a pair of monoclonal antibodies conjugated with different fluorescent dyes, they developed a flow-cytometric assay for the detection of erythrocyte mutants lacking expression of the M or N allele of the glycophorin A gene.[9] The advantages of this assay are its requirement of only a small volume of blood (1 mL), that fixed erythrocytes can be stored for several weeks before the measurements, that blood samples can be shipped from long distances to a laboratory for measurement, and that it can detect homozygous variants that are supposed to be derived from somatic recombinations in addition to mutants derived from the loss of gene functions (viz., hemizygous mutants). Results from studies on A-bomb survivors revealed that both the hemizygous and homozygous variant frequencies increased with radiation dose, although the slope of the latter increase was about 2 times smaller.[10,11] Somatic recombination has been recognized as one of the mechanisms associated with loss of function of suppressor oncogenes such as RB, p53, and DCC. Unfortunately, mature erythrocytes do not contain nuclei and hence molecular confirmation of the mutants is not possible.

Thus, an assay method was needed that could detect and confirm both specific locus mutations and somatic recombinations. The HLA-A locus of PBLs was chosen for this purpose along with the previously established flow-cytometric

technique. The rapid enumeration of mutant* lymphocytes can be achieved by the use of a flow cytometer and confirmation of mutations can be performed after sorting and clonal propagation of the mutant lymphocytes. Because both specific locus mutations and somatic recombinations have been demonstrated to be induced by radiation exposure in mice and in *Drosophila,* the present assay was applied to A-bomb survivors to see whether radiation effects may be detected even many years after the radiation exposure.

Materials and Methods

Donors

Fifty-eight RERF employees and 168 Adult Health Study (AHS) participants were the subjects of the present study. The AHS members sampled mainly consisted of persons whose DS86 doses were 0 Gy or >1 Gy, so that radiation effects could be clearly observed if they remained.

Lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by glass-bead defibrination and Ficoll-Hypaque separation, as described previously.[12] After washing twice with Earle's balanced salt solution containing 2.5% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin, mononuclear cells were used for the experiments after counting the number of cells by staining with Türk's solution.

Purification of monoclonal antibodies

Mouse hybridoma cell line BB7.2 was obtained from American Type Culture Collection (Rockville, Md.). The monoclonal antibody produced by this cell line reacts to HLA-A2 and A28 allele products. Another hybridoma cell line, HU-49, developed by one of the authors (A.W.), produces a monoclonal antibody that reacts to HLA-A24 allele products. Both hybridoma cell lines produce immunoglobulin IgG2b subclass.

The hybridoma cells were inoculated intraperitoneally in Balb/c or Balb/c nude mice (1×10^7 cells/mouse) pretreated with Pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemical Co., Inc., Milwaukee, Wis.). Ten to 14 days later, serum and ascites were collected and were subjected to the following purification procedures: dialysis against 40% ammonium sulfate, dialysis against 0.015 M phosphate-buffered saline (PBS, pH 7.4) overnight, and isolation of the IgG2b fraction using a protein A affinity column (Pharmacia Fine Chemicals, Uppsala, Sweden). The IgG2b fraction thus obtained was quantitated by measurement of OD280.

Biotin labeling of purified monoclonal antibodies

Monoclonal antibodies prepared at a concentration of 1 mg/mL or more were

**In this report, we use the term "mutants" in a broad sense to represent the rare lymphocytes lacking expression of specific HLA-A allele products, a frequent usage in this field. The term includes both somatic recombinations and gene mutations, although the confirmation of the latter awaits further studies.*

mixed with 1/4th volume of 0.015 M PBS containing 0.01% NaN_3 and 1/5th weight of *N*-hydroxysuccinimide-*d*-biotin (Sigma Chemical Co., St. Louis, Mo.) dissolved in dimethylsulfoxide (2 mg/mL), and the mixture was kept for 4 hr at room temperature. Subsequently, it was dialyzed against PBS containing 0.01% NaN_3 overnight and was used as biotinylated monoclonal antibodies at a concentration of 100 μg/mL after quantitation by measurement of OD280.

Staining of PBMCs by monoclonal antibodies

About 3 million PBMCs were centrifuged and the supernatant was discarded. To the cell pellet was added 120 μL of biotinylated anti-HLA-A2 or A24 antibody and 60 μL of FITC-conjugated anti-CD3 antibody (Leu 4 antibody, Becton Dickinson Immunocytometry Systems, Mountain View, Calif.), followed by gentle pipetting, and the tube was then kept in ice for 30 min. After washing the cells with PBS, the cells were suspended with 120 μL of phycoerythrin (PE)-labeled streptavidin (Sigma) and the tube was kept in ice for 30 min to label biotinylated monoclonal antibody with PE. The cells were washed again by centrifugation and were suspended with 250 μL of PBS for flow-cytometric analysis. Labeling of lymphocytes by monoclonal antibodies against surface antigens other than CD3 followed the same procedure, namely anti-CD5 (Leu 1), anti-CD8 (Leu 2a), anti-CD4 (Leu 3a), anti-CD2 (Leu 5b), and anti-CD20 (Leu 16) (Becton Dickinson).

Measurement of the frequency of mutant T lymphocytes lacking expression of HLA-A2 or A24 alleles

Flow-cytometric detection of the mutant lymphocytes was performed using a FACScan (Becton Dickinson). Both fluorescent dyes—fluorescein and PE—were excited with a 15-mW argon ion laser beam at 488 nm, and fluorescence was detected through a 530-nm band pass filter for fluorescein and a 585-nm band pass filter for PE. The spectral overlap of fluorescein and PE fluorescence was electronically compensated for using standard calibration beads (Becton Dickinson). About 3 million PBMCs stained with the pair of monoclonal antibodies were subjected to the measurement, and two-color analysis was conducted for those cells falling in the gated area of lymphocytes specified by forward and side scatters. Figure 1A shows the representative pattern of the assay in which CD3 was used as a marker of mature T cells. The Mf for HLA-A2-defective cells was calculated as:

$$\frac{\text{number of } CD3^+ \text{ HLA-A2}^- \text{ lymphocytes}}{\text{total number of } CD3^+ \text{ lymphocytes}}$$

The upper and lower limits for $CD3^+$ were arbitrarily chosen from the contour lines of 100 events per channel. The upper limit for the HLA-$A2^-$ window was generally set at the fluorescence intensity level that corresponds to 1/25th of the peak for the HLA-$A2^+$ cell population. When lymphocytes not bearing the HLA-A2 allele were subjected to flow-cytometric analysis, all the $CD3^+$ cells showed fluorescent intensity for HLA-A2 below this limit, as shown in Figure 1C.

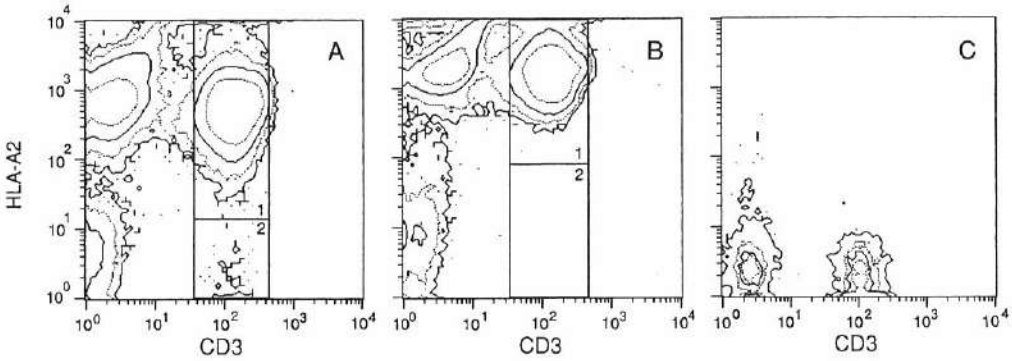

Figure 1. Flow cytograms of peripheral blood mononuclear cells (PBMCs) stained with phycoerythrin-labeled anti-HLA-A2 antibody (ordinate) and fluorescein (FITC)-labeled anti-CD3 antibody (abscissa). PBMCs from an HLA-A2 heterozygous donor (A), an HLA-A2 homozygous donor (B), and from a person who did not have HLA-A2 antigen (C) are shown. The mutant frequency is 1.42×10^{-4} in (A). Note that no mutants were detected in (B) after testing about 0.6×10^6 $CD3^+$ cells.

Cloning of mutant lymphocytes

PBMCs from heterozygotes for either HLA-A2 or A24 were subjected to flow-cytometric analysis using a FACStar (Becton Dickinson) and those cells falling in the window for HLA-$A2^-$ or $A24^-$ mutants were sorted under the conditions of full deflection envelope mode and a flow rate of 2,000–3,000 cells/sec. The sorted cells were distributed to 96-well round-bottom microplates (Costar, Cambridge, Mass.) at an average cell density of 1 cell/well. The culture medium consisted of GIT medium (Wako, Tokyo) supplemented with 1:6400 of phytohemagglutinin-P (Difco, Detroit, Mich.), 2 ng/mL of recombinant human interleukin-2 (Takeda, Tokyo), 2 mM of *L*-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% heat-inactivated FCS. As feeder cells, 5×10^4 allogeneic PBMCs and 1×10^4 OKIB cells (B-cell line) were used per well after being subjected to 50 Gy of X-irradiation.

Growing lymphocytes were successively subcultured using feeder cells to obtain 5 to 15×10^6 cells.

Southern blot analysis of the mutants

The five probes used in this analysis were pHLA2a.1 (kindly provided by Dr. H. T. Orr at the University of Minnesota), λMS29,[13] cMS605,[14] cDNA from the constant region of the T-cell receptor β gene (kindly provided by Dr. T. W. Mak at the Ontario Cancer Institute), and a 4.5-kb EcoRI-Hind III fragment from a germ-line Cμ gene[15] (a gift of Dr. T. Honjo, Kyoto University).

The probe pHLA2a.1 was derived from the 3′-untranslated region of the HLA-A gene.[16] Hind III or Bgl II digestion produces an allele-specific length of DNA fragment containing the HLA-A gene.[17] Thus it is possible to distinguish point mutations from deletions or somatic recombinations, as these latter two result in loss of the HLA-A2- or A24-specific restriction fragment.

The probe λMS29 detects the minisatellite locus DNF21S1 located at the distal portion of chromosome 6's short arm (6pter-p25),[13] whereas the HLA-A locus is more proximal, viz., 6p21.3. Since the minisatellite locus consists of tandemly repeated core sequences and the repeat number is highly polymorphic, it serves as a good marker to distinguish one chromosome from its homologue in diploid somatic cells. When the genotype of the donor is heterozygous for the DNF21S1 locus and the HLA-A2 mutant cells have lost their heterozygosity, two alternative mechanisms are possible, namely large deletions and somatic recombinations. Since the two loci are quite apart from each other on the metaphase chromosome map, deletions including both HLA-A and DNF21S1 loci could be clearly recognized by chromosome analysis and hence distinguished from somatic recombinations. This probe also hybridizes to the sequences on chromosome 16 but it can be distinguished from those on chromosome 6 since the polymorphism frequency is quite low in chromosome 16 and the majority of the cases give 4.7-kb Hinf I fragments.[18]

Another minisatellite locus probe, cMS605, detects the polymorphic locus D6S86 at chromosome 6's long arm (6q).[14] This probe was used to show that the loss of heterozygosity at the HLA-A and DNF21S1 loci at chromosome 6p does not accompany the loss of heterozygosity at chromosome 6q, and hence confirms that these are the recombinants.

cDNA of the T-cell receptor gene β was used to identify the clonality of the mutants.[19]

To isolate DNA, frozen cells were thawed and suspended in 0.3 mL of PBS, to which was added 1–5 mL of digestion buffer (20 mM Tris-HCl, pH 8.0; 100 mM NaCl, 5 mM EDTA, and 0.5% SDS) containing 400 μg/mL of proteinase K (Boehringer Mannheim, Mannheim, Germany), and the mixture was incubated at 37°C for 4 hr. The solution was phenol-chloroform (2:1) extracted, then dialyzed against 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA and stored at 4°C.

For the Southern blot, 5 μg of DNA was digested to completion with Hind III, Bgl II, or Hinf I restriction enzyme (Toyobo, Tokyo), fractionated on a 0.7%–1.0% agarose gel in Boyer's buffer (50 mM Tris-HCl, pH 8.0, 20 mM sodium acetate, 2 mM EDTA, and 10 mM NaCl), and transferred to nitrocellulose filters (Schleicher and Shuell, Keene, N.H.) using 20 × standard saline citrate (SSC) (3 M NaCl, 0.3 M trisodium citrate dihydrate, pH 7.0). Prehybridization was conducted for 30 min at 65°C in 3 × SSC; for 1 hr at 65°C in 10 × Denhardt's solution, 3 × SSC; and for 30 min at 65°C in 50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 1 M NaCl, 10 × Denhardt's solution, and 0.1% SDS.

Hybridization was carried out for 12–16 hr at 65°C in 2 mL of the same solution used in prehybridization, containing 100 mg/mL of denatured salmon sperm DNA. The probes were labeled with ^{32}P-dCTP (Amersham, Amersham, U.K.) to a specific activity of 10^8–10^9 cpm/μg using a random primer method (Amersham kit), and 15–30 ng of a probe was added to one filter. Washing of hybridized filters was performed as follows. The pHLA2a.1 was washed in 2 × SSC, 0.1% SDS for 15 min at room temperature and for 30 min at 60°C, followed by washing for 10 min at 70°C in 0.1 × SSC, 0.1% SDS. The other probes were washed for 5 min at room temperature in 2 × SSC, 0.1% SDS, followed by two to three washings for 15 min at 65°C in 0.1 × SSC, 0.1% SDS. Autoradiography was performed at –80°C with an intensifying screen (Fuji, Tokyo).

In vitro X-irradiation

PBMCs from an HLA-A24 heterozygous donor were X-irradiated with 0, 1, and 2 Gy. At each dose level, 1 to 2×10^7 cells were cultured for 11 days in the presence of feeder cells using the medium described for mutant cloning. Thereafter, cells were stained with PE-conjugated anti-HLA-A24 antibody and FITC-labeled anti-CD3 antibody for the measurement of the Mf. Propidium iodide was also used to exclude dead cell contamination from the mutant window. The mutant cells were sorted and cloned as described above for chromosome and Southern blot analyses.

Statistical analysis

A linear regression model was used to evaluate the effects of sex, age, and A-bomb radiation dose on Mf. Namely,

$$ln\mathrm{Mf} = \alpha_0 + \alpha_1 I_s + \alpha_2 a + \alpha_3 d \ ,$$

where α_0 is the natural log of the background Mf at dose 0 and age 0 for males, $I_s = 1$ for females and 0 for males, a is donor age, d is radiation dose, and α_1, α_2, and α_3 are unknown parameters. Tests concerning these parameters were made using the usual regression F test. We found no obvious departures from the assumptions underlying these tests. Although Poisson methods might seem more appropriate in this context, they are not because of measurement errors present in the denominator of Mf.

Results

Identification of heterozygous donors for HLA-A2 or A24

Figure 1 shows the flow cytograms of PBMCs stained with anti-HLA-A2 and anti-CD3 antibodies. It was found that HLA-A2$^-$ mutants were detected at a frequency on the order of 10^{-4} for heterozygous donors (Figure 1A) but not at all for HLA-A2 homozygous donors (Figure 1B). PBMCs from donors who do not carry the HLA-A2 allele showed distinctively different patterns, as expected (Figure 1C). The same results were obtained for HLA-A24 (data not shown). These results prompted us to use flow cytograms to identify appropriate donors, viz., heterozygotes for HLA-A2 or A24.

PBMCs from 226 individuals were typed for HLA-A2 and it was found that 84 were heterozygotes (37%), 8 were homozygotes (4%), and 134 were negative (59%). Similarly, 147 persons were tested for HLA-A24 by flow cytometry. It was found that 77 were heterozygotes (52%), 17 were homozygotes (12%), and 53 were negative (36%).

A fraction of these examinees was also typed by a standard NIH assay using a battery of antisera, and the results provided additional information that monoclonal antibody produced by hybridoma BB7.2 (anti-HLA-A2) does not react to HLA-A1, A11, A24, A26, A31, and A33. Likewise, we confirmed that monoclonal antibody produced by HU-49 (anti-HLA-A24) does not react to HLA-A1, A2, A11, A26, A31, and A33.

Table 1. Frequencies of HLA-A2$^-$ mutants (donors M.I. and H.M.) and HLA-A24$^-$ mutants (donors J.K. and K.T.) per 10^4 cells of different lymphocyte subsets

	Lymphocyte subsets					
Donors	CD2$^+$	CD3$^+$	CD4$^+$	CD5$^+$	CD8$^+$	CD20$^+$
H.M.	1.11	0.87	1.26	0.73	1.11	1.65
J.K.	0.64	1.10	0.81	0.21	0.91	3.59
K.T.	1.55	1.91	2.01	2.07	0.87	1.60
M.I.	1.21	1.37	1.16	1.22	1.34	0.75

Mutant frequency among lymphocytes of different subsets

By using a combination of anti-HLA-A2 or A24 antibody and a series of antibodies against various lymphocyte subsets, it was possible to measure the Mf for lymphocytes of various subsets. Table 1 summarizes the results for two donors each for HLA-A2 and A24. A number of markers were used: CD2 and CD5 for pan-T cells, CD3 for mature T cells, CD4 for helper/inducer T cells, CD8 for suppressor/cytotoxic T cells, and CD20 for pan-B cells. It is apparent that no specific subset consistently showed a higher or lower Mf compared with the others. Therefore, the CD3 marker was chosen for the following studies since CD3$^+$ cells are the most predominant among PBMCs, viz., about 70% on average.

Reconstruction experiments

To measure the precision of the present assay, PBMCs from an HLA-A24$^-$ donor were stained with PE-labeled anti-HLA-A24 and FITC-labeled anti-CD3 antibodies and were mixed with PBMCs from an HLA-A24 homozygous donor, similarly stained with the two antibodies, at ratios ranging from 3×10^{-5} to 1×10^{-3}. The mixed-cell samples were subsequently subjected to flow-cytometric analysis. The results are shown in Figure 2A. Because the Mf is based on CD3$^+$ cells and the CD3$^+$ fraction among PBMCs is different depending on the donor, the expected Mf is expressed as the mixed ratio multiplied by the ratio of the CD3$^+$ fraction among PBMCs for HLA-A24$^-$ donors to that for HLA-A24 homozygous donors. In this experiment, the ratio was 0.74/0.53 = 1.40 and the expected Mf was shown by the straight line in Figure 2A. It is evident that the measured Mfs are in close agreement with the expected values. Similar results for HLA-A2 are shown in Figure 2B. In this case, the ratio of the CD3$^+$ fractions was 0.71/0.70 = 1.01 and the observed Mfs were slightly lower on average than the expected values.

Taking into account the experimental errors associated with cell counting and dilution processes, the results appear to be satisfactory, and the assay does not often falsely miscount normal cells as mutants.

Reproducibility of the assay

Blood samples were repeatedly obtained from 14 individuals, 9 of whom were heterozygous for HLA-A2 and 5 of whom were heterozygous for HLA-A24, and their Mfs measured. These results are shown in Figure 3. It is evident that the

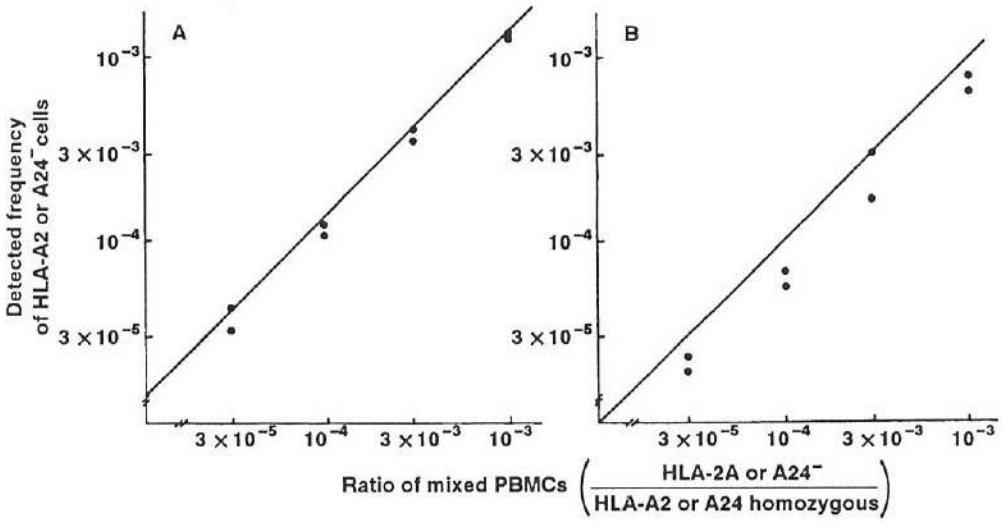

Figure 2. Results of a reconstruction experiment to evaluate the accuracy of HLA mutant cell detection. The straight lines represent theoretical values when differences in the $CD3^+$ lymphocyte fraction in peripheral blood mononuclear cells (PBMCs) from different donors are taken into account (see text for details). A: HLA-A24 locus; B: HLA-A2 locus.

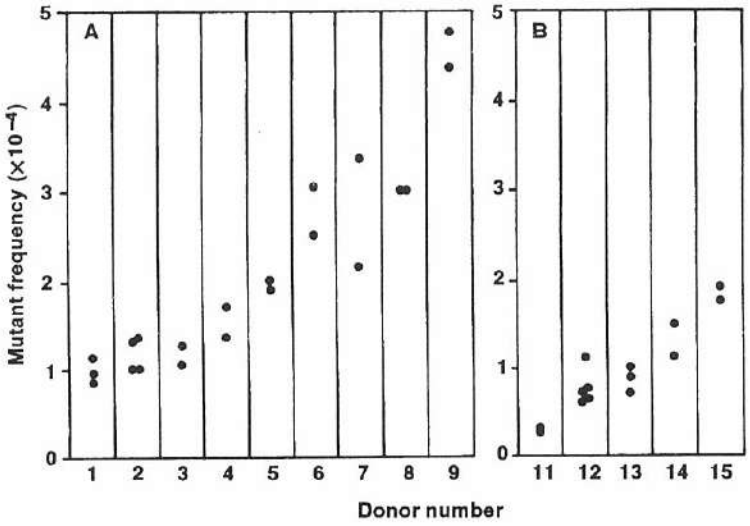

Figure 3. Results of tests of the reproducibility of the assay for detecting $HLA\text{-}A2^-CD3^+$ (A) and $HLA\text{-}A24^-CD3^+$ (B) lymphocytes.

reproducibility is good. Samples measured three times or more showed an average standard deviation of 0.18×10^{-4}, which is quite satisfactory as a measurement of somatic mutations.

Effects of radiation exposure, donor age, and sex

The Mf of lymphocytes from 38 AHS participants who were heterozygous for HLA-A2 and 31 individuals who were heterozygous for HLA-A24 was measured to evaluate the effect of previous radiation exposure (Figure 4). Contrary to our expectation, statistical analysis did not show any significant increase in Mf with radiation dose (testing $\alpha_3 = 0$ vs. $\alpha_3 \neq 0$, $p = .81$ for HLA-A2, $p = .87$ for HLA-A24). It was also noted that the donor age or sex did not affect Mf among these people. However, there is a substantial amount of data indicating an age-related increase of Mf in somatic cells. Since A-bomb survivors are all over the age of 40, it was thought that the absence of a correlation between Mf and donor age might be due to the lack of younger individuals in the population. Therefore, an additional group of younger individuals was studied so that a donor age range of 20–80 years could be obtained. As shown in Figure 5, the inclusion of younger individuals resulted in a statistically significant increase of Mf with age as reflected in the parameter α_2 of the regression model ($p < .01$). When this age effect is taken into account, however, the effect of exposure to A-bomb radiation was still not statistically significant (data not shown). The mean Mf ($\pm SD$) of adults aged 20 to 50 was $1.50 \times 10^{-4} \pm 0.97 \times 10^{-4}$ for HLA-A2 ($n = 15$, mean age = 34) and $0.71 \times 10^{-4} \pm 0.27 \times 10^{-4}$ for HLA-A24 ($n = 17$, mean age = 38).

Molecular analyses of mutant lymphocytes

Using the FACStar sorting system, possible mutant cells lacking expression of the HLA-A2 or A24 allele were isolated and clonally propagated. The phenotypes of these clonal cells were subsequently analyzed using flow-cytometric analysis.

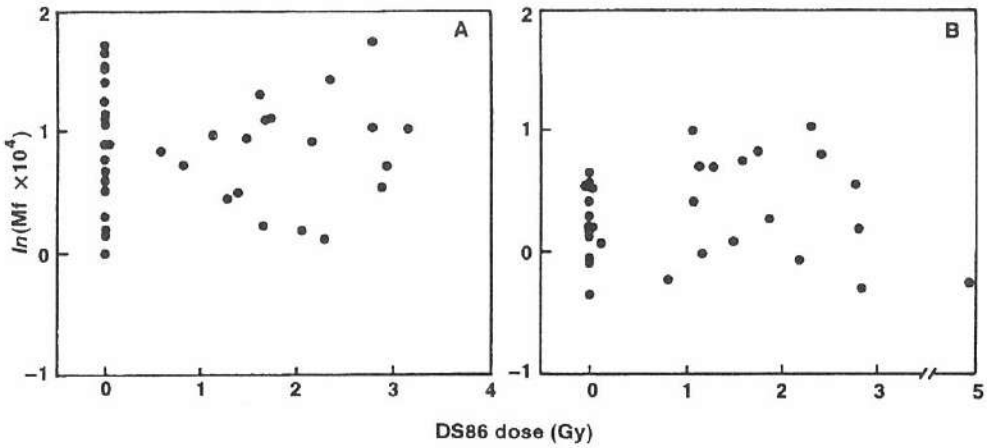

Figure 4. Natural log-transformed mutant frequency (Mf) of the HLA-A locus plotted against atomic bomb radiation dose. A: HLA-A2 locus; B: HLA-A24 locus.

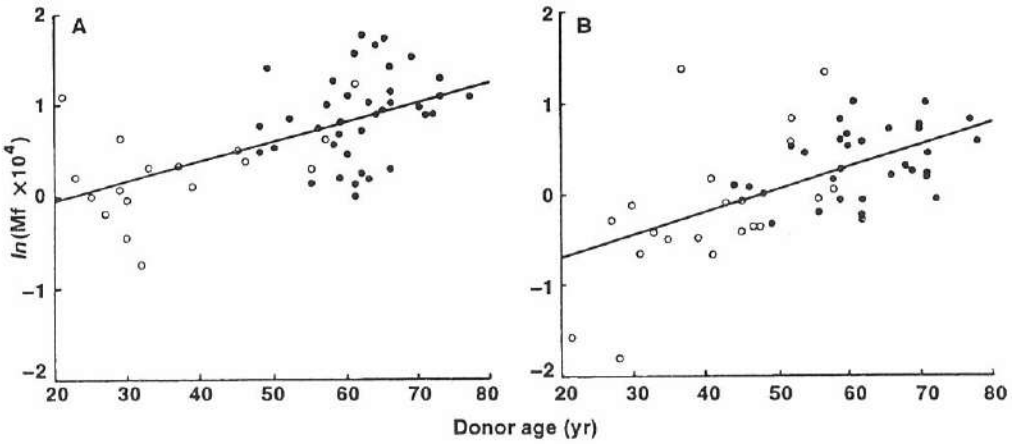

Figure 5. Natural log-transformed mutant frequency (Mf) of the HLA-A locus plotted against donor age. Open circles: laboratory volunteers; closed circles: Adult Health Study participants. A: HLA-A2 locus; B: HLA-A24 locus.

Eighty clones derived from three donors were examined and it was found that 77%–100% of them, depending on the experiment, were indeed defective in the expression of the selected HLA-A alleles (Table 2). The remaining nondefective cells most probably were due to errors associated with the sorting processes.

Table 3 shows the results on lymphocyte surface markers for the mutant clones. As has been known for mutant lymphocytes at the HPRT locus,[20] >80% of the clones consisted of $CD4^+$ cells and the remainder were mostly $CD8^+$ cells.

Southern blot hybridization analyses were undertaken for 50 mutant clones from three donors to detect gross structural alterations of DNA. Using the probe pHLA2a.1, it was found that 17 out of 50 clones had lost the restriction fragment specific to the selected HLA-A allele. The remaining 33 clones did not show any detectable changes (Table 4). Figures 6–8 show representative patterns of Southern blotting for the mutant clones derived from each donor. Since loss of the HLA-A2- or A24-specific band may result either from simple deletion of the HLA-A locus or from somatic recombination, the minisatellite locus DNF21S1 located at the distal region of the HLA-A locus, viz., 6p21.3 for HLA-A and 6pter-p25 for DNF21S1, was tested for the retention of heterozygosity. Representative results are shown in Figure 9. It was concluded that 16 out of the 17 mutant clones that had lost the restriction fragment specific to the selected HLA-A allele had also lost heterozygosity at the DNF21S1 locus.

However, all 16 clones were found to retain their heterozygosity at the D6S86 minisatellite locus located at chromosome 6q after Southern hybridization probed with cMS605 (data not shown). Metaphase chromosome analyses revealed no detectable deletions in any of the mutant clones irrespective of the retention or loss of HLA-A2- or A24-specific bands (data not shown). Because the HLA-A and DNF21S1 genes are located in distinctively separate regions of the metaphase chromosome, it was concluded that 16 out of 50 mutant clones lacking HLA-A2- or A24-specific bands and with lost heterozy-

Table 2. Results of cloning of sorted $CD3^+$ mutant T lymphocytes from three donors

Donors	Selected HLA-A alleles	No. of mutant clones (*A*)	Total no. of clones tested (*B*)	Fraction of mutant clones (*A/B*)
K.M.	HLA-A2	33	43	0.77
M.I.	HLA-A2	16	20	0.80
J.K.	HLA-A24	17	17	1.00

Table 3. Number of mutant T-cell clones bearing CD4 and/or CD8 surface markers

Donors	Surface markers				
	$CD4^+8^-$	$CD4^-8^+$	$CD4^-8^-$	$CD4^+8^+$	Total
K.M.	25	4	0	0	29
M.I.	15	0	0	1	16
J.K.	11	4	1	1	17
Total	51	8	1	2	62
	(82%)	(13%)	(2%)	(3%)	

Table 4. Characteristics of mutants lacking expression of the HLA-A2 or A24 allele

Characteristics			Origin of mutants			
Southern blot analysis		Cytogenetic analysis of chromosome 6p	In vivo	X-ray exposure (Gy)		
HLA-A	DNF21S1			0	1	2
+	H	No change	33	4	4	0
–	H	Visible deletion	0	0	8	4
		No change	1	0	1	0
		ND	0	0	2	1
–	LOH	Visible deletion	0	0	1	0
		No change	16	0	3	0
ND	ND	Visible deletion	0	0	2	2

NOTE: Different colonies of the same TCR rearrangement pattern were counted as one. "+" = the presence of the selected allele-specific band; "–" = its absence. H = heterozygous; LOH = loss of heterozygosity; and ND = not done.

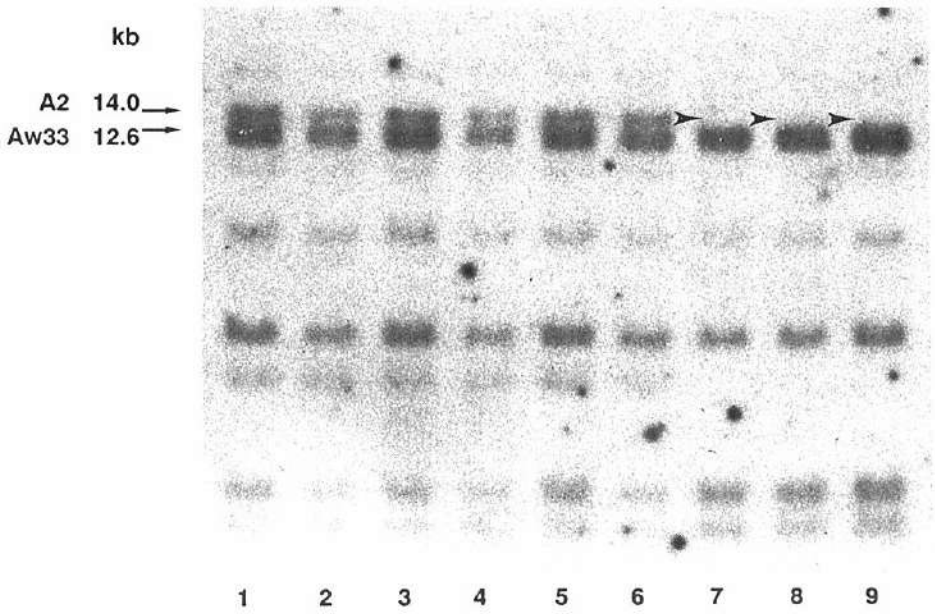

Figure 6. Southern blot hybridization of Bgl II-digested DNA from lymphocyte clones of donor K.M. (HLA-A2, Aw33) probed with pHLA2a.1. The 14.0-kb and 12.6-kb bands correspond to HLA-A2 and Aw33 alleles, respectively. Lane 1: normal clone; lanes 2–9: HLA-A2$^-$ mutant clones. The HLA-A2-specific band is absent in three of the mutant clones (lanes 7–9), as shown by the arrowheads.

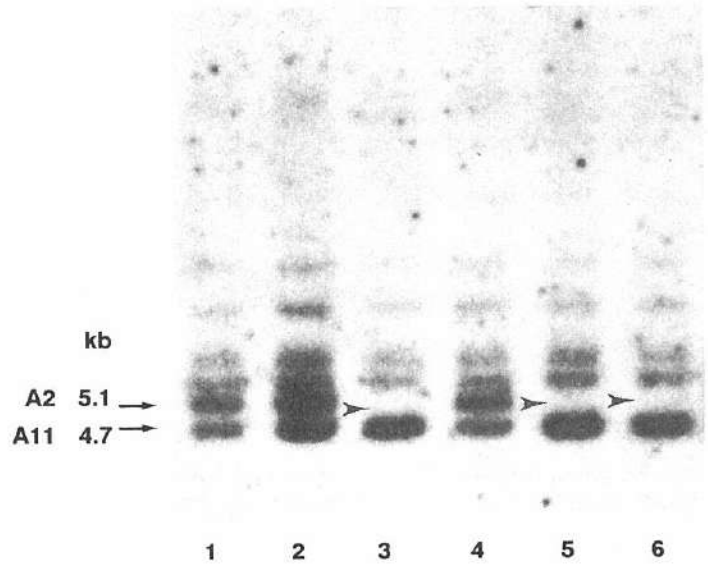

Figure 7. Southern blot hybridization of Hind III-digested DNA from lymphocyte clones of donor M.I. (HLA-A2, A11) probed with pHLA2a.1. The 5.1-kb and 4.7-kb bands correspond to HLA-A2 and A11 alleles, respectively. Lane 1: normal clone; lanes 2–6: HLA-A2$^-$ mutant clones. The 5.1-kb band is absent in three of the mutant clones (lanes 3, 5, 6), as shown by the arrowheads.

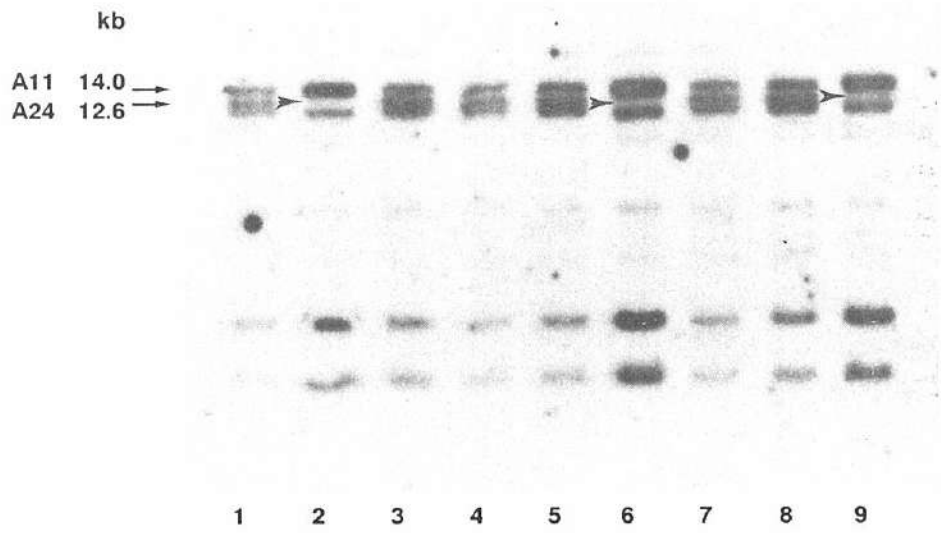

Figure 8. Southern blot hybridization of Bgl II-digested DNA from lymphocyte clones of donor J.K. (HLA-A11, A24) probed with pHLA2a.1. The 14.0-kb and 12.6-kb bands correspond to HLA-11 and A24 alleles, respectively. Lane 1: normal clone; lanes 2–9: The HLA-A24$^-$ mutant clones. HLA-A24-specific band is absent in three of the mutant clones (lanes 2, 6, and 9), as shown by the arrowheads.

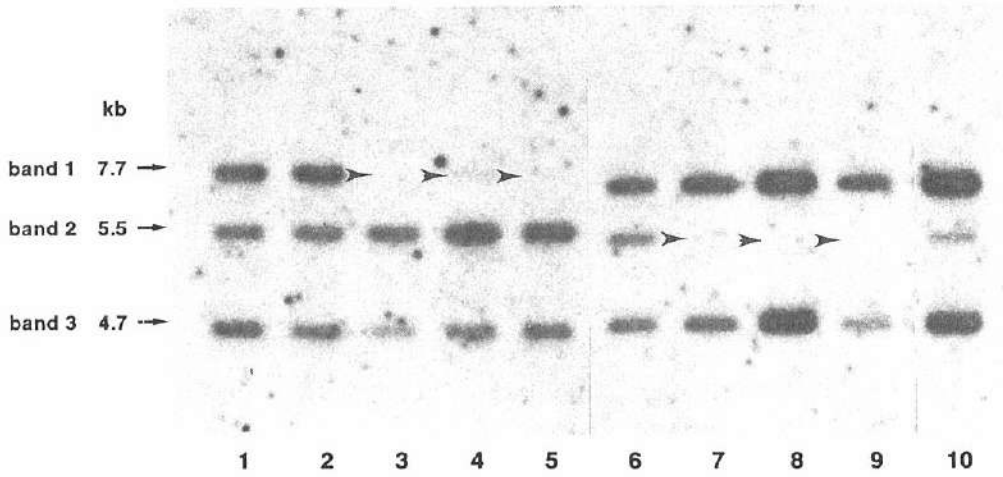

Figure 9. Southern blot hybridization of Hinf I-digested DNA from lymphocyte clones of donor K.M. (HLA-A2, Aw33; lanes 1–5) and donor J.K. (HLA-A11, A24; lanes 6–10) probed with λMS29. Lane 1: normal clone; lane 2: HLA-A2$^-$ mutant clone retaining the HLA-A2-specific band; lanes 3–5: HLA-A2$^-$ clones lacking the HLA-A2-specific band; lane 6: normal clone; and lanes 7–10: HLA-A24$^-$ mutant clones lacking the HLA-A24-specific band. The 7.7-kb and 5.5-kb bands correspond to the DNF21S1 locus on chromosome 6, and the 4.7-kb band corresponds to the DNF21S2 locus on chromosome 16.[16] Heterozygosity of the DNF21S1 locus was lost in three HLA-A2$^-$ clones (lanes 3–5) and in three HLA-A24$^-$ clones (lanes 7–9), as shown by the arrowheads.

gosity at the distally located minisatellite locus DNF21S1 were caused by somatic recombinations, not large deletions. The remaining 33 mutant clones retaining the HLA-A2- or A24-specific bands were found to be heterozygous at the two minisatellite loci, as would be expected if the mutations occurred as a result of subtle changes such as base substitutions or small deletions or insertions at the HLA-A locus.

Additional confirmation for the somatic recombination origin of the mutants was obtained by direct measurement of radioactivity at bands specific to the nonselected HLA-A allele, which is predicted to be doubled in such recombinants. Since the total amount of DNA subjected to each lane of electrophoresis might not be equal, the same filter was rehybridized using a probe for immunoglobulin Cμ gene, and the ratio of radioactivity at bands for the nonselected HLA-A allele to immunoglobulin was taken as an indicator of the number of HLA-A genes remaining. These results are summarized in Figure 10. One normal clone and three mutant clones retaining the selected HLA-A2 allele showed a ratio of about 0.2 while four mutant clones that lacked the selected HLA-A2 allele and had lost heterozygosity at the DNF21S1 locus showed a ratio that was 2 times higher, viz., about 0.4. One mutant clone retaining the selected allele showed a ratio of >0.4 (shown with brackets in Figure 10). In this case, however, the ratio of HLA-A2, the selected allele, to immunoglobulin bands was also 2 times larger and hence it is most probably caused by unusually low radioactivity at the immunoglobulin gene-specific band and not by duplication of the HLA-A locus, for example.

Phenotypes of the mutant lymphocytes from donor K.M. were examined for HLA-A and B loci with the standard NIH method using polyclonal antisera. This donor has been typed as HLA-A2, w33, B48, w51. Among the six mutant clones that had lost both the HLA-A2 allele and heterozygosity at the DNF21S1 locus (Table 5, mutant clones 1–6), five were found to have lost expression of B48 antigen whereas one mutant clone retaining the HLA-A2 allele (mutant clone 7) and one wild-type clone were both positive for HLA-Aw33, B48, and Bw51. Thus, it is most probable that in this person the HLA-A2 and B48 alleles are located on

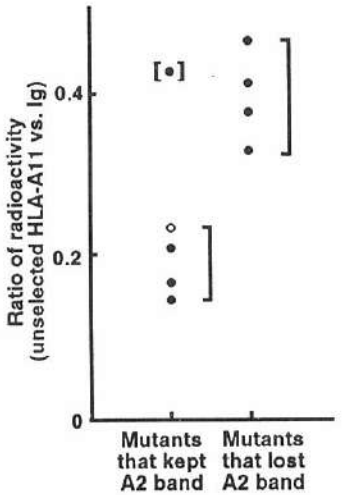

Figure 10. Ratio of the radioactivity of the unselected HLA-A11-specific band with that for the immunoglobulin (Ig) band in the Southern hybridized filter. One normal clone (open circle) and eight mutant clones (closed circles) were analyzed using a Bio Image Analyzer (Fuji). Mean ± *SD* = 0.200 ± 0.028 for the clones that retained the A2 band and 0.394 ± 0.057 for the clones that lost the A2 band.

Table 5. Phenotypes of HLA-A and B loci of mutant lymphocyte clones from donor K.M. determined by the standard NIH method using polyclonal antisera

Mutant clone	Phenotypes				Characteristics of mutants after Southern blot analysis
	A2	Aw33	B48	Bw51	
1	−	+	+	+	Loss of HLA-A2 and DNF21S1 alleles
2	−	+	−	+	Loss of HLA-A2 and DNF21S1 alleles
3	−	+	−	+	Loss of HLA-A2 and DNF21S1 alleles
4	−	+	−	+	Loss of HLA-A2 and DNF21S1 alleles
5	−	+	−	+	Loss of HLA-A2 and DNF21S1 alleles
6	−	+	−	+	Loss of HLA-A2 and DNF21S1 alleles
7	−	+	+	+	Retention of HLA-A2 and DNF21S1 alleles
Wild type	+	+	+	+	

one chromosome 6 and HLA-Aw33 and Bw51 on the other chromosome 6. Somatic recombination at some point proximal to the HLA-B locus in the chromosome 6 short arm resulted in loss of heterozygosity at both the HLA-A and B loci.

One exceptional case (mutant clone 1 in Table 5) bearing B48 antigen but that has lost the HLA-A2 allele and heterozygosity at the DNF21S1 locus can be explained as having resulted from a recombination that took place at some point between the HLA-A and B loci since the HLA-B locus is more proximally located. Analyses of T-cell receptor gene rearrangements showed that all the mutant clones tested had different rearrangement patterns and hence no indication was observed for the clonal propagation of mutant lymphocytes in vivo (data not shown).

In vitro mutagenesis study

X-irradiated PBMCs, cultured for 11 days after irradiation, were subjected to flow-cytometric analysis for the measurement of Mfs at the HLA-A24 locus.

In this experiment, the flowgram showed the presence of dully stained cells and the upper limit for the *y*-axis was moved upward from the ordinary setting of 1/25th to 1/10th of the peak value so that as many mutants as possible would fall in the window. Because such manipulations were expected to increase the chance of normal cells falsely falling in the mutant window, the Mf was obtained as the frequency of potential mutant cells in the window multiplied by the fraction of mutant colonies confirmed among all of the colonies generated after sorting and clonal culture of these potential mutants (Table 6).

A large fraction of the colonies derived from the sorted cells were from normal cells in the control cell population, viz., 13 out of 20 tested, and in one subpopulation exposed to 1 Gy, 12 out of 27 tested. In another subpopulation exposed to 1 Gy and in a population exposed to 2 Gy, the fraction of contaminating normal cells was very low. The results shown in Table 6, column E, suggest that the Mf increases on the order of 10^{-4} per Gy, which is about one order of magnitude greater than that reported for the X-chromosomal HPRT locus in lymphocytes.[21–23]

Table 4 contains a summary of the characteristics of these mutants along with those of in vivo-derived mutants. While only 1 out of 50 in vivo-derived mutants showed possible small deletions at the HLA-A locus without any

chromosomal abnormality, the vast majority of mutants derived from in vitro X-irradiation bore chromosomally detectable deletions, viz., 16 confirmed cases out of 24 successfully analyzed for the chromosome 6p. Detailed results for the deletions will be described in an accompanying paper.[24] The actual numbers of mutant colonies derived from the in vitro study were 7, 22, and 9 for 0, 1, and 2 Gy, respectively. Because the same TCR rearrangement patterns and the same deletions with or without specific chromosome changes occurred in other chromosomes, it was concluded that some of these mutants were clonal derivatives that had emerged during the 11-day postirradiation incubation period. Namely, one twin and one triplet in the 0 Gy population, one twin in the 1 Gy population, and two twins in the 2 Gy population. These colonies derived from a single mutant cell were counted as one in Table 4.

Discussion

The present HLA-A locus assay is the third flow-cytometric assay available for quantitative measurement of somatic mutations. It is remarkable that the Mf at the HLA-A locus was on the order of 10^{-4}. This frequency is several times higher than that for erythrocyte glycophorin A locus mutations[9] or for X-chromosomal HPRT gene mutations in lymphocytes[6–8] but is similar to that for T-cell receptor gene mutations.[25]

Morley's group has reported a mutation assay for the same HLA-A locus by means of a complement-dependent cell cytotoxicity (CDC) assay, namely, by combining monoclonal antibody and complement to kill cells bearing the epitope recognized by the antibody.[26,27] Their results show that the Mfs at the HLA-A2 and A3 loci were mostly on the order of 10^{-5} and the average Mfs for adults (mean age of 35) at these loci were 2.6×10^{-5} and 3.2×10^{-5}, respectively.[26] When compared with the present results, an average Mf of 1.50×10^{-4} at the HLA-A2

Table 6. Summary of in vitro mutagenesis experiments

Dose (Gy)	No. of cells in mutant window (*A*)	Total no. of $CD3^+$ cells ($\times 10^4$) (*B*)	No. of $A24^-$ colonies (*C*)	No. of $A24^+$ colonies (*D*)	No. of colonies failed to be tested	Mutant frequency ($\times 10^{-4}$) $\left(\frac{A}{B} \times \frac{C}{C+D}\right)$ (*E*)
0	200	64.8	7	13	3	1.08
1(a)	173	59.3	15	12	0	1.62
1(b)	184	55.4	15	0	1	3.32
2	148	21.5	11	2	0	5.82

NOTE: Peripheral blood mononuclear cells were irradiated with 0, 1, and 2 Gy of X rays and were subsequently cultured for expression of the mutant phenotype. Eleven days after the irradiation, the cells were stained with a pair of monoclonal antibodies and were subjected to flow-cytometric analysis. Cells that fell into the mutant window were sorted and 96 cells were replated in microwell plates at an average concentration of 1 cell/well. The growing colonies were tested for the expression of HLA-A24 antigen, and the mutant frequencies of the irradiated populations were assessed as (*E*). Cloning efficiencies were 0.27 (0 Gy), 0.33 (1 Gy [a]), 0.18 (1 Gy [b]), and 0.15 (2 Gy).

locus for 15 individuals of the corresponding ages (mean age of 34), the flow-cytometric data gave an Mf about 5 times higher than that of the CDC assay. Such a discrepancy would most probably be attributable to the different assay techniques, although the same monoclonal antibody was used. On the other hand, results at the HLA-A24 locus in the present study showed an Mf about one-half of that at the HLA-A2 locus (average Mf = 0.71×10^{-4} for 17 individuals of corresponding ages). The difference would be a reflection of the different specificities of the antibodies used, since there is no reason to expect that the HLA-A2 allele mutates more frequently than the HLA-A24 allele.

The fraction of somatic recombinants among the total number of mutants that had lost the HLA-A allele selected was about one-third, in close agreement with the results obtained with the CDC assay.[26,28] Both assays also showed a higher Mf in elderly persons. It remains to be known whether the age-related increase results from an equal contribution by somatic recombinations and gene mutations or from a preferential contribution by either one of the two.

It is believed that the HLA loci have evolved through extensive duplications and gene conversion-like events. If so, might they be prone to undergo mitotic recombinations even in somatic cells? Hakoda et al.[29] recently reported that among PBLs from heterozygous donors for adenine phosphoribosyltransferase (i.e., $APRT^{+/-}$), homozygous mutants ($APRT^{-/-}$) appear at a frequency on the order of 10^{-4}, of which about 80% had lost heterozygosity at the APRT gene. Likewise, in a human lymphoblastoid cell line TK-6 heterozygous for thymidine kinase gene ($TK^{+/-}$), Yandell et al. reported that about 70% of spontaneous mutants ($TK^{-/-}$) had lost heterozygosity at the TK gene.[30] Cytogenetic analysis of some of these mutants showed no evidence of detectable chromosomal changes. Since there is no evidence for tandem duplications of the APRT or TK genes, these results suggest that the HLA-A locus is not especially prone to undergo somatic recombinations. Rather, the relatively lower contribution of somatic recombinations in HLA-A locus mutations may suggest the gene-mutation-prone nature of HLA loci, since these genes have an evolutionary history of divergence, namely, creation of a large number of alleles. Alternatively, the expression of HLA loci may be prone to epigenetic down-regulational changes. To answer these questions, further studies are required on the molecular nature of mutants that did not show any gross structural changes in Southern blot hybridization analysis.

The mutation induction rate of about 2×10^{-4}/Gy obtained after in vitro irradiation experiments is approximately 10 times higher than that obtained for the X-chromosomal HPRT gene.[21–23] Further, the induction rate is most certainly an underestimate for the following reasons. First, the mutant clones obtained from the irradiated population mostly grew poorly. Thus, it is highly probable that negative selection against such mutants took place during the 11-day postirradiation expression period. Second, the discrimination of $HLA\text{-}A24^{-}$ mutant cells from normal cells was not very clear-cut under PHA-stimulated growing conditions. The upper limit of the mutant window was arbitrarily moved upward from the usual 1/25th to 1/10th of the peak fluorescent intensity, and—except for the nonirradiated cell population—a large fraction of colony-forming cells that fell in the expanded mutant window were mutants. Thus, if the upper limit of the window had been set higher, we would expect more mutant cells to have fallen in the mutant window for the irradiated cell populations, although a

higher level of normal cell contamination would have also occurred. Third, as mentioned earlier, the mutant clones from the irradiated population grew poorly. Thus, we would expect that the cloning efficiency of such cells would be lower than that of the contaminating normal cells. In fact, this is the case, as described in the legend of Table 6. Therefore, the ratio of mutant colonies among the total number of colonies produced would not be a direct reflection of the mutant cell fraction but would instead be an underestimate.

Why is the X-ray-induced Mf at the HLA-A locus so high compared with that at the X-chromosomal HPRT locus? It could be simply because, in the case of autosomal genes, cells can tolerate deletions of certain sizes because another intact homologue is present, whereas in the case of X-chromosomal markers, any deletion involving genes essential for cell multiplication is lethal even in female cells because only one X chromosome is actively transcribed.

In rodent cell lines, a large fraction of radiation-induced mutants resistant to 6-thioguanine accompany total or partial loss of the HPRT gene as detected by Southern blot analysis whereas the fraction of mutants bearing visible chromosomal changes is rather low.[31,32] On the other hand, in a rodent hybrid cell line containing one extra human chromosome 11, loss of one of the three genetic markers coded on the human chromosome has been shown to be induced by radiation with an enormously high frequency.[33] It is because any kind of deletion or even total loss of the extra human chromosome has no influence on survival in this particular cell line. Thus, these results suggest that a huge variety of deletions are induced by exposure to ionizing radiation but that only those cells capable of tolerating the loss of genetic material can survive irradiation to be detected as mutants.

Such cells bearing a large deletion in an autosome would be subjected to negative selection in vivo and hence might not be a serious threat in terms of genetic risks when they occur in male germ line stem cells. However, recent studies have revealed that many cancer cells contain mutations of suppressor oncogenes such as p53 or RB genes. If the deletions were induced in some somatic cells, growth disadvantages caused by the deletion might not be so severely subjected to negative selection as in continuously dividing spermatogonial cells since epithelial cells in liver, thyroid, or breast are believed to be mostly in a quiescent, nondividing state after some age. Further, deletions that include at least one suppressor oncogene might even provide a chance for such cells to escape from growth suppression caused by the surrounding normal cells as a result of secondary mutations of spontaneous origin, such as gene mutations in the remaining allele or somatic recombinations. Therefore, in terms of radiation-induced cancer in humans, the present results indicate the possibility that more deletions of suppressor oncogenes are involved in the radiation-induced cancers compared with so-called spontaneous ones. It will be a big challenge for us to test this hypothesis on cancer specimens derived from A-bomb survivors, and some technical developments are currently in progress toward achieving this goal.

Acknowledgments

The authors are grateful to Y. Fukuda from the Hiroshima University School of Medicine for his help in typing the lymphocytes using a standard NIH assay, N. Takahashi and M. Kodaira from RERF's Department of Genetics for their technical advice, K. Tanabe, M. Yamaoka, K. Takahashi, H. Tagawa, M. Enno, and N. Ishii for their technical assistance, S. Izumi for statistical assistance, M. Edington for editorial help, and M. Takagi for typing the manuscript.

References

1. Klein G: The approaching era of the tumor suppressor genes. Science 238:1539–45, 1987
2. Sager R: Tumor suppressor genes: The puzzle and the promise. Science 246:1406–12, 1989
3. Fearon ER, Cho KR, Nigro JM, Kern NS, Simons JW, Ruppert JM, Hamilton SR, Preisinger AC, Thomas G, Kinzler KW, Vogelstein B: Identification of a chromosome 18q gene that is altered in colorectal cancers. Science 247:49–56, 1990
4. Sankaranarayanan K: *Genetic Effects of Ionizing Radiation in Multicellular Eukaryotes and the Assessment of Genetic Radiation Hazards in Man.* Amsterdam, Elsevier Biomedical Press, 1982
5. Awa AA, Neriishi S, Honda T, Yoshida MC, Sofuni T, Matsui T: Chromosome-aberration frequency in cultured blood-cells in relation to radiation dose of A-bomb survivors. Lancet 2:903–5, 1982 (ABCC TR 27-71)
6. Albertini RJ, Castle KL, Borcherding WR: T-cell cloning to detect the mutant 6-thioguanine-resistant lymphocytes present in human peripheral blood. Proc Natl Acad Sci USA 79:6617–21, 1982
7. Morley AA, Trainor KJ, Seshadri RS, Ryall RG: Measurement of in vivo mutations in human lymphocytes. Nature 302:155–6, 1983
8. Hakoda M, Akiyama M, Kyoizumi S, Awa AA, Yamakido M, Otake M: Increased somatic cell mutant frequency in atomic bomb survivors. Mutat Res 201:39–48, 1988
9. Langlois RG, Bigbee WL, Jensen RH: Measurement of the frequency of human erythrocytes with gene expression loss phenotypes at the glycophorin A locus. Hum Genet 74:353–62, 1986
10. 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
11. 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
12. Akiyama M, Bean MA, Sadamoto K, Takahashi Y, Brankovan V: Suppression of the responsiveness of lymphocytes from cancer patients triggered by co-culture with autologous tumor-derived cells. J Immunol 131:3085–90, 1983

13. Wong Z, Wilson V, Patel I, Royle NJ, Povey S, Jeffreys AJ: Characterization of a panel of highly variable minisatellites cloned from human DNA. Cytogenet Cell Genet 46:719, 1987

14. Armour JAL, Povey S, Jeremiah S, Jeffreys AJ: Systematic cloning of human minisatellites from ordered array charomid libraries. Genomics 8:501–12, 1990

15. Takahashi N, Nakai S, Honjo T: Cloning of human immunoglobulin μ gene and comparison with mouse μ gene. Nucleic Acids Res 8:5983–91, 1980

16. Koller BH, Sidwell B, DeMars R, Orr H: Isolation of HLA locus-specific DNA probes from the 3'-untranslated region. Proc Natl Acad Sci USA 81:5175–8, 1984

17. Koller BH, Ward FE, DeMars R, Orr HT: Comparison of multiple HLA-A alleles at the DNA level by using Southern blotting and HLA-A-specific probes. J Immunol 135:4229–34, 1985

18. Wong Z, Royle NJ, Jeffreys AJ: A novel human DNA polymorphism resulting from transfer of DNA from chromosome 6 to chromosome 16. Genomics 7:222–34, 1990

19. Nicklas JA, O'Neill JP, Albertini RJ: Use of T-cell receptor gene probes to quantify the in vivo *hprt* mutations in human T-lymphocytes. Mutat Res 173:67–72, 1986

20. Hakoda M, Akiyama M, Kyoizumi S, Kobuke K, Awa AA, Yamakido M: Measurement of in vivo HGPRT-deficient mutant cell frequency using a modified method for cloning human peripheral blood T-lymphocytes. Mutat Res 197:161–9, 1988

21. O'Neill JP, Sullivan LM, Albertini RJ: In vitro induction, expression and selection of thioguanine-resistant mutants with human T-lymphocytes. Mutat Res 240:135–42, 1990

22. Sanderson BJS, Dempsy JL, Morley AA: Mutations in human lymphocytes: Effects of X- and UV-radiation. Mutat Res 140:223–7, 1984

23. Vijayalaxmi, Evans HJ: Measurement of spontaneous and X-irradiation induced 6-thioguanine resistant human blood lymphocytes using a T-cell cloning technique. Mutat Res 129:283–8, 1984

24. Kodama Y, Kushiro J, Hirai Y, Kusunoki Y, Nakamura N, Akiyama M, Awa AA: Chromosome aberrations in mutant T lymphocytes at the HLA-A locus. RERF TR manuscript in preparation.

25. 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 71:1981–99, 1990

26. Janatipour M, Trainor KJ, Kutlaca R, Bennett G, Hay J, Turner DR, Morley AA: Mutations in human lymphocytes studied by an HLA selection system. Mutat Res 198:221–6, 1988

27. McCarron MA, Kutlaca A, Morley AA: The HLA-A mutation assay: Improved technique and normal results. Mutat Res 225:189–93, 1989

28. Morley AA, Grist SA, Turner DR, Kutlaca A, Bennett G: Molecular nature of in vivo mutations in human cells at the autosomal HLA-A locus. Cancer Res 50:4584–7, 1990

29. Hakoda M, Yamanaka H, Kamatani N, Kamatani N: Diagnosis of heterozygous states for adenine phosphoribosyltransferase deficiency based on detection of in vivo somatic mutants in blood T cells: Application to screening of heterozygotes. Am J Hum Genet 48:552–62, 1991

30. Yandell DW, Pryja TP, Little JB: Molecular genetic analysis of recessive mutations at a heterozygous autosomal locus in human cells. Mutat Res 229:89–102, 1990

31. Fuscoe JC, Ockey CH, Fox M: Molecular analysis of X-ray-induced mutants at the HPRT locus in V79 Chinese hamster cells. Int J Radiat Biol 49:1011–20, 1986

32. Vrieling H, Simons JWIM, Arwert F, Natarajan AT, van Zeeland AA: Mutations induced by X-rays at the HPRT locus in cultured hamster cells are mostly large deletions. Mutat Res 144:281–6, 1985

33. Waldren C, Jones C, Puck TT: Measurement of mutagenesis in mammalian cells. Proc Natl Acad Sci USA 76:1358–62, 1979