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Evidence for Increased Somatic Cell Mutations at the Glycophorin A Locus in Atomic Bomb Survivors

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A recently developed assay for somatic cell mutations was used to study survivors of the atomic bomb at Hiroshima. This assay measures the frequency of variant erythrocytes produced by erythroid precursor cells with mutations that result in a loss of gene expression at the polymorphic glycophorin A (GPA) locus. Significant linear relations between variant frequency (VF) and radiation exposure were observed for three different variant cell phenotypes. The spontaneous and induced VFs agree with previous measurements of radiation-induced mutagenesis in other systems; this evidence supports a mutational origin for variant cells characterized by a loss of GPA expression and suggests that the GPA assay system may provide a cumulative dosimeter of past radiation exposures. VFs for some survivors differ dramatically from the calculated dose response, and these deviations appear to result primarily from statistical fluctuations in the number of mutations in the stem-cell pool. These fluctuations allow one to estimate the number of long-lived hemopoietic stem cells in humans.

THERE IS CONTINUING INTEREST IN evaluating the long-term health effects of human exposure to ionizing radiation (*1*) and in developing methods for assessing genetic damage in exposed individuals. The survivors of the atomic bombs at Hiroshima and Nagasaki provide a population particularly well suited for quantitative studies of this type. Researchers at the Radiation Effects Research Foundation (RERF) in Japan are accumulating extensive clinical and experimental data on large numbers of exposed and control survivors and their progeny (*2*). The exposure history of survivors is relatively well defined; dose estimates are available for individual survivors, and their exposure is dominated by a single, acute, whole-body, radiation dose. Previous studies of chromosome aberrations have revealed persistent genetic damage in somatic cells of these individuals (*3*). This study of specific locus mutations in somatic cells should further enhance our understanding of radiation-induced health effects.

The present study uses a newly developed specific locus mutation assay to detect the loss of gene expression at the glycophorin A locus in human somatic cells (*4*). Glycophorin A (GPA), a cell-surface glycoprotein on erythrocytes, occurs in two allelic forms, M and N, and is the product of codominantly expressed alleles on chromosome 4. In the GPA expression-loss assay, pairs of monoclonal antibodies specific for individual allelic forms are each conjugated with a different fluorescent dye and used to label fixed erythrocytes from heterozygous MN donors. Flow cytometry and sorting are used to enumerate and purify rare, single-color cells that lack the expression of one of the two GPA alleles. Presumably these cells lack expression because they are progeny of mutated erythroid precursor cells. Two of three previously described assay systems [1W1 and 2W2 (*4*)] were used in this study to determine the frequency of four variant cell types: those that lack the M-form of GPA (hemizygous NØ and homozygous NN variants) and those that lack the N-form of GPA (MØ and MM variants).

Blood samples were obtained from heterozygous MN donors in the RERF Adult Health Study and from a standard donor. One cohort of 43 donors (exposed) was composed of Hiroshima A-bomb survivors with revised tentative 1965 radiation dose estimates (T65DR) (*5*) ranging from 14 to 884 cGy (1 cGy = 1 rad). Repeat samples were obtained from 27 of these donors. A second cohort of 20 donors (control), with age and sex ratio similar to the exposed cohort, was composed of Hiroshima survivors with T65DR dose estimates of less than 1 cGy, with repeat samples from 10 donors. The standard donor (standard), a 29-year-old Japanese volunteer, was sampled 27 times during the study to monitor assay reproducibility. All samples were coded and then analyzed blind within 2 weeks of collection.

Variant frequencies (VFs) from all measurements for both assay systems are shown in Fig. 1; the mean VFs for each cohort are given in Table 1. Consistently low VFs were observed for three of the four variant cell types (NØ, MØ, and MM) in samples from the standard and control donors. The mean VFs for these donors ranged from 7×10^{-6} to 15×10^{-6}, which agreed with previously determined values of 9×10^{-6} to 21×10^{-6} for normal donors (*4*). From multiple measurements of the standard donor and replicate measurements on repeat blood samples from control and exposed donors, we determined a measurement coefficient of variation (CV) of about 60%, which was also comparable to previous estimates. The scattered VFs for NN variants were probably caused by staining artifacts previously described for this cell type (*4*).

Among the exposed donors, VFs of the NØ, MØ, and MM cell types varied widely from control levels to a maximum of 1312×10^{-6}. We confirmed the significance of the differences between the control and exposed donors with the nonparametric Mann-Whitney test (*6*) (Table 1). The mean frequencies of NØ and MØ variants were similar for the exposed donors, data that suggest that radiation-induced loss of GPA expression occurs at comparable frequencies for both the M and N alleles. The elevated VF of MM variants in exposed donors suggests that radiation induces events that lead to homozygosity [for example, chromosome missegregation or mitotic recombination (*4*)], although these events appear to be induced at a lower level than gene-loss events. Radiation-induced missegregation or recombination events have been reported for yeast (*7*), *Drosophila* (*8*), and mammalian cells (*9, 10*).

To relate GPA-assayed VFs to each individual's estimated dose, we assumed that T65DR doses approximate bone marrow doses since current revisions in exposure and organ doses are not yet available (*11*). Because the data set is too small to allow a rigorous test of a linear versus quadratic dose response, we also assumed a linear

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Table 1. Frequencies of four variant cell phenotypes for the three donor types. Standard donor (S), control cohort (C), and exposed cohort (E).

Variant cell type	Variant frequency* ($\times 10^{-6}$)			b† ($\times 10^{-6}$ per cGy)	P‡
	S	C	E		
NØ	9	15	78	0.26	0.001
MØ	7	9	63	0.23	0.001
MM	9	9	32	0.10	0.004
NN	28	64	69	—	0.43

*Mean variant frequencies of all donors in each population for each end point. The mean dose for the exposed population was 240 cGy (T65DR dose estimates). †Mean frequency of induced variant cells per centrigray for each end point; b = (exposed − control)/240 cGy. ‡Probabilities based on the Mann-Whitney nonparametric test that the VF of the exposed cohort is higher than that of the control cohort.

increase of VF with dose (D) such that (VF = $a + bD$). While dose-response shapes vary among cell types and exposure conditions, linear models have been reported for radiation-induced mutations in a number of other systems (*12–14*). We performed separate linear regressions of VF versus dose for each of the three cell types NØ, MØ, and MM, and all three showed significant positive relations with dose [slope (± SE) b = 0.18 (± 0.10), 0.16 (± 0.06), and 0.08 (± 0.04) × 10^{-6}/cGy; significance of the regression P = 0.037, 0.005, and 0.024, respectively (*15*)].

To simplify the analysis and reduce the effects of measurement errors, we calculated a mean hemizygous VF for each donor using VFs from replicate blood samples. Mean values included both NØ and MØ VFs since the two cell types showed similar spontaneous and induced frequencies. Figure 2a shows hemizygous VFs for the control and exposed donors. Whereas there was a highly significant relation with dose (P = 0.004) and most points clustered around the linear regression line, some individual values differed substantially from the calculated fit. These outlying values will be discussed below.

Previous studies of chromosome aberrations in A-bomb survivors also showed considerable scatter in aberration frequencies for individual donors, but these studies indicated a clear dose response when average frequencies were calculated for groups of donors in different dose intervals (*3*). A similar grouping of the data for exposed donors, with the mean VF and dose plotted for each group, is shown (Fig. 2b). The grouped data fit remarkably well with a linear regression, supporting a linear dose-response model for the exposed population. The linear-fit parameters for hemizygous variants reported in Table 1 and Fig. 2 agree to within about a factor of 2 (spontaneous VF, a = 9 × 10^{-6} to 23 × 10^{-6}; and induced VF per centigray, b = 0.15 × 10^{-6} to 0.26 × 10^{-6}).

There is extensive documentation of radiation-induced specific locus mutations in cultured mammalian cells (*9, 12, 14, 16*), in somatic cells in mice and humans (*17*), and in mouse germ cells (*12, 13, 18*). Most of these data can be fit to a linear dose response

Table 2. Replicate analyses on selected exceptional donors.

Donor No.	T65DR (cGy)	Observed VF* ($\times 10^{-6}$)		Expected VF† ($\times 10^{-6}$)
		NØ	MØ	
72	173	478	42	53
		882	60	
77	388	16	175	100
		13	175	
29	463	1312	251	117
		1045	65	
60	672	34	44	163
		13	22	
30	884	17	ND	209
		14	15	

*For each donor, the top row contains results from the initial blood sample, whereas the second row contains results from a replicate blood sample; ND indicates not done. Time intervals between the two samples for each donor (from top to bottom): 3, 1, 7, 1, and 8 months. †Variant frequencies calculated from the T65DR dose with the linear fit parameters in Fig. 2b.

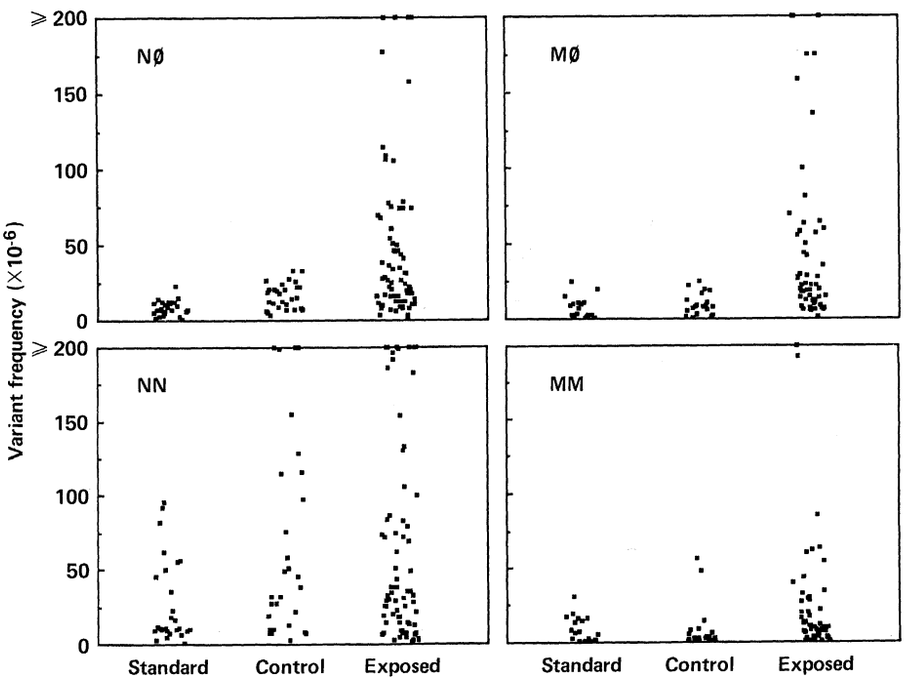

Fig. 1. Variant cell frequencies in all blood samples from the standard donor, the control cohort, and the exposed cohort. Variant frequencies greater than 200 × 10^{-6} are plotted at the top of each panel and include values from 225 × 10^{-6} to 1312 × 10^{-6}.

with spontaneous frequencies of a = 2 × 10^{-6} to 30 × 10^{-6} and induced frequencies per centigray of b = 0.06 × 10^{-6} to 0.66 × 10^{-6}. Thus, the similarity in fit parameters between these other systems and the GPA assay system 40 years after exposure supports a mutational origin for GPA variants, and suggests that the mutational lesions must be stably integrated in hemopoietic stem cells with no selective disadvantage for mutant cells. Persistent cytogenetic lesions have also been observed in hemopoietic stem cells of A-bomb survivors (*19*). In contrast to GPA variants and cytogenetic lesions, there appears to be an in vivo selective disadvantage for lymphocytes lacking hypoxanthine phosphoribosyltransferase activity ($HPRT^-$ lymphocytes) (*20*), and this may explain the small increase reported for $HPRT^-$ lymphocytes in A-bomb survivors (*21*).

Since other studies of genetic damage and health risk in A-bomb survivors generally involve pools of donors, comparisons with present results can best be made from ratios of values for high-dose pools (T65DR > 400 cGy, mean ~ 600 cGy) to values for low-dose pools (T65DR dose < 1 cGy). The 13-fold increase we have observed in GPA variants is similar to the 14-fold increase previously reported for exchange-type chromosome aberrations (*3*). Although increased risk for cancer in A-bomb survivors varies with time since exposure, cumulative mortality data from 1950 to 1984 show increased relative risks of 16-fold for leukemia, 4-fold for breast and colon cancer, 2-fold for stomach and respiratory cancer, and 2-fold for all cancers other than leukemia (*22*). Thus, although risks differ in each case, relative increases in risk are generally similar in magnitude to relative increases in the

frequency of chromosome aberrations and GPA expression–loss variants.

Although the VFs of most donors are consistent with a linear dose response, the values for some exposed donors differ dramatically from those predicted by the linear fit in Fig. 2a. Even though the limited precision of this assay (pooled CV = 60%) might explain the scatter at low doses, it is unlikely that measurement errors are responsible for the large deviations consistently observed in some donors. The results in Table 2 illustrate the size and reproducibility of these deviations from expected values. These exceptional donors appear to fall into two categories. For moderate-dose donors (170 to 500 cGy) large fluctuations in VFs were seen among donors with similar exposures and between alleles in individual donors, while for high-dose donors (> 500 cGy) uniformly low VFs were observed (Table 2 and Fig. 2a).

Since M-loss and N-loss events are two similar but genetically independent end points, a comparison between MØ and NØ frequencies for individual donors may explain these unexpected VFs. A donor's MØ frequency should equal his NØ frequency, but both frequencies will differ from the expected value if (i) the donor's exposure history differs from his T65DR dose, or (ii) he is unusually susceptible to radiation damage. This explanation is clearly not compatible with the large differences in VF between alleles in moderate-dose donors. Although this explanation could account for the uniformly low VFs in high-dose donors, it seems unlikely that doses were overestimated for all of these donors.

A second explanation is that large deviations from expected values result from statistical fluctuations, such as Poisson uncertainty in the number of induced mutations recorded in a limited number of stem cells or uncertainty in the number of mutations expressed by a limited number of stem cells that are contributing to the erythrocyte pool at any given time. In this case MØ and NØ frequencies would not be correlated for a given donor, and, if expression fluctuations dominate, then both frequencies could vary with time. Moderate-dose donors show large and reproducible differences between NØ and MØ frequencies that are consistent with Poisson fluctuations in the recording of induced mutations. Time-dependent expression fluctuations appear to be less important, as results from initial and replicate blood samples were highly correlated [slope (± SE) 1.01 (± 0.08), $P < 0.001$; typical sampling interval, 2 to 3 months]. Even exceptional VFs persisted for up to 8 months (Table 2).

Poisson fluctuations alone, however, do not explain the consistently low VFs of both alleles in high-dose donors. One additional factor may clarify the response of the GPA assay for all exposure groups. It has been shown that the number of surviving hemopoietic stem cells decreases exponentially with radiation dose [$N/N_0 = e^{-D/100}$; where N/N_0 is the fraction of surviving cells and D is dose in centigrays (*23*)] so that high-dose exposures could dramatically reduce the number of stem cells available for recording induced mutations. We have modeled the effect of stem-cell kill on the recording of induced mutations at specific doses by estimating the number of long-lived stem cells in normal donors, and then calculating (i) the number of surviving stem cells (from the equation above), (ii) the induced VF for mutations (from the fit parameters in Fig. 2b), and (iii) the number of induced mutations per donor [the product of (i) and (ii)]. To be consistent with our observations, this model requires a long-lived stem-cell pool size in the range of 10^6 to 10^7 for a normal individual. Whereas this estimate is somewhat low compared to estimates based on colony-formation assays (*24*), only a small fraction of colony-forming cells may have sufficient self-renewal potential to continue to contribute to the erythrocyte pool after 40 years (*25*).

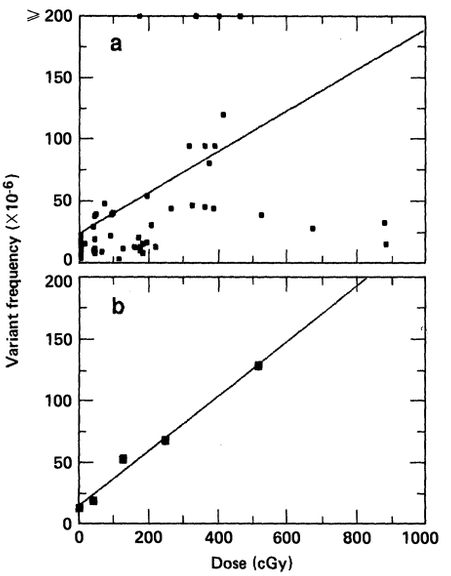

Fig. 2. Plots of hemizygous variant frequency (VF) versus T65DR dose for control and exposed donors. The VF calculated for each donor is the mean value of all NØ and MØ measurements performed on that donor. (**a**) Individual variant frequencies for all control and exposed donors. VFs greater than 200 are plotted at the top of the panel. These VFs are ($\times 10^{-6}$) 336, 330, 244, and 668 (from low to high dose). The line corresponds to a linear regression on these data yielding the following fit parameters (± SE): a = 23 (± 16) × 10^{-6}, b = 0.17 (± 0.06) × 10^{-6}/cGy; significance of the regression, P= 0.004. A linear fit neglecting the four values on the top and the four highest dose values yielded similar fit parameters [a = 10 (± 3) × 10^{-6}, b = 0.15 (± 0.02) × 10^{-6}/cGy; $P < 0.001$] which show that the fit is not dominated by outlying values. (**b**) Variant frequencies of pooled groups of control and exposed donors. Exposed donors were divided into four equal-sized subgroups (each containing 10 or 11 donors) on the basis of estimated exposure. The dose intervals resulting from this grouping were 14–62, 69–174, 180–359, and 361–884 cGy. Control donors made up a fifth, zero-dose group. Each point corresponds to the mean hemizygous VF and the mean dose for each group. The line corresponds to a linear regression on these data yielding the following fit parameters: a = 15 (± 4) × 10^{-6}, b = 0.22 (± 0.02) × 10^{-6}/cGy; $P < 0.001$.

A low-dose exposure of 50 cGy would produce minimal cell kill and induce a VF of about 10 × 10^{-6}. The number of stem-cell mutations per donor would be 10 to 100, so Poisson fluctuations would be relatively unimportant. An exposure to 400 cGy would reduce the stem-cell pool to about 10^4 to 10^5 cells, and induce a VF of 100 × 10^{-6}. At this dose, the number of mutations per donor would be 1 to 10, so significant Poisson fluctuations would be expected among donors and between alleles. Also, the magnitude of these statistical fluctuations would be large because each additional mutation in a stem-cell pool of this size would increase the VF by 10 × 10^{-6} to 100 × 10^{-6}. An exposure of 800 cGy would reduce the stem-cell pool to about 10^2 to 10^3 cells, so that even with an expected VF of 200 × 10^{-6}, no mutations would be recorded in most individuals. Thus our model provides an explanation for the large fluctuations in VF observed for moderate-dose donors and for the consistently low VFs seen for high-dose donors.

Ultimately, the usefulness of the GPA expression loss assay for determining the exposure history or health risk of individual donors will depend on the precision and stability with which rare mutational lesions are recorded in a finite number of stem cells. This analysis of data from A-bomb survivors shows that VFs can differ substantially from expected values among people who received large acute radiation exposures. This presumably is a result of statistical recording fluctuations in the small number of surviving stem cells. Our model suggests that statistical fluctuations should be less significant for low-dose or chronic exposures because of minimal perturbation of the stem-cell pool size. These fluctuations could also be less significant for assays performed a short time after exposure since the target pool might contain short-lived erythroid precursors as well as stem cells. It should be possible to determine the significance of statistical factors directly by quantitative comparisons of MØ and NØ frequencies in donors with different exposure histories.

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Cells in Temporal Cortex of Conscious Sheep Can Respond Preferentially to the Sight of Faces

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To investigate whether the temporal cortex of a nonprimate species contains cells responsive to the sight of faces, a study was made in conscious sheep of the responses of neurons in this brain region to the sight of faces. Of 561 cells from which responses were recorded, 40 responded preferentially to faces. Different categories of these cells were influenced by dominance (presumably indicated by the presence and size of horns), breed and familiarity, and threatening faces such as those of humans and dogs. These results demonstrate that cells that respond preferentially to faces are present in the temporal cortex of a nonprimate species, and that the responses of these cells are influenced by factors relevant to social interaction.

IN MONKEYS, A REGION OF THE TEMPORAL lobe contains some cells that respond preferentially to monkey and human faces (*1*). These cells also respond selectively to specific faces (*2*) or facial expressions (*3*). Damage to this region is associated with social disturbance, including reduced aggression (*4*), impaired visual discrimination (*5*), and in humans facial recognition may be impaired (*6*). In primates, therefore, this region of the brain is thought to play a role in individual recognition and appropriate social behavior, although its function in nonprimate species is unknown.

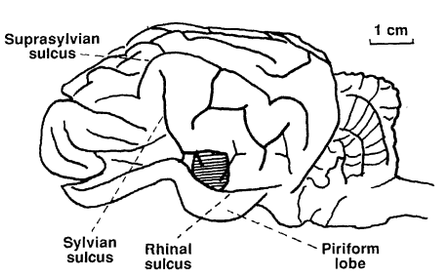

Fig. 1. Schematic drawing of a sagittal view of a sheep brain showing the location (shaded area) of cells that responded to the sight of faces.

Relatively little electrophysiological work has been done on the visual system of sheep but it has been shown, in anesthetized animals, that cells in their visual cortex respond to visual stimuli in the same way as similarly located cells in primates (*7*). As in primates, facial recognition is socially important to sheep since the face conveys breed and individual identity. Sheep prefer to interact socially with members of their own breed (*8*), and ewes visually recognize their own lambs by their faces rather than by other body features (*9*). Also, in horned sheep (*10*), and other horned ungulates (*11*), the size of the horns conveys information on both gender and position in the dominance hierarchy. The present study provides the first demonstration that cells showing preferential responses to the sight of facial stimuli are present in the sheep's temporal cortex. Further, different categories of these cells are distinguished by their responsiveness to dominance (the presence and size of horns), breed, familiarity, and stimuli associated with potential threat, such as humans and dogs.

Five Dalesbred sheep (a horned breed) were used. The methods for conscious single-unit electrophysiological recordings were as previously described (*12*) except that the temporal cortex was the brain region investigated. Recordings were made with the animals conscious and comfortably suspended in a canvas hammock. Head movement was prevented by anchoring the insensitive portion of the sheep's horns to the frame of the hammock. By means of a slide projector, visual stimuli were present binocularly with the use of a back-projection screen (0.6 m wide and 0.4 m high) placed with its center at eye level, 1 m directly in front of the animal. Approximately life-size stimuli were presented for 5 seconds, either stationary or slowly oscillating (0.2 Hz) horizontally. Although eye movements were not monitored the screen was positioned so that the stimuli fell on the area centralis of each eye when the sheep was looking forward. Since stimuli were presented in the dark, the screen represented the only major area of visual change for the animal and thus it probably focused on this for most of the

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