Influence of Sex, Smoking and Age on Human *hprt* Mutation Frequencies and Spectra

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ABSTRACT

Examination of the literature for *hprt* mutant frequencies from peripheral T cells yielded data from 1194 human subjects. Relationships between mutant frequency, age, sex, and smoking were examined, and the kinetics were described. Mutant frequency increases rapidly with age until about age 15. Afterward, the rate of increase falls such that after age 53, the *hprt* mutant frequency is largely stabilized. Sex had no effect on mutant frequency. Cigarette smoking increased mean mutant frequency compared to nonsmokers, but did not alter age *vs.* mutant frequency relationships. An *hprt in vivo* mutant database containing 795 human *hprt* mutants from 342 individuals was prepared. No difference in mutational spectra was observed comparing smokers to nonsmokers, confirming previous reports. Sex affected the frequency of deletions (>1 bp) that are recovered more than twice as frequently in females ($P = 0.008$) compared to males. There is no indication of a significant shift in mutational spectra with age for individuals older than 19 yr, with the exception of A:T → C:G transversions. These events are recovered more frequently in older individuals.

AGING occupies a central position in health concerns. While the causes of aging are undoubtedly complex, it has been suggested that somatic mutations play a central role in the process (for review see Morley 1995). Recent models of aging suggest a role for free radicals (Slagboom and Vijg 1989; Gutteridge 1992; Harman 1992) and the diminishing efficiency of DNA repair with age (for review see Holmes *et al.* 1992). The DNA damage and repair theory of aging assumes that the total number of DNA lesions increases as a function of time and that DNA repair is unlikely to accurately correct them. In addition, the model asserts that mechanisms of DNA surveillance are likely to decrease with age (Warner and Price 1989). The catastrophic model is perhaps the most extreme. This model suggests that mutations accumulate with age to the point where the accuracy of molecular processes progressively deteriorates, and at the end of our lives, mutation rates escalate dramatically (Holmes *et al.* 1992).

Another potential mechanism of aging involves DNA methylation (Mazin 1994). Deamination of 5-methylcytosine (m^5C) to thymine that occurs at CpG sites results in the mispairing of thymine with guanine. The result is replication-mediated C/G → T/A transitions. Approximately 7×10^6 C → T transitions per human genome per division occur (Mazin 1994). Numbers of this type of mutation accumulate with cell division and, consequently, with age. O'Neill and Finette (1998) recently reported that mutations at CpG sites in newborns (cord blood) represent 33% of the single-base substitutions (6/18), while in adult populations such mutations represent only 5.1% (13/253). Their data indicate a dramatic age-related shift in mutational specificity.

More than 150 human genetic diseases have been characterized by Martin (1997) as in some way related to the normal process of human aging. However, as each of them differs significantly from "normal aging," Martin (1997) concluded "that there is no single gene that accelerates or decelerates the normal aging process." In other organisms, genes seemingly related to aging have been identified. An age-1 mutation in *Caenorhabditis elegans* has been observed to increase life span (Johnson 1990). This mutation increases the levels of superoxidase dismutase and catalase in an age-dependent manner (Larsen 1993). When either enzyme is overexpressed in Drosophila, no effect is noted on the life span. However, in concert they increase life span by one-third (Orr and Sohal 1994). This is one pathway of evidence to suggest that oxygen radicals play a major role in the aging process. Specifically, oxidative damage that causes 8-oxo-7-hydro-2′-deoxyguanosine has been shown to increase with age both *in vitro* and *in vivo* (Bohr and Anson 1995). Common sources of oxidative stress include metabolism, mitochondrial oxidative phosphorylation, and radiation.

Current techniques permit the assessment of *in vivo* human somatic mutation frequencies. In addition, sequence characterization of mutations provides insight into their origin. With these advances, it has become possible to assess the influence of aging on both muta-

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tion frequency and mutational spectra. One of the targets of choice for monitoring mutations in humans is the *hypoxanthine-guanine phosphoribosyltransferase* (*hprt*) gene. Used in peripheral T cells, the *hprt* gene is nonessential, and all classes of mutation can be recovered. *In vivo*, T cell *hprt* mutants can be selected and quantified by their resistance to the base analogue 6-thioguanine ($\sim$10 μm), as used in the T cell clonal assay (Albertini *et al.* 1982).

Human populations demonstrate *hprt* mutant frequency (MF) values that range over two orders of magnitude (Branda *et al.* 1993; Cole and Skopek 1994). Accordingly, studies that use MF values to monitor mutation in human populations require large sample sizes to account for this high degree of variation. Sources of MF variation are reviewed elsewhere, but they are primarily age, cloning efficiency (CE), and clonal expansion (Cole and Skopek 1994). Recent studies suggest that dietary components may also affect individual variability observed in MFs (Branda and Albertini 1995; Duthie *et al.* 1995). No evidence for any sex influence has been reported (Cole *et al.* 1988; Tates *et al.* 1991; Branda *et al.* 1993; Cole and Skopek 1994; Huttner *et al.* 1995).

Consequences of tobacco smoking on MF have been widely studied, with enigmatic results. Cole *et al.* (1988) observed that smokers had a 56% higher MF than nonsmokers. They later reported that the smoking effect was age related. The observed increase in MF for smokers was 2.9% per year compared to only 0.8% in nonsmokers (Cole *et al.* 1990, 1991). This observation differs from that of Davies *et al.* (1992) and Albertini *et al.* (1988), who found no effect, and Sala-Trepat *et al.* (1990), who found smokers to have lower MF than nonsmokers. A time-dependent effect of smoking was reported by Jones *et al.* (1993), where MF increased 10% per year of smoking compared to an increase of 1% per year by age. Another article reports that a statistically significant difference between MF values of smokers and nonsmokers could be observed only for female subjects (Huttner *et al.* 1995). In a more recent study, maternal cigarette smoking failed to demonstrate any significant effect on the MF of newborns (cord blood; Finette *et al.* 1997).

Many investigators report a linear increase of *hprt* MF with subject age (Morley *et al.* 1982; Trainor *et al.* 1984; Cole *et al.* 1988, 1991; Tates *et al.* 1991; Davies *et al.* 1992; Branda *et al.* 1993; Jones *et al.* 1993; Robinson *et al.* 1994). Overall, these studies suggest a 1–3% increase in MF per year at this locus. Most studies had limited numbers of adolescent subjects. Later comparison of MF values between adolescents and adults demonstrated a nonlinear response in MF with age (Finette *et al.* 1994). Adolescents were observed to have a rapid increase in MF with age as compared to adult values (Finette *et al.* 1994).

Other studies using different loci have also demonstrated linear age *vs.* MF relationships. Frequency of mutations at the HLA-A locus were found to increase with age from a mean of 0.71×10^{-5} in neonates to 6.5×10^{-5} in the elderly (age >60 yr, Grist *et al.* 1992). In addition to demonstrating a linear age relationship for MF at the *hprt* loci, Akiyama *et al.* (1995) resolved similar linear relationships using the glycophorin A and α/β T-cell receptor (TCR) genes.

While the effect of age on mutant frequency has been the subject of several studies, the effect of aging on the mutational specificity of spontaneously arising mutants has not been well studied. The mutational spectrum of *hprt* is affected by age (O'Neill and Finette 1998). Studies using Southern blot and PCR techniques have demonstrated a shift in the frequency of V(D)J recombinase-mediated deletion events at the *hprt* locus, which account for $\sim$40% of the T cell mutations in cord blood samples (Fuscoe *et al.* 1991) but only 2% of the events in adults (Fuscoe *et al.* 1992). It is assumed that during the ontogeny of T cells, when V(D)J recombinase is most active, the enzyme complex occasionally works on more than just TCR sequences. As the frequent exon II–III deletion events in cord blood are flanked by V(D)J recombinase consensus sequences, the production of this particular class of events is clearly age related. Both CpG transitions and V(D)J recombinase-mediated deletions were found to be dramatically altered when compared between newborns (cord blood) and adults. Alterations to mutational specificity that may occur later in life have yet to be formally explored.

MATERIALS AND METHODS

Mutant frequency analysis: A database containing published *hprt* MF data was constructed, including unpublished MF data from this laboratory. A total of 1194 individual subjects ranging in age from 0 to 85 yr were identified from the available literature. The MF dataset is available upon request. An overview of the MF dataset by author is given in Table 1. Pretreatment cancer patients were included, as those studies (Karnaoukhova *et al.* 1997, L. G. Karnaoukhova, K. S. Wilson and B. W. Glickman, unpublished data) and previous studies (Dempsey *et al.* 1985; Caggana *et al.* 1991) report no difference in MF compared to normal populations. Where multiple MF determinations were made for a single subject sample, the average MF was determined and used in the MF database.

Cloning efficiencies in the mutant frequency dataset were found to vary substantially between laboratories (n = 1194, mean CE = 40.5 $\pm$ 21.6%). Many factors are likely to contribute to this wide variation. Analysis of the effect of smoking status, sex, or age on cloning efficiency did not reveal any significant trends. For this reason, CEs are not considered during any of the age and MF analyses and are not reported henceforth.

Mutation frequency (MuF) is the frequency of independent mutational events that occur in a subject rather than the frequency of mutants that may arise from one mutational event by way of *in vivo* T cell clonal expansion. Within our own data, we corrected two subjects' mutant frequencies to estimated mutation frequencies by correcting for clonality using unique TCR rearrangements (Curry *et al.* 1995). These two corrections were found not to have any bearing on the subsequent analysis as a whole. Such corrections were not possible in the larger dataset. However, mutant frequency values do reflect a reasonable approximation of the actual mutation frequen-

TABLE 1

Summary of the assembled mutant frequency dataset by author

Reference	Subjects	Male/female	Smoker/nonsmoker[a]	Mean age ± SD (years)	Mean MF ± SD × 10^{-6}
Bachand *et al.* (1991)	25	17/8	6/19	51.6 ± 8.8	15.0 ± 9.9
Branda *et al.* (1993)	232	77/155	106/101/25[b]	52.6 ± 16.3	14.5 ± 11.2
Cole *et al.* (1988)	17	8/9	5/12	34.9 ± 11.8	5.9 ± 2.9
Curry *et al.* (1993)	1	1/0	1/0	37.0	26.0
Curry *et al.* (1995)	1	0/1	1/0	50.0	12.0
Curry *et al.* (1997)	12	12/0	2/10	29.3 ± 4.7	19.3 ± 10.5
Davies *et al.* (1992)	41	21/20	23/18	36.1 ± 12.0	2.8 ± 2.2
Finette *et al.* (1994)	49	29/20	0/49	5.6 ± 4.7	2.3 ± 2.2
Finette *et al.* (1997)	63	37/26	0/63	0.0 ± 0.0	0.9 ± 1.0
Hakoda *et al.* (1988)	17	7/10	0/0/17	58.6 ± 7.4	3.3 ± 2.0
Henderson *et al.* (1986)	23	7/7/9[c]	4/19	21.0 ± 18.3	2.6 ± 2.2
Hirai *et al.* (1995)	84	25/59	14/68	64.0 ± 7.7	10.7 ± 8.6
Hou *et al.* (1995)	76	76/0	0/76	43.4 ± 12.8	8.6 ± 4.6
Huttner *et al.* (1995)	44	26/18	20/24	39.8 ± 8.8	7.2 ± 5.6
Jones *et al.* (1993)	120	68/52	58/62	30.5 ± 6.9	8.5 ± 5.9
Karnaoukhova *et al.* (1997)	12	9/3	8/2/2[b]	74.8 ± 6.0	46.4 ± 37.5
L. G. Karnaoukhova, K. S. Wilson and B. W. Glickman (unpublished data)	11	7/4	4/6/1[b]	70.8 ± 9.1	45.9 ± 36.0
McGinniss *et al.* (1990)	45	38/7	6/37/2[b]	0.0 ± 0.0	0.7 ± 0.4
O'Neill *et al.* (1989)	6	3/3	0/6	26.3 ± 4.3	5.4 ± 1.0
Robinson *et al.* (1994)	145	71/61/13[c]	52/92/1	34.0 ± 16.7	9.0 ± 7.4
Saddi *et al.* (1996)	8	4/4	0/8	10.8 ± 4.4	3.9 ± 4.6
Sala-Trepat *et al.* (1990)	42	0/42	17/21/4[b]	50.4 ± 9.1	19.0 ± 14.0
Skandalis *et al.* (1997)	9	3/6	4/5	30.8 ± 14.0	15.5 ± 7.9
Tates *et al.* (1991)	111	111[c]	47/64	40.2 ± 10.9	11.2 ± 12.0
Totals	**1194**	**546/515/133**[c]	**378/762/54**[b]	**38.2 ± 21.6**	**10.1 ± 11.8**

[a] Smokers are considered those actively smoking tobacco.
[b] Smoking status unknown.
[c] Sex of subjects not reported in literature.

cies. In general, correction for clonality does not exceed ~20–30% of MF, with the possible exception of subjects where the MF is $> 40 \times 10^{-6}$ (O'Neill *et al.* 1994). Following the suggestions of Morley (1996), such cases have not been excluded from this analysis, despite the possibility that MF values $>40 \times 10^{-6}$ may reflect clonal runs of single mutational events.

Analyses were performed using the commercial statistical software packages Statistica (Statsoft, Tulsa, OK) and SAS (V6.12, SAS Institute, Cary, NC). Regression analyses of mutant frequencies are based upon their natural logarithms, as these data are skewed (Henderson *et al.* 1986). Four subjects demonstrated null mutant frequencies; hence, the natural logarithms could not be calculated, and they were excluded from further analysis. Relationships between ln MF and age were determined using linear regression. Standard deviations (in brackets) are given after all regression terms. To evaluate age trends, the dataset was partitioned into three age groups and then reanalyzed. Differences between the Pearson's correlation coefficients were tested for significance using a Fisher's *z*-transformation as described by Neter *et al.* (1989). Following the conservative Bonferroni procedure, the level of significance was lowered ($P = 0.05$/number of two-way comparisons) when more than two two-way comparisons were performed (Wassertheil-Smoller 1995).

***hprt* mutational spectra analysis:** *Mutational database construction:* Release five of the *hprt* mutant database was obtained from Mutabase Software (Durham, NC). All somatic mutants from human subjects were identified, but only mutants characterized by cDNA or multiplex PCR methods were selected from the *hprt* mutant database. Unpublished mutant spectra characterized by this laboratory ($n = 76$; L. G. Karnaoukhova, K. S. Wilson and B. W. Glickman (unpublished data) were also included.

Recent publications not yet included in the *hprt* mutant database were also added. Records from smokers, nonsmokers, and those with unknown smoking status were included, and these records were then reorganized to include three new fields: age, sex, and subject ID. This additional information required examination of the original publications, and it was necessary to determine the actual number of individuals from which the data were derived. Each mutant was also checked for accuracy against the original publication. Records with no age data were excluded from the *hprt* mutant dataset.

Mutants containing more than one mutation are described solely as complex. The original *hprt* database describes such mutations with multiple records. Mutants identified with truncated cDNAs or aberrant exon splicings were identified as splice mutations. Splice mutations for which the actual mutation had been determined by genomic DNA sequencing were identified by the presumed causative mutation. Uncharacterized splice mutations, though reported in the following tables, were excluded from further analysis ($n = 188$).

Analysis and the elimination of reporting bias: Mutations collected for the analyses were obtained from several laboratories

using either cDNA or multiplex PCR methods for the molecular characterization of mutants. As differences in these two methods may result in the unequal reporting of mutation classes or the unequal classification of mutations, a potential for bias exists. An Adams Skopek Monte Carlo test (Adams and Skopek 1987) was used to test each subset of data (by author and/or by methodologies) used against the remainder of the mutant dataset.

Mutational spectra for smokers and nonsmokers were tested for differences. Similar mutational spectra for males and females were generated and tested. Finally, mutational spectra for several age groups were identified. For example, the dataset was partitioned into two age groups (4–37 and 38–80 yr) and further into three and four age groups, with roughly equal numbers of mutants where possible. As the mutant database contained only two mutant sequences obtained from a young individual (4 yr old), no spectra analysis for individuals <19 yr old was attempted.

Mutational spectra were compared using the Adams Skopek Monte Carlo test. Mutational spectra were further evaluated using Fisher's exact test to examine specific mutational classes. Chi-square analysis was used in comparisons where the Fisher's exact test could not be performed.

RESULTS

Mutant frequency analysis: *Influence of sex on ln MF:* Differences in mean age and ln MF between males and females were tested using a *t*-test. As noted in Table 1, the sex of some subjects was not reported in the literature. Distribution of age between males and females was significantly different ($P < 10^{-6}$). Cord blood samples (age = 0) are overrepresented in the male population ($n = 47$) as compared to the female population ($n = 9$), and result in a lower mean age for males. To obtain similarly distributed ages for the comparison of ln MF, the cord blood samples were excluded from the analysis. Although the mean age of female subjects remained significantly higher ($P = 0.001$) than that of males, ln MF was not significantly different between the sexes. As sex had no influence on ln MF, both sexes were combined to assess any effects of smoking.

Influence of smoking on ln MF: Differences between the ln MF of individuals who smoke tobacco and nonsmokers were tested for significance. Mean age was significantly different between smokers and nonsmokers ($P < 10^{-6}$). Subjects 16 yr old or younger were excluded from this analysis, as they mainly comprised nonsmokers and significantly lowered the mean age of the group. With this exclusion, no significant difference in the mean age between the smoking and nonsmoking groups was observed. However, the smoking population had a significantly higher mean ln MF compared to the nonsmoking group ($P = 0.0004$).

To further explore the difference in the mean ln MF between smokers and nonsmokers, these two groups were subdivided by sex. After subjects 16 yr old or younger were excluded from the analysis, mean ages were not significantly different, except between male and female nonsmokers ($P = 0.0001$). Significant differences in the mean ln MF were observed only between male smokers and nonsmokers ($P = 0.005$), as well as between male nonsmokers and female smokers ($P = 0.001$). Male nonsmokers were noted to have the lowest ln MF among the four groups. Surprisingly, no significant difference was detected between the female smokers and nonsmokers.

Influence of age on ln MF: Results of several linear regression analyses are reported (Table 2). In the first regression, all data were included in the analysis of age *vs.* ln MF. The dependent variable was ln MF, and age was the independent variable. Correlation coefficients of the linear age-ln MF relationships for males and females are significantly different ($P = 0.007$). As previously noted, the mean age of male subjects was significantly lower than that of females. The male group contains significantly more MF determinations from cord blood samples (males $n = 84$, females $n = 35$), which partially accounts for the lower male mean age as compared to females. After excluding cord blood samples from both male and female linear age ln MF regressions, there was no significant difference in the correlation coefficients. The regression lines are nearly identical, as indicated by the intercepts and slopes (Table 2).

Linear regressions for smokers and nonsmokers revealed no significant differences in the correlation coefficients, but only after subjects 16 yr old or younger were excluded from the analysis. Smoking and nonsmoking age *vs.* ln MF relationships produce identical slopes, but the smoking group intercept is ~8% higher (Table 2).

Consistent with previous observations (Finette *et al.* 1994), the data in this study were partitioned into a child group (ages 0–15 yr) and two adult groups (ages 16–52 and 53–85 yr; Table 2). The rationale for the further subdivision of the adult group is as follows. The adult population was divided at the point where the oldest partition (ages 53–85 yr) no longer provided a significant relationship between age and ln MF (Figure 1). A comparison of the correlation coefficients for the effect of age on ln MF in the three age partitions and for the entire dataset (Table 2) was performed. All three of the partitioned relationships are significantly different from the overall regression line. In addition, the regression lines of the three age group partitions are all significantly different from one another.

Mutational spectra analysis: A total of 795 independent mutations were collected from 342 individuals, and a summary of the mutational data assembled is listed by publication (Table 3). The complete *hprt* mutant dataset is available upon request. The mean age of these 298 individuals (excludes subjects that only presented uncharacterized splice mutants) is 40.3 ± SD 15.2 yr. Mutations were from individuals older than 18 yr, with the exception of two mutations from a 4-yr-old. Hence, the analysis is limited to the examination of changes in

TABLE 2

Summary of regression analysis for the influence of sex and smoking on the age *vs.* ln MF relationships

	Regression line with SD	n^a	r^b	P
All data	ln MF = 0.264 (0.052) + 0.039 (0.001) age	1190	0.690	$P < 10^{-6}$
Males	ln MF = 0.239 (0.069) + 0.039 (0.002) age	544	0.716	$P < 10^{-6}$
Females	ln MF = 0.411 (0.091) + 0.035 (0.002) age	514	0.625	$P < 10^{-6}$
Males aged >0 yr	ln MF = 0.927 (0.088) + 0.026 (0.002) age	462	0.527	$P < 10^{-6}$
Females aged >0 yr	ln MF = 1.064 (0.103) + 0.022 (0.002) age	479	0.436	$P < 10^{-6}$
Smokers aged >16 yr	ln MF = 1.276 (0.122) + 0.022 (0.003) age	370	0.406	$P < 10^{-6}$
Nonsmokers aged >16 yr	ln MF = 1.202 (0.091) + 0.019 (0.002) age	576	0.389	$P < 10^{-6}$
Ages 0–15 yr	ln MF = −0.513 (0.072) + 0.139 (0.016) age	187	0.541	$P < 10^{-6}$
Ages 16–52 yr	ln MF = 1.007 (0.111) + 0.026 (0.003) age	675	0.316	$P < 10^{-6}$
Ages 53–85 yr	ln MF = 1.831 (0.442) + 0.010 (0.007) age	328	0.084	$P = 0.13$

[a] As the sex and/or smoking status of some subjects is not reported for all subjects, the *n* values do not sum to total sample size.
[b] Pearson correlation.

mutational specificity that occur exclusively in adults. As the majority of mutations ($n = 513$) have been obtained from the 236 male subjects, this unfortunately limits the analysis of the spectra for the effect of sex. Summary information regarding the subject samples by spectra groupings is shown (Table 4).

Test for dataset bias: When each author dataset was tested against the remainder of the *hprt* mutant dataset

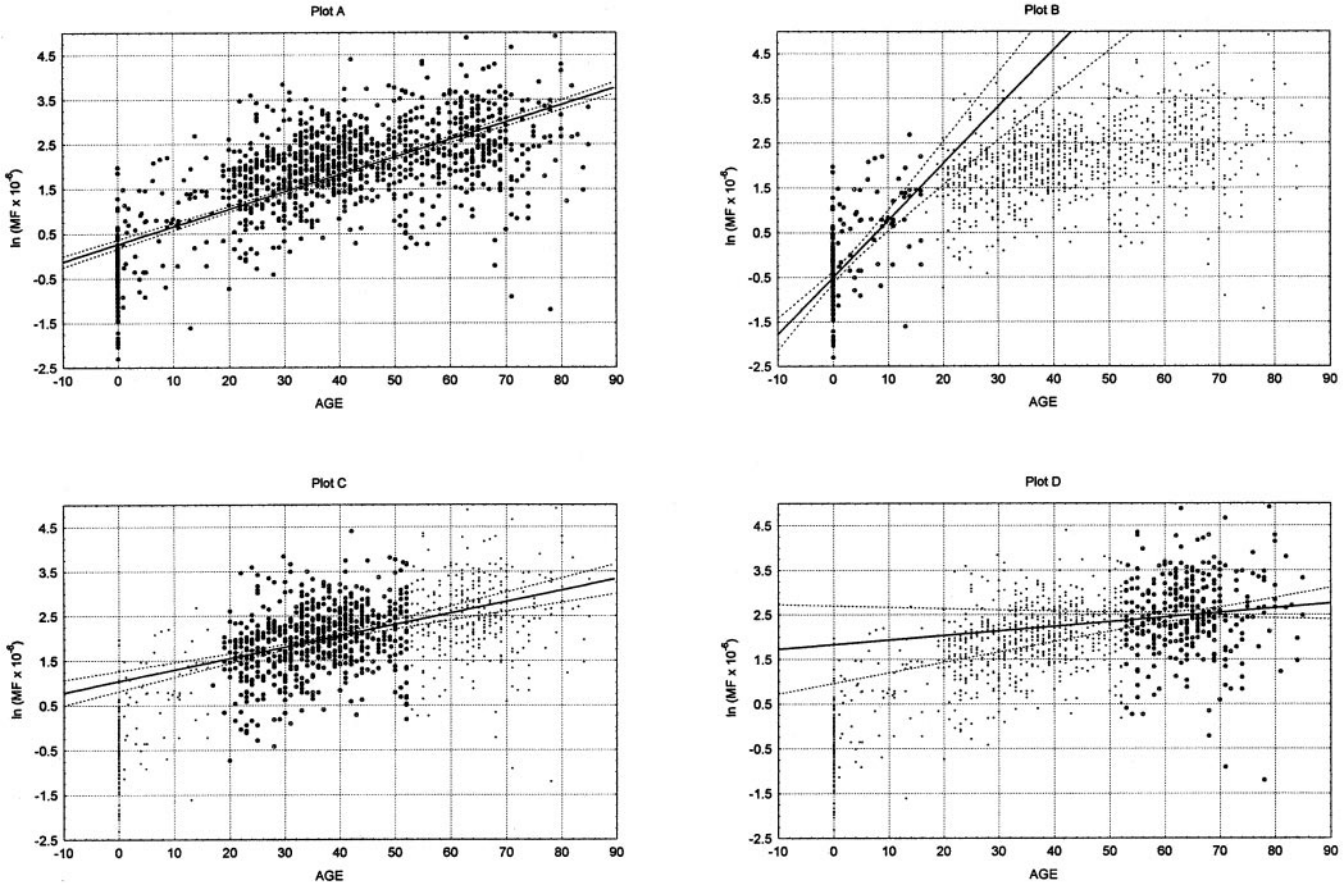

Figure 1.—Linear regression lines for the ln MF = *f*(age) relationships for the entire dataset (Plot A) and three age groups: ages 0–15 (Plot B), ages 16–52 (Plot C), and ages 53–85 (Plot D), where no relationship with age exists. Large circles indicate the data that is used for the respective regressions. The actual equations, *r* values, and probability values for the equations are shown in Table 2. All four lines are significantly different from one another.

TABLE 3

Summary of mutant dataset for molecular analysis

Reference	Subjects[a]	Sex F/M	Nonsmoker/ smoker	Mutants sequenced	Mean age ± SD
Andersson *et al.* (1992)	4	0/4	4/0	12	25.8 ± 3.8
Burkhart-Schultz *et al.* (1993)	52[b]	17/35	25/27	52	35.5 ± 8.7
Burkhart-Schultz *et al.* (1996)	52	17/35	25/27	102	28.9 ± 7.2
Burkhart-Schultz and Jones (1997)	46	8/41	18/31	49	35.0 ± 9.6
Curry *et al.* (1993)	1	1/0	0/1	8	37.0 ± 0.0
Curry *et al.* (1995)	1	0/1	0/1	38	50.0 ± 0.0
Hou *et al.* (1993)	6	0/6	6/0	14	36.3 ± 15.9
Karnaoukhova *et al.* (1997)	11	2/9	2/9	58	74.4 ± 6.1
L. G. Karnaoukhova, K. S. Wilson and B. W. Glickman (unpublished data)	10	3/7	4/6	76	70.0 ± 8.9
Osterholm and Hou (1998)	30	0/30	30/0	58	43.3 ± 13.0
Podlutsky *et al.* (1998)	38	0/38	38/0	115	43.2 ± 11.1
Recio *et al.* (1990)	1	0/1	1/0	27	23.0 ± 0.0
Rossi *et al.* (1990)	9	0/9	9/0	31	37.4 ± 6.4
Shimahara *et al.* (1995)	15	0/15	15/0	34	66.3 ± 8.1[c]
Skandalis *et al.* (1997)	9	6/3	4/5	31	31.1 ± 14.7
Steingrimsdottir *et al.* (1993)	4	0/4	2/2	34	41.5 ± 14.0
Vrieling *et al.* (1992a)	12	0/12	3/9	56	36.5 ± 6.8
Totals	**342**	**62/280**	**191/151**	**795**	**39.6 ± 14.8**

[a] Subject totals corrected for subjects appearing in more than one publication.
[b] Number of subjects reflects only those listed in release 5 of the *hprt* data base (Mutabase Software, Durham, NC).
[c] Exact ages could not be determined for the 34 individual mutants collected from the 15 subjects, but the mean age of these male subjects was 65.7 ± 7.9 yr, with a range of 58–81 yr, and all mutants are later placed into the oldest age partition.

using the Monte Carlo test, several demonstrated significant differences. In particular, the data of Burkhart-Schultz *et al.* (1996) were significantly different ($P < 10^{-6}$). In this particular dataset ($n = 102$), the authors reported only point mutations. After mutational spectra combined from all three of the Burkhart-Schultz *et al.* (1993, 1996; Burkhart-Schultz and Jones 1997) articles were compared to the remaining

TABLE 4

Subject summary data for spectra

	Female	Male	Smokers	Nonsmokers
Total individuals/mutants	62/104	236/513	132/248	164/369
Smokers/nonsmokers	33/27	99/137	132/0	0/164
Age ± SD yr	36.1 ± 14.2	41.4 ± 15.3	39.5 ± 13.8	40.9 ± 16.3
	19–37 yr	38–83 yr		
Total individuals/mutants	157/282	139/335		
Smokers/nonsmokers	70/87	62/77		
Age ± SD yr	29.1 ± 5.6	52.9 ± 12.5		
	19–31 yr	32–45 yr	46–83 yr	
Total individuals/mutants	101/179	108/206	87/232	
Smokers/nonsmokers	39/62	62/46	31/56	
Age ± SD yr	26.0 ± 4.2	37.8 ± 3.7	60.1 ± 10.4	
	19–31 yr	32–40 yr	41–54 yr	55–83 yr
Total individuals/mutants	101/179	80/159	59/132	56/147
Smokers/nonsmokers	39/76	43/37	35/24	15/41
Age ± SD yr	26.0 ± 4.2	36.1 ± 2.4	46.3 ± 4.1	66.0 ± 8.2

TABLE 5

Mutational spectra for smoking status, sex, and age (two partitions)

Mutation class	Smokers (%)	Nonsmokers (%)	Female (%)	Male (%)	19–37 yr (%)	38–83 yr (%)
GC → AT	55 (22.2)	103 (27.9)	17 (16.3)	141 (27.5)	70 (24.8)	88 (26.3)
AT → GC	22 (8.9)	36 (9.8)	11 (10.6)	47 (9.2)	30 (10.6)	28 (8.4)
Transitions	77 (31.0)	139 (37.7)	28 (26.9)	188 (36.6)	100 (35.5)	116 (34.6)
GC → TA	21 (8.5)	29 (7.9)	12 (11.5)	38 (7.4)	28 (9.9)	22 (6.6)
GC → CG	24 (9.7)	34 (9.2)	15 (14.4)	43 (8.4)	25 (8.9)	33 (9.9)
AT → TA	22 (8.9)	34 (9.2)	4 (3.8)	52 (10.1)	25 (8.9)	31 (9.3)
AT → CG	25 (10.1)	24 (6.5)	10 (9.6)	39 (7.6)	14 (5.0)	35 (10.4)
Transversions	92 (37.1)	121 (32.8)	41 (39.4)	172 (33.5)	92 (32.6)	121 (36.1)
Total substitutions	169 (68.1)	260 (70.5)	69 (66.3)	360 (70.2)	192 (68.1)	237 (70.7)
Deletion[a]	32 (12.9)	49 (13.3)	22 (21.2)	59 (11.5)	38 (13.5)	43 (12.8)
Frameshift (−)	26 (10.5)	35 (9.5)	7 (6.7)	54 (10.5)	30 (10.6)	31 (9.3)
Frameshift (+)	6 (2.4)	7 (1.9)	1 (1.0)	12 (2.3)	7 (2.5)	6 (1.8)
Complex	12 (4.8)	8 (2.2)	5 (4.8)	15 (2.9)	6 (2.1)	14 (4.2)
Tandem	2 (0.8)	6 (1.6)	0 (0.0)	8 (1.6)	5 (1.8)	3 (0.9)
Insertion	1 (0.4)	4 (1.1)	0 (0.0)	5 (1.0)	4 (1.4)	1 (0.3)
Other classes	79 (31.9)	109 (29.5)	35 (33.7)	153 (29.8)	90 (31.9)	98 (29.3)
Grand total	**248 (100)**	**369 (100)**	**104 (100)**	**513 (100)**	**282 (100)**	**335 (100)**
Uncharacterized splice	88	90	34	144	54	124

Mutational classes demonstrating significance are underlined and described in text.
[a] Involving more than a single base pair.

mutational spectrum, no significant difference was detected. Their most recent article (Burkhart-Schultz and Jones 1997) includes mutants not reported in Burkhart-Schultz *et al.* (1996). Other datasets that were found to be significantly different from the remainder contained <50 mutations, and the comparison of such small mutational spectra likely accounts for the observed differences. When these smaller datasets were grouped together, by laboratory where possible, and the analyses were repeated, no significant differences were observed.

Differential characterization of exon splice mutations represents the most consistent bias. Where causative mutations responsible for exon splice mutations were reported in the literature, the causative mutation was listed in the mutant data base. As some authors did not further characterize exon splice mutants while others did, this class of mutation could possibly bias the dataset. For example, a group of mutants where the causative mutations were determined would be different from a group where no further characterization of exon splice mutants was attempted. To avoid this potential bias, splice mutants that have not been further characterized are not considered. Finally, the spectrum of mutations obtained from fully characterized splice mutants could differ from those mutations that do not affect splicing. However, comparison of the mutational spectra from characterized exon splice mutations against mutations that do not affect splicing revealed that these spectra are identical in terms of the other classes of mutations.

Influence of smoking on spectra: The smoking and nonsmoking groups were nearly identical in terms of age distribution (Table 4). Mutants were sorted into those from smokers (248 independent mutants) and nonsmokers (369 independent mutants), and the two spectra were compared (Table 5). Results of the Monte Carlo test revealed no significant differences. A barely significant difference in the frequency of G:C → A:T between smokers and nonsmokers was detected using Fisher's exact test (two-tailed, $P = 0.05$).

A total of 87 independent mutants (not including the uncharacterized splice mutations; 85 from smokers) were obtained from lung cancer patients (Karnaoukhova *et al.* 1997). Mutants from the treated patients did not differ from the pretreatment patients (same individuals; Karnaoukhova *et al.* 1997), so the data were pooled. With these additional data, no significant difference in the frequency of G:C → A:T transitions between smokers and nonsmokers could be detected. As a result, the smoking and nonsmoking mutational spectra datasets have been combined for further analysis.

Influence of sex on spectra: Few datasets are large enough to examine the influence of sex on mutational specificity. Subject age distribution (Table 4) was essentially equal; however, relatively few mutants were recovered from females subjects ($n = 104$) as compared to males ($n = 513$). Monte Carlo analysis did demonstrate a significant difference between the two spectra (Table 5; $P = 0.008$; 95% confidence limits 0.006–0.01). Each mutation class was tested alone using Fisher's exact test (two-tailed). There were significant differences in the frequency of G:C → A:T ($P = 0.02$) and A:T → T:A

($P = 0.04$) base substitutions between males and females. Caution is warranted, however, when considering the limited set of mutants obtained from female subjects. These differences were tested again with the addition of 87 mutants (67 from males) obtained from the etoposide study (Karnaoukhova *et al.* 1997). In both cases, significance was lost after this addition.

Deletion frequency in females is almost twice as high as in males (Table 5). Mutants from the etoposide study were added, and the data were reanalyzed. Addition of the etoposide mutants further strengthened the significance of the conspicuously uneven distribution of deletions between the sexes (Table 6). An analysis of the regions surrounding the endpoints of these deletions in male and female subjects did not reveal an obvious difference with regard to prevalence of deletions flanked by repeated sequences. Lengths of these intragenic deletions were calculated, and the means were determined. Females had a mean deletion length of 24 ± 65 bp compared with males, who had a mean of 34 ± 84 bp.

Characterized splice mutations were obtained disproportionately from males ($n = 99$) as compared to females ($n = 16$). This bias likely results from the relative ease of characterizing the single mutant allele in males. To ensure that the bias did not affect the analysis, it was repeated with the exclusion of all splice mutations. The increased frequency of deletions from female over male subjects was confirmed.

Effect of age on spectra: To facilitate the analysis of age-related effects on mutational specificity, data were subdivided into three different age groups, as described (Table 4), with nearly equal numbers of mutants. Group mean ages were all significantly different from one another. The Monte Carlo test was performed on mutational spectra for the two (spectra shown in Table 5), three, or four partitioning groups (spectra not shown). No differences in spectra were observed for any of the age partitions. Fisher's exact test was used to compare all the classes individually (2×2 tables). The A:T → C:G class demonstrated a significant difference in the two (d.f. = 1, two-tailed, $P = 0.015$), three (d.f. = 2, two-tailed, $P = 0.004$), and four (d.f. = 3, two-tailed, $P = 0.012$) age partitions. In each case, frequency of A:T → C:G mutations was significantly lower for the youngest age partition than for the older partitions. For all three sets of comparisons, the observation remained significant after the addition of the 87 etoposide mutants.

TABLE 6

Analysis of the frequency of deletion between females and males

	Male: deletions/total	Female: deletions/total	Fisher's *P* value
All data	59/513	22/104	0.008
Etoposide mutants[a]	7/67	3/20	0.42
All data + etoposide	66/580	25/124	0.008

[a] The etoposide mutant data (Karnaoukhova *et al.* 1997) include 10 deletion events although the publication refers to one mutant involving 2 separate deletions as a complex mutation.

Analysis of the distribution of mutation: Distribution of mutation within the *hprt* gene was examined for age-related differences. This analysis was based solely upon the distribution of single-base-pair substitutions. Using the Monte Carlo test, the influence of smoking and age in the two-age group partition was examined. No significant differences between spectra were observed during either the smoking or age analysis (results not shown). However, several frequently mutated single-base-pair substitution sites were identified in the mutant dataset. Sites recovered at least eight times were assembled (data not shown), and the effects of smoking, sex, and age were examined using the Monte Carlo test. Several of these frequently mutated sites have been previously identified as hotspot sites for mutation (positions 197, 508, and 617; Burkhart-Schultz *et al.* 1996). No significant differences were observed in the smoking, sex, or age partitions.

Sites were then tested individually with Fisher's exact test (cell counts for nonevents were dependent upon the site nucleotide). Comparing the spectra obtained from males and females, base substitutions at position 611 are more frequently recovered in females than in males (16 *vs.* 3%; $P = 0.02$). The addition of the etoposide data maintained the significance ($P = 0.01$). Addition of these data was permissible because the etoposide subject group comprised both males and females.

CpG site mutation analysis: The frequency of G:C → A:T transition mutations occurring at CpG sites was analyzed. The *hprt* coding sequence contains eight CpG dinucleotides that yield 12 nucleotide positions where a mutation will cause an amino acid substitution or stop codon. From this collection of mutants, only five CpG sites, all coding for arginine, were found mutated. No significant differences (Fisher's exact test) were observed in the frequency of mutations occurring at CpG sites for the smoking/nonsmoking, sex, or age partition comparisons. Previous investigations found a significant strand bias for mutations arising by deamination of the methylated cytosine in the human *hprt* gene on the nontranscribed strand (Skandalis *et al.* 1994). Their analysis used the hprt database (release 3) and included all mutations collected from both *in vitro* and *in vivo* human sources. This analysis, which limits the mutant collection to only *in vivo* spontaneous sources, confirms the nontranscribed stand bias ($\chi^2 = 10.8$, d.f. = 1, $P < 0.001$).

DISCUSSION

T lymphocyte biology and mutant frequency: The *hprt* T cell clonal assay depends upon peripheral T cells because of ease of acquisition and because they can be cloned *in vitro*, which permits mutant selection. As a result, the assay is intimately linked to the biology of T cells (for review see Cole and Skopek 1994). Composition of the available pool of T lymphocytes changes with age. At birth, nearly all the T lymphocytes display CD45RA isotypes, indicating that they are "naive" or virgin T cells. They have not met antigen and, thus, have not yet responded through clonal expansion. Later in adult life, a second isotype called CD45RO is borne by nearly half of the T lymphocyte population (Michie *et al.* 1992). These responder or "memory" T cells have encountered antigen and responded by clonal proliferation. Clonal expansion requires cell division that provides the opportunity for both DNA replication errors and their fixation. It is reasonable to suggest that the majority of spontaneous mutations arising in T lymphocytes are the result of clonal expansion (Nicklas *et al.* 1988). Observations that CD45RO+ T cells have two to five times higher mutant frequency than the naive CD45RA+ T cells further substantiates this assertion (Baars *et al.* 1995).

Observed *hprt* mutant frequency is dependent on a number of factors. The rate at which mutation occurs is estimated at 5×10^{-7} mutations per nominal cell division (Greene *et al.* 1995). Sizes of T lymphocyte pools fluctuate greatly over time, but are estimated at $1–5 \times 10^{-11}$ cells for a healthy adult. During the course of an immunological challenge, a single "responding" T cell undergoes an estimated 20–50 divisions. It is likely that more than one T cell responds to antigenic challenge. The number of challenges met both recently and over a lifetime must, as a result, influence the frequency of *hprt* mutants in an individual.

Clonal expansion of mutant T cells increases the potential for recovery in the T cell clonal assay. Single mutations, which have not clonally expanded, are nearly undetectable in the current assay, which uses 10–40 $\times 10^6$ cells. Prevalence of mutant clonal expansion in subjects is only evident when sufficient numbers of mutants have been isolated and characterized at the *hprt* locus and subsequently when mutant characterization indicates a possible clonal run at a TCR locus. This laboratory has previously recognized one large and two smaller clonal runs in a single subject by using such methods (Curry *et al.* 1995), as have others (Nicklas *et al.* 1988; Albertini *et al.* 1990). O'Neill *et al.* (1994) concluded that mutant clonal expansion is a frequent occurrence after observing that of 58 individuals, 35 had some degree of expansion. These observations indicate that a large component of MF is contributed by the clonal expansion of single mutational events.

The kinetics at which clonal expansions are reversed remain unclear. Also unclear is the size of the original clonal pool, which remains as memory cells, and how those memory cells are maintained. An additional complication is the possible selection against *hprt* mutants that may have a reduced proliferation rate (Podlutsky *et al.* 1996). Current estimates for the half-life of T cells vary greatly, but they are likely to be in the range of 2–3 yr (da Cruz *et al.* 1996). In summary, the observed mutation frequency at the *hprt* locus in T cells is dependent upon the nature and life cycle of this complex tissue.

Mutant frequency analysis: *Influence of sex and smoking on hprt MF:* No effect of sex on ln MF was observed, confirming previous reports. Smoking was related to elevated mean ln MF values when compared to nonsmokers. Further examination of the effect of smoking revealed that the increase in ln MF was detectable only in male smokers. This contradicts the findings of Huttner *et al.* (1995), who noted a smoking-related increase in MF for females but not males.

The effect of smoking on *hprt* MF remains troublesome. Smoking has a pronounced effect on T lymphocyte populations. Smoking increases the number of peripheral white blood cells by ~30% (Howell 1970; Corre *et al.* 1971). Increased leukocyte counts have been associated with the number of cigarettes smoked. Leukocyte counts decrease after the cessation of smoking, but only after 5 yr (Parry *et al.* 1997). Increase in size of neutrophil and CD4+ subpopulations is strongly associated with smoking (Freedman *et al.* 1996; Parry *et al.* 1997; Schaberg *et al.* 1997; Tanigawa *et al.* 1998). Mutants recovered with the T cell clonal assay are mainly CD4+ (McGinniss *et al.* 1990).

Several suggestions have been presented concerning the smoking-related increase in CD4+ T cells. Smoking may alter cell trafficking by decreasing the ability of the cells to adhere to cellular tissues (marginal pool), thus increasing their relative yield in the peripheral pool (Parry *et al.* 1997). Others suggest that a continuous local inflammation in the respiratory tract is responsible or that some component of tobacco smoke may act as antigen by stimulating T cell proliferation (Tanigawa *et al.* 1998). Smoking-related increases in peripheral T cell populations may be related to increases in *hprt* MF. Should smoking serve to increase the peripheral pool by proliferation, an increased *hprt* MF could also be expected. Smokers are observed to be more susceptible to common colds than nonsmokers (Cohen *et al.* 1993), which may also explain the increased peripheral T cell pools through proliferation against antigen.

Influence of age on hprt MF: The results here demonstrate that despite a highly significant overall (ages 0–85 yr) ln MF *vs.* age linear relationship, the data do provide for an alternative model. As has been demonstrated previously, ln MF increases rapidly with age in children (Finette *et al.* 1994), and this increase significantly differs from the relationship found for adult populations

(Branda *et al.* 1993). The results here confirm that observation. At about the age of 15, the rate at which *hprt* mutants accumulate falls to a level maintained for another 40 yr, after which few *hprt* mutants appear to accumulate (Figure 1).

Rapid increases in MF in children must be related to the nature of their immune systems. Specifically, a juvenile immune system is one that is growing and learning with every encounter with antigen. A newborn child enters an antigen-rich environment, where it must respond to numerous immunological challenges. During growth, the lymphoid tissues increase dramatically in size. These factors must affect *hprt* MF. In midlife, when the increase in *hprt* MF is observed to decrease as compared to juveniles, that decrease can be accounted for by both changes in the immune system and reduced antigen encounters. Children are challenged more frequently compared to adults, who encounter relatively fewer novel antigens and have already developed immunity to those encountered previously.

Changes to the immune system, most notably the involution of the thymus (for review see Bodey *et al.* 1997), may have a role in the complex relationship between *hprt* MF and age. Thymus involution begins in late childhood (age 15), and by late adulthood (approximately age 60), thymic tissues have been completely replaced by fat and are no longer maturing naive T cells (George and Ritter 1996). After this point, the individual is completely dependent on the T cell pool generated earlier in life. Of particular relevance are changes in the aging immune system that specifically affect T lymphocytes. T cells of aged individuals demonstrate impaired responses to antigen and decreased clonal expansion (McCarron *et al.* 1987; Globerson 1995; Grubeck-Loebenstein 1997; Pawelec *et al.* 1997). Loss of naive T cell production and age-related T cell impairment is conspicuously related to the last age partition, where *hprt* MF no longer varies with age. A clear demonstration of thymus involution and the lack of an MF-age correlation after the age of 53 are beyond the reach of this study, but they clearly warrant further investigation.

Mutational spectra analysis: *Influence of smoking:* Using two spectra containing 43 and 55 independent mutants collected from smokers and nonsmokers, respectively, Vrieling *et al.* (1992a) reported finding no significant difference between the two spectra. The authors did note that both their spectra were void of any G:C → T:A transversions, which are produced efficiently by the metabolite benzo[*a*]pyrene, a tobacco carcinogen (Yang *et al.* 1991). Their relatively small sample size limited the chance for recovering such events. In the larger sample collection analyzed here, there was no difference in the distribution of G:C → T:A transversions between the smoking and nonsmoking spectra.

The liability of comparing mutational spectra of limited size is that apparent differences between the spectra may disappear as the spectral size increases. For instance, the apparent mutational "hotspot" (exclusively G:C → A:T transitions at position 617) reported by Vrieling and his colleagues (1992b) in humans occupationally exposed to ethylene oxide (mutants = 18) is frequently mutated in unexposed populations, and some transversions have been recovered at this particular site (Burkhart-Schultz *et al.* 1993, 1996; Andersson *et al.* 1992).

To date, this study is the largest comparison of smoking and nonsmoking mutational spectra. Analysis of smoking and nonsmoking spectra using the Adams Skopek Monte Carlo test did not reveal any difference, confirming reports of Vrieling *et al.* (1992a) and Burkhart-Schultz *et al.* (1993, 1996). This conclusion confounds the observation that numerous smoking-related DNA adducts are formed in various tissues, including T lymphocytes (Van Schooten *et al.* 1997).

Burkhart-Schultz *et al.* (1996) concluded that smoking yielded no observable change in spectrum despite an increase in MF. A plausible explanation for an enhanced MF but no change in spectrum between smokers and nonsmokers relates to the fact that smoking increases the numbers of T cells *in vivo.* As smokers are more prone to and suffer more respiratory illnesses, they may have relatively more active immune systems with higher T cell turnover than nonsmokers. It follows that the smoking-related increase in MF might be a reflection of more rounds of replication experienced in the T cells of smokers. This would also explain why no change in spectrum is observed. Finally, the possibility exists that the *hprt* locus or T cells are simply a poor biomarker for the study of the effects of smoking.

Influence of sex: The mutant dataset of males is eightfold larger than that of females. This reflects the relative ease of molecular analysis resulting from the male hemizygous state. Despite the disparity in sample size, comparison of base substitution mutational spectra from males and females did not reveal any significant difference. Among 104 mutants recovered from females, a statistically significant (Fisher's test, $P = 0.008$) increase in the frequency of deletions over that of males was observed. Analysis of the deletion endpoints for both males ($n = 59$) and females ($n = 22$) reveals a similar distribution of the breakpoints occurring at repeat and inverted repeat sequences.

An increase in the recovery of small deletions from female subjects is intriguing. It is possible that the difference reflects recombination-mediated repair, as a second copy of the gene is available in females. However, a larger number of mutants from females is needed to better understand the mechanisms involved.

Influence of age: The question of how age affects mutational specificity was examined by coupling the *hprt* mutant dataset with subject age. Analysis of several different modes of age partitioning using the Monte Carlo test revealed no significant differences in the complete spec-

tra; however, an increase in the frequency of A:T → C:G transversions with age was observed.

In general, A:T → C:G transversions are relatively rare in mutational spectra. However, several chemical agents have been reported to increase the frequency of this class of transversion. Benzo(*a*)pyrene's ultimate carcinogenic metabolite, [(+)-BPDE], was found to increase the frequency of this type of transversion in the *hprt* gene of Chinese hamster cells, but only at low doses (Wei *et al.* 1993). In the Salmonella/microsome assay, *N*-nitrosomethylaniline induced predominantly this type of transversion (Zielenska and Guttenplan 1988). Eight purine base analogues are noted to induce A:T → C:G transversions in *Escherichia coli* (5- to 124-fold). The proposed mechanism is the reaction of these hydroxylaminopurines at the O^4 position of thymine (Murray 1987). *N*-Ethyl-*N*-nitrosourea, a potent monofunctional, ethylating agent capable of alkylating the O^4 position of thymine, has also been reported to induce these transversions (Eckert *et al.* 1988; Guttenplan 1990). Involvement of these agents in the age-related increase of A:T → C:G transversions cannot be determined.

Another potential mechanism for the origin of A:T → C:G transversions may be linked to oxygen radicals and the polymerase most active in dividing cells. *In vitro* oxygen-radical-induced mutagenesis was eloquently demonstrated to be DNA polymerase specific (Feig and Loeb 1994). Using oxygen-radical-damaged M13 viral DNA in a forward mutation assay, DNA polymerase α demonstrated a 14-fold increase in the frequency of A → C transversion as compared to undamaged templates. By comparison, DNA polymerase β yielded only a 1.5-fold increase. DNA polymerase α accounts for >85% of total DNA polymerase activity in dividing cells, but only 5% in quiescent cells (Kornberg and Baker 1991). The involvement of this type of mechanism in the age-dependent increase in A:T → C:G transversions can be only speculative at this time.

CONCLUSIONS

The major contribution to *hprt* mutation occurs early in life even though mutations continue to accumulate at a diminishing rate until the early 50s. After this age, there is no further significant accumulation of mutation. Despite the overall increase in mutation in adults over time, the spectrum of mutation was relatively constant, with the single exception of the frequency of A:T → C:G transversions that increase with age. No evidence was recovered to support any model of aging that predicts mutation rate to accelerate with age. Either most mutations do occur at an early stage in life, or these observations are peculiar to T cells. As the stem cells for the production of T cells are less prolific in old age and T cells themselves apparently divide less vigorously, there are fewer opportunities for mutation. Thus, while the HPRT clonal assay may be the most prominent technique in use for the monitoring of mutation in humans *in vivo*, it is not necessarily well suited for the purpose. Indeed, neither long-term exposure, such as in the case of tobacco smoking (this study), nor treatment with a powerful chemotherapeutic agent, such as etoposide (Karnaoukhova *et al.* 1997), produced any observable impact on mutational spectra. We are thus forced to conclude that further insights into the life cycle and fundamental biology of T cells are required to properly appreciate the advantages and limitations of this system for environmental monitoring purposes.

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