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Evidence for an Elevated Frequency of In Vivo Somatic Cell Mutations in Ataxia Telangiectasia

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Summary

Somatic cell mutation frequency in vivo was measured in individuals with high cancer risk who were from ataxia telangiectasia (A-T) families. The assay for somatic mutation measures the frequency of variant erythrocytes which are progeny of erythroid precursor cells with mutations that result in a loss of gene expression at the polymorphic glycophorin A (GPA) locus. Samples from 14 of 15 A-T homozygotes showed high frequencies of GPA gene expression–loss variant cells with normal expression of only one of the two alleles at the GPA locus (i.e., GPA hemizygous variant cells). The mean elevation of the frequency of hemizygous variant cells over those in normal controls and unaffected family members was 7–14-fold. A-T homozygotes also showed an increase in the frequency of cells in which one allele at the GPA locus had lost expression and in which the remaining allele was expressed at a homozygous level (i.e., GPA homozygous variant cells). Family members who are obligate A-T heterozygotes did not appear to have a significantly elevated frequency of GPA hemizygous or homozygous variant cells. These indications of elevated in vivo frequencies of variant erythrocytes in A-T homozygotes support a causal link between susceptibility to somatic mutation and susceptibility to cancer.

Introduction

Ataxia telangiectasia (A-T) is an inherited autosomal recessive syndrome, characterized by progressive cerebellar ataxia and oculocutaneous telangiectasia (Boder 1985), in which homozygotes have a very high cancer risk (Spector et al. 1982). Heterozygotes, estimated to comprise 0.68%–7.7% of the U.S. population (Swift et al. 1986), also show a significant excess of cancer and may represent an important proportion of cancer-prone individuals in the general population (Swift et al. 1987). We hypothesized that cancer susceptibility of A-T homozygotes and heterozygotes could be related to an increased rate of in vivo somatic mutation. To directly assess in vivo mutability, we measured the expression of mutations in erythroid cells of members of A-T families by enumerating the frequency of glycophorin A (GPA) variant erythrocytes in peripheral blood samples (Langlois et al. 1986).

The assay is based on the autosomal GPA locus which codes for the M and N blood group erythrocyte cell-surface antigens. Because the MN blood phenotype results from codominant allelic expression at the GPA locus, nonlethal null mutations in either the M or the N allele of erythroid precursor cells can be detected independently in our assay as hemizygous variant erythrocytes of Nϕ or Mϕ phenotype in the peripheral blood of individuals with MN blood type. Variant cells of this phenotype may arise by several genetic mechanisms, e.g., amino acid substitutions, deletions/insertions, as well as chromosome aneuploidy or nonhomologous somatic recombination. With this same assay, homologous mitotic recombination, chromosome missegregation, or gene conversion also can be detected as homozygous variant cells of MM or NN phenotype (Langlois et al. 1986). Our assay for detecting variant erythrocytes that presumably result from in vivo mutations at the GPA locus has demonstrated an increase in somatic cell mutations both in humans exposed to

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mutagenic chemotherapy (Bigbee et al. 1987) and in Hiroshima A-bomb survivors exposed to whole-body high-energy radiation (Langlois et al. 1987). The persistence of effects in A-bomb survivors who were exposed in 1945 indicates that stem cells in the bone marrow were mutated at the GPA locus by ionizing radiation and that these mutations continue to be expressed 40 years later as a high frequency of GPA variant erythrocytes in peripheral blood. The assay has also demonstrated very high frequencies of GPA hemizygous and homozygous variant erythrocytes in samples from Bloom syndrome homozygotes (Langlois et al., in press).

Material and Methods

Blood Samples

Blood samples were obtained with informed consent from members of 19 unrelated A-T families. Samples were drawn into EDTA anticoagulant and were transported immediately to Livermore. As required for this assay, only samples of MN blood type (approximately half of the total) were analyzed. All experimental analyses were performed without knowledge of the A-T status of the donors. A set of concurrent control samples was analyzed together with the samples from the A-T families. Most of the concurrent control samples were obtained from local healthy individuals, although three control blood samples were included in the coded shipments of samples from the A-T families. Nϕ and NN variant cell frequencies were obtained for samples from 24 concurrent control individuals, ages 29–61 years (mean age 43 years). The ratio of smokers to nonsmokers in this group was approximately 2:1. Mϕ and MM frequencies were obtained for samples from a subset of 4 of these individuals, one of whom was a smoker. Also included are variant-cell frequency data from an independent set of historic control samples from healthy individuals studied previously (Jensen et al. 1988). Nϕ and NN frequencies were obtained for samples from 54 historic control individuals, ages 1–84 years (mean age 37 years). Mϕ and MM frequencies were obtained for samples from a subset of 23 of these individuals. The ratio of smokers to nonsmokers in the historic control group was approximately 1:5.

GPA Analysis

GPA variant-cell frequencies were determined using the GPA in vivo somatic cell mutation assay as described elsewhere (Langlois et al. 1986). In brief, fixed peripheral blood erythrocytes are simultaneously immunolabeled with two GPA-specific monoclonal antibodies coupled to one of the distinguishable fluorophors, fluorescein (green fluorescence) or Texas Red™ (red fluorescence). Normal cells bind both antibodies and display two-color fluorescence, whereas variant cells, which fail to bind a GPA allele-specific antibody, are only singly labeled. Hemizygous Nϕ and homozygous NN variant cells are enumerated by labeling with a fluoresceinated GPA-specific "public" monoclonal antibody, 10F7, which recognizes both allelic forms of GPA, and with a biotinylated GPA(M)-specific monoclonal antibody, 6A7, followed by Texas Red–conjugated avidin. Cells of hemizygous Nϕ phenotype (loss of expression of the M allele) fail to bind 6A7, exhibit only background Texas Red fluorescence, and have one-half the normal 10F7 fluorescein fluorescence resulting from expression of the single N allele. Homozygous NN cells also fail to bind 6A7 but display normal 10F7 fluorescein fluorescence resulting from homozygous expression of the N allele. Hemizygous Mϕ and homozygous MM variant cells are enumerated by labeling with a fluoresceinated GPA(M)-specific monoclonal antibody, 9A3, and with a biotinylated GPA(N)-specific monoclonal antibody, NN-3, followed by Texas Red–conjugated avidin. Cells of hemizygous Mϕ phenotype (loss of expression of the N allele) fail to bind NN3, exhibit only background Texas Red fluorescence, and have 9A3 fluorescein fluorescence characteristic of the expression of a single M allele. Homozygous MM cells also fail to bind NN3 but exhibit 9A3 fluorescein fluorescence at a homozygous level of expression of the M allele. The antibody-labeled cell population is analyzed using a dual laser–beam flow sorter which provides individual excitation and detection of each fluorophor. Objects with single-color fluorescence intensities characteristic of hemizygous or homozygous variant erythrocytes are identified and sorted onto microscope slides for visual confirmation. For each sample, approximately 10^6 cells are analyzed; the variant cell frequency (V_f) is reported as the number of microscopically verified single-color erythrocytes per million total cells.

Statistical Analysis

On decoding, a preliminary statistical analysis of the distributions of the A-T family and of the control group V_f's revealed that the distributions were not normally distributed (Kolmogorov-Smirnov test). Therefore the distribution-free, nonparametric, two-sided, Mann-Whitney test was used to estimate the probability, *P*, of the identity of each A-T family group with each of the normal control groups.

Results

Control Groups

Concurrent with the measurement of GPA V_f's in samples from the A-T families, data were also obtained for samples from normal individuals. These samples were obtained from 21 local donors and from 3 individuals whose blood was included in shipments of samples from the A-T families. The V_f's observed for these three samples fell within the range observed for fresh samples from local controls. Thus, as we found elsewhere (Langlois et al. 1987), storage and shipment of blood does not appear to affect V_f's in normal samples. Since the group of concurrent control samples was rather small, V_f's determined in previous studies of 54 normal donors were also used for comparison with the A-T families. The concurrent and historic control populations are similar in age distribution (mean ages of 43 and 37 years, respectively). The concurrent control group contains a higher proportion of cigarette smokers, 2:1, versus 1:5 for the historic control group. Our studies suggest a small smoking effect in the GPA assay; the mean hemizygous V_f appears to be increased by ~20% in smokers over that in nonsmokers, although the increase is not statistically significant (W. L. Bigbee, R. G. Langlois, and R. H. Jensen, unpublished results). As shown in figure 1 and table 1, the distributions of Nϕ, NN, and MM V_f's are very similar for the two control groups and do not differ significantly (P = .572, .972, and .354, respectively); however the Mϕ distributions appear to differ (P = .006). This indication of a difference in Mϕ V_f's between the two control groups must be viewed with caution given the small number of samples (four) in the concurrent control group.

A-T Families

As shown in figure 1, the majority of blood samples from A-T homozygotes contain a very high frequency of Nϕ and Mϕ variant erythrocytes. Mean hemizygous

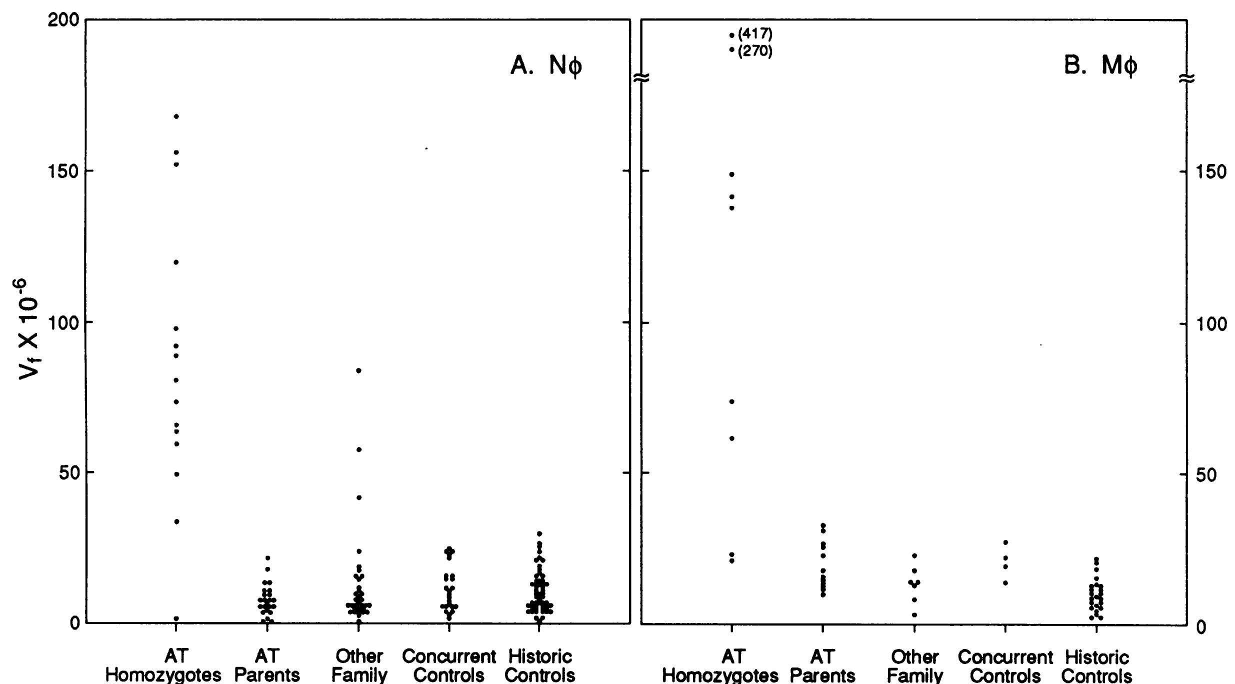

Figure 1 GPA hemizygous V_f's in peripheral blood of individuals from A-T families and of normal controls. Blood samples were simultaneously immunolabeled with two GPA-specific monoclonal antibodies and were analyzed by flow cytometry, and the variant erythrocytes were enumerated using cell sorting as described in Material and Methods. *A*, GPA hemizygous Nϕ variant cells; *B*, GPA hemizygous Mϕ variant cells (about half the MN blood samples were analyzed by this second, independent assay). V_f's are plotted for A-T homozygotes, for A-T parents (=mothers or fathers of A-T homozygotes), for other family(=brothers, sisters, aunts, uncles, grandmothers, or grandfathers of A-T homozygotes), for concurrent controls, and for historic controls. Concurrent controls are healthy individuals sampled and analyzed concurrently with the samples from the A-T families. Historic controls are healthy individuals sampled and analyzed in previous studies.

Table 1

Frequencies of GPA Variant Erythrocytes in Ataxia Telangiectasia (A-T) Families, Compared with Those in Controls

Donors (*N*)	Range	Median	Mean (SD)
Nϕ Variant Frequency per 10^6 Cells			
A-T homozygotes (15)	2–168	89	94 (50
A-T parents (23)	1–22	8	8 (5)
Other family (33)	1–84	7	13 (17)
Concurrent controls (24)	2–25	10	12 (7)
Historic controls (54)	1–30	10	11 (7)
Mϕ Variant Frequency per 10^6 Cells[a]			
A-T homozygotes (9)	22–417	138	144 (128)
A-T parents (12)	10–33	17	20 (8)
Other family (7)	3–23	14	13 (6)
Concurrent controls (4)	14–27	20	20 (5)
Historic controls (23)	2–21	9	10 (5)
NN Variant Frequency per 10^6 Cells			
A-T homozygotes (15)	0–140	14	43 (52)
A-T parents (23)	0–114	9	17 (25)
Other family (33)	1–161	10	23 (38)
Concurrent controls (24)	3–137	16	28 (32)
Historic controls (54)	1–155	14	28 (29)
MM Variant Frequency per 10^6 Cells[a]			
A-T homozygotes (9)	2–100	33	41 (35)
A-T parents (12)	0–66	4	10 (18)
Other family (7)	0–6	1	2 (2)
Concurrent controls (4)	3–23	10	12 (8)
Historic controls (23)	1–36	4	10 (11)

[a] A subgroup of the A-T samples that were analyzed for Nϕ and NN V_f's also were analyzed for Mϕ and MM V_f's.

V_f's are elevated 8-fold (for Nϕ) and 7–14-fold (for M0) over the levels in the two groups of controls (table 1), and both elevations are highly significant (Nϕ vs. historic and concurrent controls $P < .001$; Mϕ vs. historic controls $P < .001$, vs. concurrent controls $P = .016$). Nϕ and Mϕ V_f's in the A-T homozygote samples are also significantly elevated over those observed in samples from the A-T parents and other family members ($P < .002$ for all).

While 12 of the 15 A-T homozygotes had clearly high Nϕ and Mϕ V_f's, 3 showed a Nϕ or Mϕ V_f within the normal range (fig. 1, table 1): for donor 167, Nϕ = 89/million and Mϕ = 22/million; for donor 266, Nϕ = 34/million and Mϕ = 23/million; and for donor 234, Nϕ = 2/million and Mϕ was not measured. The low Mϕ V_f in donor 167 may reflect experimental error in the single measurement performed on this sample, as our experience with the assay indicates the coefficient of variation of individual measurements can be as large ~60% (Langlois et al. 1987); for the other two A-T homozygotes, however, the normal V_f's were confirmed by replicate measurements on independently fixed and labeled cell aliquots. Alternatively, the A-T phenotype is genetically heterogeneous (Paterson et al. 1977; Inoue et al. 1981; Murnane and Painter 1982; Jaspers and Bootsma 1982; Chen et al. 1984), and thus it is possible that these normal V_f's reflect genetic heterogeneity in susceptibility of A-T homozygotes to somatic mutation, with high susceptibility being common but not universal.

Parents of A-T homozygotes, who are obligate A-T heterozygotes, show a statistically significant ($P < .001$) elevation of Mϕ V_f's over the levels in the historic controls; however, no significant increase over the levels in the smaller set of concurrent controls was observed. There was also no significant elevation of Nϕ V_f's over the levels in either historic or concurrent controls. While more than half of the other family members are expected to be A-T heterozygotes, this group showed no difference from controls in either Nϕ or Mϕ V_f's, nor is there evidence for a bimodal distribution of Nϕ or Mϕ V_f's in these samples. However, three individuals in this group showed Nϕ V_f's above the normal range (42, 58, and 84/million; Mϕ V_f's were not measured).

The frequencies of GPA homozygous NN and MM variant cells were also determined (table 1). The mean MM V_f in A-T homozygotes is elevated fourfold over that observed in A-T family members, and controls. This elevation is significant compared with levels in the A-T parents ($P = .017$), other family members ($P = .002$), and historic controls ($P = .008$) but is not significant compared with those in the small set of concurrent controls ($P = .190$). The NN V_f's in samples from the A-T families and in those from controls were quite variable, owing in large part to antibody-labeling artifacts which, in this version of the assay, produce high NN frequencies in occasional samples (Langlois et al. 1986). Given this dispersion of the data, there are no significant differences in NN V_f's between any of the A-T family groups and controls, although the mean NN V_f in the A-T homozygotes appears to be elevated.

Discussion

Compared with controls, A-T homozygotes have a significantly elevated level of hemizygous Nϕ and Mϕ

variant erythrocytes. In addition, these individuals may have an increased frequency of homozygous NN and MM variant cells. These results provide clear evidence for a high background frequency of gene expression–loss somatic mutations in vivo in A-T homozygotes. Previous reports of elevated background levels of chromosome aberrations (for review, see McKinnon 1987) and of micronuclei in exfoliated cells (Rosin and Ochs 1986) are also consistent with spontaneous genetic instability in vivo in these individuals. In vitro studies, in contrast, have suggested near-normal background mutation frequencies for A-T cells (Arlett and Harcourt 1981; Simons 1982) and hypomutability of these cells by ionizing radiation (Arlett and Harcourt 1978; Arlett 1980; Tatsumi and Takebe 1984). Since spontaneous in vitro mutation frequencies are affected by time in culture and by the rate of cell doublings (Simons 1982), it is unclear whether A-T cells differ from normal cells in spontaneous mutation rate. Fluctuation experiments may be needed to clarify this point with regard to cultured cells (Arlett and Harcourt 1981). While our results do not provide any new information on induced mutations in A-T, it seems possible that the genetic defect(s) in these individuals could produce a high background mutation frequency but no increase in induced mutability by radiation.

A-T heterozygotes do not display a consistent elevation of V_f's in the GPA assay. Some of the data suggest an increase; $M\phi$ V_f's of the A-T parents are significantly elevated over those in historic controls, and three samples from other family members showed $N\phi$ V_f's above the normal range. However, the $M\phi$ V_f's in the A-T parents are not significantly elevated over those in the concurrent controls, and the $N\phi$ V_f's in the A-T parents are not elevated over those in either control group. The three high V_f's observed in the other family members may indicate that there are A-T heterozygotes who are particularly susceptible to mutagenesis or who may have experienced higher incidental mutagenic exposure than did other family members who were assayed. One of these individuals had received previous radiation therapy for breast cancer. No genetic evidence of differences in susceptibility are apparent, as no exceptionally high V_f's were observed for other members of these A-T families. Thus, A-T heterozygotes may have a background mutation frequency higher than that in normal individuals, but the difference is too small to detect with this assay.

Other studies, however, suggest that A-T heterozygotes, while lacking the clinical features of A-T, have subtle cytological abnormalities and increased cancer risk. A-T heterozygotes display elevated levels of micronuclei in exfoliated epithelial cells (Rosin and Ochs 1986). Cultured fibroblasts and PHA-stimulated lymphocytes from A-T heterozygotes generally show an increased level of background chromosome aberrations and hypersensitivity to killing by X-irradiation (Gropp and Flatz 1967; Schmid and Jerusalem 1972; Harnden 1974; Rary et al. 1974; Cohen et al. 1975; Oxford et al. 1975; Levitt et al. 1978; Al Saadi et al. 1980). However, compared with normal cells, in these cells X-irradiation does not induce an increase in cytogenetic abnormalities, except for one report of increased chromatid damage (Parshad et al. 1985). Also, in sensitivity to killing, inhibition of DNA synthesis, and level of cytogenetic damage by DNA-damaging agents to which homozygous A-T cells are hypersensitive, heterozygous A-T cells appear to be similar to normal cells (for review, see McKinnon 1987). Thus, A-T heterozygotes appear to have a spectrum of subtle cytological defects, possibly including mutation, which could be responsible for the increased risk of cancer seen in epidemiologic studies of these individuals (Swift et al. 1987).

The significance of our GPA-based mutational endpoint in estimating cancer risk has not yet been determined. However, it is clear that mutagenesis is an initiation event in some neoplasia (for review, see Borg 1985), and oncogene activation by mutation has been demonstrated (Brodeur 1986). In addition, loss of wild-type alleles of "tumor supressor genes" by several genetic mechanisms, including gene deletion or inactivation, recombination, and chromosome loss, has been observed in a wide variety of human tumors (Friend et al. 1988). The GPA mutation assay may provide a measure of each individual's initiation susceptibility; an estimate of overall risk will also require a means for assessing individual susceptibility to other steps in neoplasia, such as stimulation of tumorigenic growth by promoters.

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