

The frequency of HPRT-deficient T-cells was calculated similarly from the cloning efficiency of TG^r cells in the peripheral blood T-cells.

APRT Enzyme Assay. Nonselected and DAP^r T-cell clones were washed twice in phosphate-buffered saline, frozen, and thawed 3 times in saline solution at a cell concentration of $10^6/100 \mu\text{l}$. After centrifugation at 6000 rpm for 10 min, the supernatants were collected and used for enzyme assays. APRT activity was determined as described by Thomas *et al.* (26) using [8-¹⁴C]adenine (54 mCi/mmol; Amersham, Buckinghamshire, England). Protein concentration was determined by the method of Lowry *et al.* (27).

RESULTS

Selection and Characterization of DAP^r T-Cell Clones. We were able to obtain a number of DAP^r T-cell clones from the peripheral blood of the heterozygotes. According to our experience with T-cells from individuals with various genotypes at the *aprt* locus, only homozygously deficient cells are resistant to the concentration of DAP used here (28). In order to reconfirm this, we determined APRT activities in the resistant clones. Since *APRT^J* codes for a mutant APRT which shows *in vitro* activity (28), measuring APRT levels in the extracts of T-cell clones is useful for the confirmation of homozygosity only when the heterozygotes contain *APRT^{Q0}*, which is associated with a complete deficiency. Therefore, we determined APRT levels in the extracts of DAP^r T-cell clones as well as nonselected clones from a heterozygote with a genotype of *APRT¹/APRT^{Q0}* (*APRT¹* represents a normal allele) (3). As shown in Fig. 1, all seven DAP^r clones showed virtually no enzyme activity, while nonselected clones from the heterozygous subject exhibited high levels of enzyme activity.

In Vivo Frequency of DAP^r and TG^r T-Cells in Heterozygotes. The apparent frequency of DAP^r T-cells (F_a calculated as de-

scribed in "Materials and Methods") in the four heterozygotes ranged from 8.8×10^{-6} to 31.6×10^{-6} (average, 19.0×10^{-6}) and always 2 to 4 times higher than that of TG^r T-cells determined in parallel experiments (statistically not significant), using samples from the same individuals (Table 1). The frequency of TG^r T-cells obtained in this study is similar to values reported by our group (25) and those of other workers (13, 14, 29, 30). The frequency of *aprt^{-/-}* cells should be larger than F_a as F_a was lower than 1 (average, 0.15) when T-cells obtained from individuals with the genotype of *aprt^{-/-}* were used (Table 2). In contrast, experiments using T-cells from a HPRT-deficient patient (Lesch-Nyhan syndrome) have shown that, unlike DAP, thioguanine does not significantly reduce the cloning efficiency (data not shown), thereby supporting data presented by Dempsey *et al.* (31). Such being the case, comparison between frequencies of DAP^r and TG^r T-cells requires adjustment by different cloning efficiencies if genetic events at *aprt* and *hprt* loci are in question. After making the adjustment described in "Materials and Methods," the corrected frequency of *aprt^{-/-}* cells (F_b) in PBMC from *aprt^{+/-}* individuals (5.9×10^{-5} to 2.1×10^{-4}) is a 10- to 40-fold higher than the frequency of *hprt⁻* cells from the same individuals. The average frequency of *aprt^{-/-}* T-cells in *aprt^{+/-}* individuals was 1.3×10^{-4} , while the average frequency of *hprt⁻* T-cells was 6.7×10^{-6} . The difference between these frequencies was significant ($P < 0.05$) when tested by the nonparametric rank test.

Characterization of a DAP^r T-Cell Clone in a Normal Subject. Cloning of DAP^r T-cells (*aprt^{-/-}*) from normal individuals (*aprt^{+/+}*) is expected to be extremely difficult, since such cells would be produced only through two separate events. We examined a total number of 3.0×10^9 PBMC from 310 control subjects in search of DAP^r cells and found one resistant clone. APRT enzyme activity in the cell extract of this clone was zero, thus confirming that this clone was a real homozygously deficient clone (Fig. 1). Taking into account the average cloning efficiency of normal T-cells without DAP (0.44) and that of known *aprt^{-/-}* T-cells with DAP, as described in "Materials and Methods," the frequency of *aprt^{-/-}* T-cells (F_b) in normal PBMC was calculated to be 5.0×10^{-9} . This value calculated from data having only one positive well (one clone) is, of course, subject to error, but still is the best estimate available.

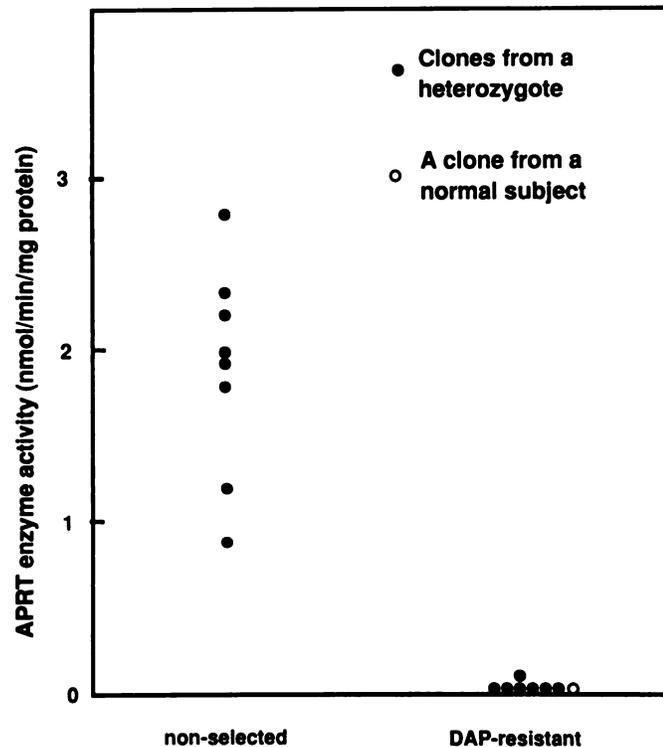


Fig. 1. APRT activities in the extracts of nonselected and DAP-selected T-cell clones. T-cells were cloned from the peripheral blood of an obligate heterozygote with the genotype of *APRT¹/APRT^{Q0}* in the presence or absence of 100 μM DAP, as described in the "Materials and Methods." APRT activities in the cell extracts were determined as described in "Materials and Methods." APRT activity in the cell extract from a DAP^r T-cell clone from a normal subject is also shown.

DISCUSSION

We obtained evidence that somatic mutations at the *aprt* locus, previously identified in *in vitro* cultured cells (5-12), also occur *in vivo* in humans. Although we cannot completely exclude the possibility that such enzyme-deficient T-cells had been induced *in vitro*, such an occurrence is unlikely as we added DAP to the cells at the initiation of culture. Several cell divisions are usually necessary for the expression of the mutant genotype after mutational events occur (32). Similar arguments have been made in the case of somatic HPRT-deficient T-cells, but the data from subsequent studies indicated that the mutations occurred *in vivo* (33).

Our data show that the two-step process (*aprt^{+/+}* \rightarrow *aprt^{+/-}* \rightarrow *aprt^{-/-}*) leading to the complete deficiency of APRT as proposed in previous *in vitro* studies is also true of *in vivo* mutations. DAP^r clones were easily selected from each of 4 heterozygous individuals, but only one DAP^r clone was selected from 310 normal subjects, clearly showing that two-step mutations are necessary for somatic T-cells to become DAP^r. Thus, each DAP^r cell in the heterozygotes has undergone a mutation

Table 1 Frequency of DAP^r and TG^r T-cells in heterozygotes (*aprt*^{+/-})

PBMC from 4 heterozygotes were cloned with or without 100 μM DAP after distributing the indicated numbers of cells. Cloning efficiencies were calculated for control plates (CE) and +DAP plates (values not shown) by assuming a Poisson distribution for the number of colony-forming cells per well. From these cloning efficiencies, the frequency of DAP^r T-cells (F_a) was calculated as described in "Materials and Methods." The frequency of TG^r T-cells was similarly calculated from cloning efficiencies in a control plate and +TG plates.

Heterozygotes	Age (yr)	Sex	Control plate with the following no. of cells/well		CE ^a	+DAP plate with the following no. of cells/well		Frequency of DAP ^r T-cells (F _a)	+TG plate with 2 × 10 ⁴ cells/well	Frequency of TG ^r T-cells
			1	2		2 × 10 ⁴	4 × 10 ⁴			
KM	27	F	39/96 ^b	61/96 ^b	0.51	45/288 ^b	31/96 ^b	18.0 × 10 ⁻⁶	16/384 ^b	4.3 × 10 ⁻⁶
KF	31	M	40/96	68/96	0.58	32/288	15/96	8.8 × 10 ⁻⁶	16/384	3.8 × 10 ⁻⁶
YO	31	F	24/96	42/96	0.29	20/192	16/96	17.6 × 10 ⁻⁶	20/360	9.9 × 10 ⁻⁶
TE	33	F	30/96	ND	0.37	20/96	ND	31.6 × 10 ⁻⁶	12/192	8.7 × 10 ⁻⁶

^a CE, calculated cloning efficiency; TG, thioguanine; ND, not determined.

^b Data are expressed as the number of positive wells/number of total wells.

Table 2 Cloning efficiency of APRT-deficient T-cells with or without DAP

PBMC were obtained from 4 patients with homozygous APRT deficiency. T-cells were cloned in microtiter plates after distributing the indicated numbers of fresh PBMC per well in the presence or absence of 100 μM DAP. Cells were cultured in 96 wells for each cell density. The cloning efficiency was calculated as in Table 1. Data are expressed as calculated cloning efficiencies and number of positive wells among a total of 96 wells.

Homozygotes	-DAP plate with the following no. of cells/well ^a		+DAP plate with the following no. of cells/well				
	1	2	1	2	5	10	20
AK	0.30 (25) ^b	0.23 (34)	0.040 (4)	0.060 (11)	ND ^c	ND	ND
TY	0.26 (22)	0.30 (43)	ND	0.050 (9)	0.052 (22)	0.063 (45)	0.055 (64)
SG	0.29 (24)	0.27 (40)	ND	0.040 (7)	0.016 (8)	0.038 (30)	0.033 (45)
OD	0.24 (21)	0.22 (34)	ND	ND	0.024 (11)	0.025 (21)	0.025 (37)

^a Number of fresh PBMC per well.

^b Numbers in parentheses, number of positive wells among a total of 96 wells.

^c ND, not determined.

at the somatic cell level, in addition to a germ line mutation, while a DAP^r T-cell clone from a normal subject reflects two somatic mutations which occurred *in vivo*. These processes leading to complete APRT deficiencies closely resemble mechanisms proposed by Knudson for retinoblastomas (34). Thus, a retinal cell develops into a tumor when it becomes homozygously deficient at the *RB* locus, either by the germinal-somatic or somatic-somatic mechanism (17–22).

The frequency of *aprt*^{-/-} cells in *aprt*^{+/-} individuals (1.3 × 10⁻⁴) was much higher than the frequencies of *hprt*⁻ T-cells *in vivo* reported by various groups (3 × 10⁻⁶ to 1.2 × 10⁻⁵) (13, 14, 25, 29). Although such a high value was obtained after the adjustment by a lowered cloning efficiency of *aprt*^{-/-} cells by DAP, the nonadjusted frequency was still higher than the frequency of *hprt*⁻ cells in each individual.

A high frequency of mutational events at the *aprt* locus induced *in vitro* in cultured cells has been observed (6–8). Molecular analysis of the mutant cells obtained from heterozygous Chinese hamster ovary cells showed that such high frequency events are due to gene deletions (35–37). Using a different autosomal locus, thymidine kinase, high mutation frequency has also been observed (38, 39). Our results indicated that mutational events producing *aprt*^{-/-} cells from heterozygous *aprt*^{+/-} cells also occur frequently *in vivo* in humans.

Detection of *in vivo* mutational events at a different autosomal locus has been reported. Janatipour and coworkers (15, 16) detected T-cells lacking the surface expression of the product of the HLA-A locus. The mechanisms for the induction of mutant cells in their system may be more complicated than in the *aprt* system, because mutational events affecting the intracellular transport or surface expression of the HLA-A locus products may also be detected. However, the frequency of such HLA-A-negative cells was lower but comparable to the frequency of *aprt*^{-/-} cells in heterozygous individuals.

We were also able to identify one *aprt*^{-/-} clone among a total number of 3.0 × 10⁹ cells from 310 normal subjects. The

frequency of *aprt*^{-/-} cells in *aprt*^{+/-} individuals was calculated to be 5.0 × 10⁻⁹. Since we were able to identify only one clone, the frequency value calculated here is not accurate. Nevertheless, it is important that an *aprt*^{-/-} T-cell clone was identified in a normal subject even at a very low frequency, since it clearly indicates that homozygous deficiency at the somatic cell level can be induced even without preceding germ line mutations. Of course, such a process corresponds to the mechanisms of the development of sporadic retinoblastomas without genetic backgrounds (34).

In the present study, we established a human system to investigate somatic mutations at the *aprt* locus and have shown that two different mechanisms (germinal-somatic and somatic-somatic) lead to the homozygous deficiency at this locus in somatic cells *in vivo*. Our data suggest that homozygous deficiencies induced by the two different mechanisms in somatic cells as proposed for the mechanisms of carcinogenesis in retinoblastoma (34) occur at various autosomal loci in general.

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