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# Homozygous Deficiency at Autosomal Locus *aprt* in Human Somatic Cells *in Vivo* Induced by Two Different Mechanisms[1]

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## ABSTRACT

**Associations between germinal and somatic mutations at autosomal loci play an important role in the development of some tumors, including retinoblastoma. In an attempt to determine whether equivalent events occur *in vivo* at other loci, we cloned and enumerated somatic T-cells with mutations at the *aprt* locus, by taking advantage of both the presence of a human disease caused by genetic defects at this locus and an effective selection procedure for the deficient mutants. T-cells homozygously deficient at this locus ($aprt^{-/-}$) were found in all four heterozygotes ($aprt^{+/-}$) studied, at an average frequency of $1.3 \times 10^{-4}$. From 310 normal individuals, we identified only one $aprt^{-/-}$ clone, and the calculated frequency of $aprt^{-/-}$ T-cells in $aprt^{+/+}$ individuals was $5.0 \times 10^{-9}$. These results confirm that a two-step process ($aprt^{+/+} \rightarrow aprt^{+/-} \rightarrow aprt^{-/-}$) is functional through two different mechanisms (germinal-somatic and somatic-somatic) *in vivo*. Our data suggest that the two-step mutations leading to homozygous deficiencies at the somatic cell level, as proposed for the carcinogenic mechanisms for retinoblastomas and other human tumors, generally occur at rather high frequencies at various autosomal loci in humans.**

## INTRODUCTION

The purine metabolic enzyme APRT[3] has attracted the attention of both clinicians and biological researchers. Homozygous deficiency of this enzyme causes 2,8-dihydroxyadenine urolithiasis and renal failure (1, 2). Genetically deficient alleles among Japanese have been analyzed and characterized (3). Thus, 80% of all the homozygous patients possess a common mutant allele designated *APRT*J*(3), and the defective sequence has been determined (4). All the other alleles, designated *APRT*Q0*, may possibly represent a set of different alleles having the common characteristic of being associated with complete enzyme deficiency (3).

The *aprt* locus coding for this enzyme has also been studied by somatic cell geneticists in an attempt to elucidate the nature of somatic cell mutations (5–12). As to the mutations at the somatic cell level, *hprt* coding for the enzyme HPRT has been the most extensively studied locus (13, 14). However, mutations at autosomal loci (*aprt*, for example) may be different from those at X-linked loci (*hprt*, for example) in many regards (5–12, 15, 16), and this difference may be important when tumor-related mutations are being considered (17–24).

Associations between germinal and somatic mutations have been confirmed or suggested to play important roles in the pathogenesis of retinoblastoma (17–22) and other human tumors (23, 24). Therefore, analyzing somatic events occurring at the *aprt* locus in individuals with genetic defects at the same locus may be useful for an understanding of the nature of various mutational events in humans.

Numerous studies with Chinese hamster ovary cell lines, among others, have suggested that two-step mutations are necessary to produce mutant cells completely deficient at the *aprt* locus (5–7, 9). The cells become heterozygously deficient ($aprt^{+/+} \rightarrow aprt^{+/-}$) through the first step, while through the second step, homozygously deficient cells are induced ($aprt^{+/-} \rightarrow aprt^{-/-}$) (5–7). (Sometimes, a hemizygous state in which one of the *aprt* alleles is deleted is expressed as $aprt^{+/0}$, but, throughout this article, (−) instead of (0) will be used.) It is important to ask whether these data obtained from mutation studies in cell lines have *in vivo* relevance, and our results obtained by the present investigations show that indeed they do.

## MATERIALS AND METHODS

**Subjects Studied.** The diagnosis of the homozygous state at the individual level was made by measurement of APRT enzyme activity in the hemolysates and T-lymphocyte cultures with DAP (Sigma, St. Louis, MO), as described (3). All heterozygotes ($aprt^{+/-}$) were parents of the individuals homozygously deficient in APRT ($aprt^{-/-}$). PBMC from 4 individuals with homozygous APRT deficiency and 4 heterozygous individuals were obtained from heparinized blood, using Ficoll-Hypaque density centrifugation. Two of the heterozygotes contained the *APRT*J* allele, and two contained the *APRT*Q0* allele. A total of $3.0 \times 10^9$ PBMC was obtained from 310 normal subjects.

**Cloning of APRT-deficient and HPRT-deficient Mutant T-Cells.** The cloning procedure for APRT-deficient mutant T-cells was a modification of a previously reported method in which HPRT-deficient T-cells were cloned (25). Fresh PBMC were inoculated into round-bottomed microtiter plates (Costar Corp., Cambridge, MA) at a concentration of $2 \times 10^4$ or $4 \times 10^4$ cells/well containing $10^4$ cells/well of X-irradiated (10,000 rads) Raji B-cells. The medium used for cloning was RPMI 1640 supplemented with 10% fetal calf serum, 1% human male serum, 2 mM L-glutamine, 0.5 μg/ml of phytohemagglutinin (Difco, Detroit, MI), 0.5 ng/ml of recombinant human interleukin 2 (Takeda, Tokyo, Japan), and 100 μM DAP. HPRT-deficient T-cells were cloned from the same PBMC in a similar manner, except that 2.5 μg/ml of 6-thioguanine (WAKO, Tokyo, Japan) were used instead of DAP. For the cloning of wild-type T-cells, PBMC were inoculated into microtiter wells at a concentration of 1 or 2 cells/well with X-irradiated (5000 rads) PBMC ($2 \times 10^4$ cells/well) and Raji cells ($10^4$ cells/well). After 2 wk of culture, an inverted microscope was used to determine the presence or absence of lymphocyte colonies in each well. Cloning efficiencies were calculated for wild-type, $DAP^r$, and $TG^r$ T-cells, assuming a Poisson distribution for the number of colony-forming cells per well (25). The $F_a$ of $DAP^r$ was calculated as follows.

$$F_a = \frac{\text{cloning efficiency with DAP}}{\text{cloning efficiency without DAP}}$$

Since DAP was found to decrease the cloning efficiency of even $aprt^{-/-}$ T-cells (*i.e.*, $F_a$ was lower than 1 even for samples from $aprt^{-/-}$ individuals), the $F_a$ of $aprt^{-/-}$ T-cells in each sample was calculated as follows.

$$F_b = \frac{F_a \text{ for the sample}}{F_a \text{ for cells from } aprt^{-/-} \text{ individuals}}$$

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[3] The abbreviations used are: APRT, adenine phosphoribosyltransferase; HPRT, hypoxanthine-guanine phosphoribosyltransferase; DAP, 2,6-diaminopurine; PBMC, peripheral blood mononuclear cells; $DAP^r$, DAP-resistant; $TG^r$, thioguanine-resistant; $F_a$, apparent frequency; $F_b$, corrected frequency.

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 $10^6/100\ \mu l$. 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-$^{14}$C]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 *APRT*1/APRT*Q0* (*APRT*1* 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 described in "Materials and Methods") in the four heterozygotes ranged from $8.8 \times 10^{-6}$ to $31.6 \times 10^{-6}$ (average, $19.0 \times 10^{-6}$) 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 ($5.9 \times 10^{-5}$ to $2.1 \times 10^{-4}$) 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 $1.3 \times 10^{-4}$, while the average frequency of $hprt^-$ T-cells was $6.7 \times 10^{-6}$. The difference between these frequencies was significant ($P < 0.05$) when tested by the nonparametric rank test.

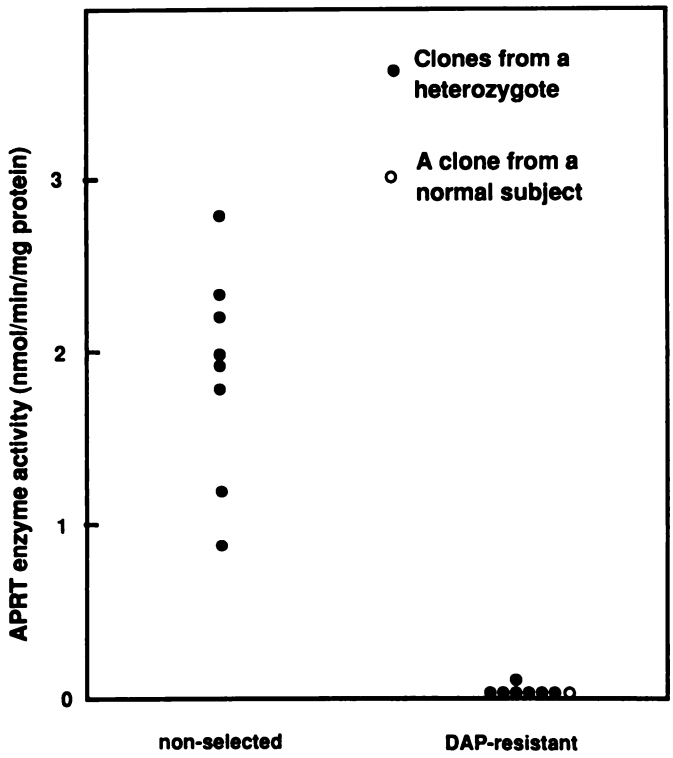


Fig. 1. APRT activities in the extracts of nonselected and DAP-selected T-cell clones. T-cells were cloned from the peripheral blood of an obligate heterozygote with the genotype of *APRT*1/APRT*Q0* in the presence or absence of 100 $\mu$M DAP, as described in the "Materials and Methods." APRT activities 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.

**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 $3.0 \times 10^9$ 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 $5.0 \times 10^{-9}$. 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.

## 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 $2 \times 10^4$ cells/well | Frequency of TG$^r$ T-cells |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | | $2 \times 10^4$ | $4 \times 10^4$ | | | |
| KM | 27 | F | 39/96[b] | 61/96[b] | 0.51 | 45/288[b] | 31/96[b] | $18.0 \times 10^{-6}$ | 16/384[b] | $4.3 \times 10^{-6}$ |
| KF | 31 | M | 40/96 | 68/96 | 0.58 | 32/288 | 15/96 | $8.8 \times 10^{-6}$ | 16/384 | $3.8 \times 10^{-6}$ |
| YO | 31 | F | 24/96 | 42/96 | 0.29 | 20/192 | 16/96 | $17.6 \times 10^{-6}$ | 20/360 | $9.9 \times 10^{-6}$ |
| TE | 33 | F | 30/96 | ND | 0.37 | 20/96 | ND | $31.6 \times 10^{-6}$ | 12/192 | $8.7 \times 10^{-6}$ |

[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 ($1.3 \times 10^{-4}$) was much higher than the frequencies of *hprt*$^-$ T-cells *in vivo* reported by various groups ($3 \times 10^{-6}$ to $1.2 \times 10^{-5}$) (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 $3.0 \times 10^9$ cells from 310 normal subjects. The frequency of *aprt*$^{-/-}$ cells in *aprt*$^{+/+}$ individuals was calculated to be $5.0 \times 10^{-9}$. 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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