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HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE WITH ALTERED SUBSTRATE AFFINITY IN MUTANT MOUSE LYMPHOMA CELLS

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Summary

Cells with altered hypoxanthine-guanine phosphoribosyl transferase (HPRT) (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) have been selected. Compared to wild type, mutant enzyme has a reduced affinity for the substrate phosphoribosyl pyrophosphate and is more labile to heat inactivation. Mutant cells are resistant to 6-thioguanine at 33—39°C and sensitive to hypoxanthine-aminopterin-thymidine at 37—39°C, but not at 33°C. We hypothesize that a single structural mutation of HPRT can explain these results.

Introduction

The cytotoxic purine 6-thioguanine (6-TG) has been widely used to select mutant cells (6-TG^r) with altered hypoxanthine-guanine phosphoribosyltransferase (HPRT) (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) activity. This enzyme converts purine bases to nucleotides by catalyzing their reaction with phosphoribosyl pyrophosphate, a requirement for the subsequent lethal incorporation of 6-TG into DNA. Although some mutants show no evidence of enzyme synthesis, biochemical, immunochemical, and physical studies have demonstrated that in some mutants structurally altered enzyme with reduced or altered activity is synthesized [1—3].

Genetic studies of HPRT have been facilitated by the combined use of

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Abbreviations: DEAE, diethylaminoethyl; EDTA, ethylenediamine tetraacetic acid; HAT, medium containing hypoxanthine, aminopterin and thymidine; HPRT, hypoxanthine-guanine phosphoribosyl transferase; 6-TG, 6-thioguanine. MNNG, *N*-methyl-*N'*-nitro-*N*-nitroguanidine.

hypoxanthine, aminopterin and thymidine (HAT) as a counter-selection system (4). Aminopterin causes a state of lethal thymidine and purine starvation by inhibition of de novo synthesis. Cells can be rescued by exogenous thymidine and hypoxanthine, but the latter can enter AMP and GTP pools only after conversion to inosinic acid by HPRT. Cells that revert from enzyme deficiency, as well as wild type cells, should, therefore, grow in HAT (designated HAT^r).

The scheme outlined above predicts the existence of but two classes of cells, wild type ($HPRT^+$, $6\text{-}TG^s$, HAT^r) and mutant ($HPRT^*$, $6\text{-}TG^r$, HAT^s). In reality, the simple model is inadequate and complex phenotypes, such as ($6\text{-}TG^r$, HAT^r), are readily demonstrated. Purine analog resistance can arise without alteration of HPRT activity [1] and HAT resistance without reversion from HPRT deficiency [5]. The biochemical alterations that underly these phenotypes are frequently obscure. We have isolated a mutant cell line that is ($6\text{-}TG^r$, HAT^s) at 39°C, but ($6\text{-}TG^r$, HAT^r) at 33°C. It is of interest that this complex phenotype can be understood as the result of a structural alteration affecting HPRT alone.

Materials and Methods

Cells and culture methods. Wild type (clone 24,3,2) cells were a subclone of the pseudodipoloid S49.1 mouse lymphoma cell line obtained from the Salk Institute [6]. The cells were grown as stationary suspension cultures in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated horse serum (Gibco). No antibiotics were used. Growth was in a humidified CO_2 incubator at 33, 37 or 39°C. The doubling time under these conditions was 16—20 h. Cells were cloned in soft agar over a feeder layer of primary mouse embryo fibroblasts and colonies counted as described elsewhere in detail [7]. Cells were counted in a Coulter Counter, Model Z_{BI}, at a lower threshold setting that eliminated pycnotic cells. Tests for mycoplasma by agar culture were negative.

Hypoxanthine-guanine phosphoribosyltransferase (HPRT) activity assay. Cells were grown to a density of about 1.5×10^6 cells/ml, harvested by centrifugation, washed once with phosphate-buffered saline and resuspended in 10 mM Tris, pH 7.4, at 1×10^8 cells/ml. After sonication for 10 sec, the lysate was centrifuged at $40\,000 \times g$ for 15 min. The supernatant was collected and Dextran Blue was added to serve as a dye marker of void volume. Substrate molecules were then removed by gel filtration at 4°C on a Sephadex G-25 column equilibrated with the same buffer. Protein content was measured according to the method of Lowry et al. [8] and all samples were diluted to the same protein concentration, usually 0.5—1.0 mg/ml. The assay was performed by adding 30 µl of cell extract to 15 µl of reaction mix and 5 µl of H_2O containing various concentrations of phosphoribosylpyrophosphate (sodium salt; Sigma). The reaction mix contained ^{14}C labelled hypoxanthine (Schwarz-Mann), $MgCl_2$ and Tris to yield final concentrations of 140 µM, 10 mM and 0.1 M respectively. The specific activity of hypoxanthine was adjusted to 26 mC/mMol. Incubation was for 1 h at 37°C. The reaction was terminated by spotting 15 µl of the incubated mixture on a diethylaminoethyl (DEAE) filter paper disc (Whatman, DE 81) that had been prewet with 20 µl of 0.1 M ethylenediamine

tetraacetic acid. The filters were dried, washed three times with 7 ml of 50% methanol and counted [2]. The reaction was linear with time and protein concentration. Controls contained either bovine serum albumin instead of cell extract or extract boiled for 10 min. There was no difference between these two control values.

Isolation of mutants with altered HPRT. S49 cells were mutagenized with 2.5 μg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Aldrich), 500 μg/ml ethyl methanesulfonate (Eastman Kodak), 1 μg/ml ICR 191 (gift of Dr. H.J. Creech) or 500-rad X-rays as described in detail elsewhere [9].

After an expression time of 6 days, $5—10 \times 10^5$ cells per 6-cm dish (Falcon) were plated at 37°C in soft agar containing 10 μg/ml 6-thioguanine (6-TG; Calbiochem). Resistant colonies were counted after 10 days. All clones from each dish were pooled by vigorously resuspending the soft agar in 20 ml of fresh medium. Cells were grown at 37°C for 2 days and, after one change of medium, transferred to 33°C for 3 days. They were then cloned at 10^6 cells/dish at 33°C in soft agar containing 5×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine (HAT, ref. 4) (all Sigma). Survivors of this counter selection were picked after 14 days and grown in suspension. Hypoxanthine and thymidine were added to the medium at the same concentrations as in HAT for several days. The resulting cultures were tested in suspension for 6-TG resistance and HAT resistance at 33 and 39°C. The $HPRT^-$ S49 clone (Table III) was selected as described [9] and expressed <0.5% of wild type enzyme activity.

Measurement of [^{14}C]hypoxanthine incorporation into cells. [^{14}C]hypoxanthine incorporation was measured according to the method of Fenwick and Caskey [2]. Cells were diluted to 5×10^5/ml and grown overnight in 0.5 ml in a well of a Falcon Multiwell dish. They were then exposed to [^{14}C]-hypoxanthine (0.5 μCi/ml; 53 Ci/mol) with or without aminopterin plus thymidine at the concentration used in HAT for 8 h, washed once with PBS, resuspended in 0.1 ml H_2O and lysed by freeze-thawing 3 times. Aliquots of lysate were spotted on DEAE filter paper discs, washed with 500 ml of H_2O and counted. Incorporation was linear to at least 24 h. Control experiments in which [^{14}C]hypoxanthine-labeled cells were treated with trichloroacetic acid and the precipitated material counted gave similar results, indicating that the DEAE filter paper assay determined incorporation into macromolecular species. Protein content of each lysate was measured by the method of Lowry et al. [8].

Measurement of phosphoribosylpyrophosphate levels in cells. Phosphoribosylpyrophosphate was measured according to the method of Kornberg et al. [10] as modified by Cohen et al. [11]. Cells were harvested by centrifugation, washed once with PBS and resuspended in 0.2 M Tris/2 mM EDTA, pH 7.0 at about 2×10^8 cells/ml. The extracts were treated with a final concentration of 0.5 M perchloric acid for 30 sec at 0°C in order to denature all enzymes that could utilize phosphoribosylpyrophosphate and then neutralized with KOH. After removal of salt and cell debris by centrifugation at 40 000 × *g* for 15 min, the supernatants were used directly or at different dilutions for the enzyme assay. For this, 100 μl of cell extract or a known concentration of phosphoribosylpyrophosphate treated in the same way were mixed with 50 μl of reaction

mix containing [*carboxyl*-^{14}C]orotic acid (50 mCi/mmol; New England Nuclear), orotidine-5′-phosphate pyrophosphorylase which is contaminated with orotidine-5′-phosphate decarboxylase (P.L. Biochemicals, Inc.) and $MgCl_2$ to yield final concentrations of 1 μCi/ml, 3.3 mg/ml and 4 mM respectively. Into each reaction tube, a smaller tube was inserted containing 50% Protosol in ethanol and a filter paper wick that was wetted by this mixture. The reaction was carried out for 3—5 h at 37°C. In a coupled reaction, the orotic acid is transformed in the presence of phosphoribosylpyrophosphate into orotidine-5′-phosphate and $^{14}CO_2$ is cleaved from this. In the presence of an excess of enzyme and orotic acid, the amount of $^{14}CO_2$ that is liberated is a direct measurement of the amount of phosphoribosylpyrophosphate present. The $^{14}CO_2$ adsorbs to the filter paper wicks and these are counted in a liquid scintillation counter. The protein content of each extract was measured according to Lowry et al. [8]. The amount of phosphoribosylpyrophosphate present in the cell extracts (in nmol/mg) was calculated by comparison with a standard curve and by correction for protein content.

Results

Selection and Counter-selection of Mutants with Altered HPRT. Wild type cultures were mutagenized with ethyl methylsulfonate, MNNG, ICR 191, or X-rays and then cloned in selective medium containing 6-TG. Individual dishes with resistant clones, one for each mutagen, were chosen and the mutant colonies on each dish individually pooled. Table I shows the frequency of 6-TG resistant mutants in each mutagenized culture and the number of colonies pooled. The resultant cultures of cells resistant to 6-TG were subsequently cloned at 33°C in soft agar containing HAT, as described in Materials and Methods, to select cells resistant to HAT as well as to 6-TG. The recovery of HAT resistant clones among cells previously treated with ethyl methylsulfonate or

TABLE I

GENERATION OF 6TG RESISTANT CELLS BY MUTAGENESIS

Mutagen	Frequency of 6-TG-resistant mutants per	Number of colonies	
	10^6 cells *	pooled **	
ethyl methyl-sulfonate 500 μg/ml	126	55	
ICR 191 1 μg/ml	865	502	
MNNG 2.4 μg/ml	277	200	
X-rays 500 rad	174	154	55

* Frequency of mutants resistant to 10 μg/ml 6-TG at 37°C. The background frequency of spontaneous mutants was $0/2 \times 10^6$ in this case.

** Number of colonies that were pooled from one dish for counter selection in HAT. Cultures derived from these colonies were grown to a cell number of about 3×10^7 and then cloned in HAT medium (see Materials and Methods).

TABLE II

GROWTH PROPERTIES OF U63/16T1 AND WILD TYPE AT 33 AND 37°C

Cells were pre-incubated at the indicated temperatures for 3 days. They were then diluted to 5×10^4 cells/ml and, after the additions indicated, grown in suspension at the same temperature for 3 days. Cells were then counted. The results are expressed as a percentage of the cell number in cultures incubated without drugs. After 5 days, survival of the mutant in HAT at 33°C was 38% because of growth inhibition (no abnormal percentage of dead cells). At 37°C, it was 4% because of extensive cell death.

	Cell number as % of untreated control			
	U63/16T1		Wild type	
Complete medium containing	33°C	37°C	33°C	37°C
No addition	(100)	(100)	(100)	(100)
10 μg/ml 6-TG	120	92	8	2
HAT	62	14	83	73
$4 \cdot 10^{-7}$ M aminopterin	2	2	n.t.	n.t.
$5 \cdot 10^{-4}$ M hypoxanthine, $4 \cdot 10^{-7}$ M aminopterin	23	5	16	5
$4 \cdot 10^{-7}$ M aminopterin, $1.6 \cdot 10^{-5}$ M thymidine	6	1	7	1
$5 \cdot 10^{-4}$ M hypoxanthine, $1.6 \cdot 10^{-5}$ M thymidine	96	80	54	64

MNNG was 100 per 10^6 and 40 per 10^6 respectively. No HAT-resistant colonies were found among the cells previously treated with ICR-191 or X-rays.

Growth properties of 6-TG-resistant, HAT-resistant clones. Five ethyl methylsulfonate-derived and three MNNG-derived HAT-resistant clones were tested in suspension culture for growth in 6-TG and in HAT at 33 and 39°C. All eight were completely resistant to 6-TG at both temperatures and also resistant to HAT at 33°C. One clone was resistant to HAT at 39°C; the remainder were sensitive. One of the latter (clone U63/16T1), and MNNG-derived clone, was further tested for its ability to grow in media containing different additives. The results (Table II) show that both hypoxanthine and thymidine were required for growth with aminopterin at 33°C. Resistance to HAT at 33°C was not, therefore, due to a second mutation that resulted in temperature sensitive resistance to aminopterin. U63/16T1 was recloned and used for the investigations described below.

TABLE III

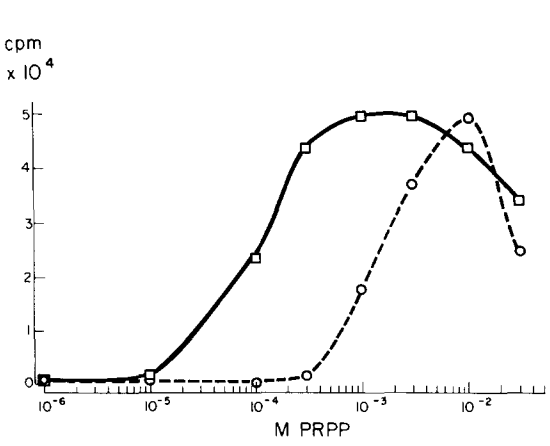
[^{14}C]HYPOXANTHINE INCORPORATED

[^{14}C]hypoxanthine incorporated into DEAE adsorbable material in three different S49 clones at two temperatures without or with addition of aminopterin and thymidine. Incorporation was measured as described in Materials and Methods.

	cpm per culture	
	33°C	39°C
Wild type — aminopterin/thymidine	7297	11827
Wild type + aminopterin/thymidine	7782	10794
U63/16T1 — aminopterin/thymidine	115	129
U63/16T1 + aminopterin/thymidine	1067	551
HPRT$^-$ — aminopterin/thymidine	150	112
HPRT$^-$ + aminopterin/thymidine	93	73

Incorporation of [^{14}C]hypoxanthine into wild type, U63/16T1 and HPRT$^-$ cells at 33 and 39°C. U63/16T1, wild type and HPRT$^-$ cells were tested for their ability to incorporate [^{14}C]hypoxanthine in the presence and absence of aminopterin and thymidine (Table III). There was an increase in ^{14}C incorporation into U63/16T1 cells with the addition of aminopterin and thymidine. The increase was about nine-fold at 33°C and four-fold at 39°C, indicating that in the presence of HAT medium at least part of these cells' HPRT could function. As expected, incorporation into wild type cells was substantial and into HPRT$^-$ cells minimal. This latter result was unaffected by temperature or by the addition of aminopterin or thymidine.

Properties of mutant and wild type HPRT. Both U63/16T1 and wild type cells were found to have HPRT activity. These enzymes differed in several properties. The cellular level of mutant enzyme was temperature-dependent; cells grown at 37°C contained only 48% as much enzyme activity/mg protein as cells grown at 33°C. This amount may be insufficient to allow growth and survival in the presence of aminopterin. The mutant enzyme had a reduced affinity for phosphoribosylpyrophosphate, as shown in Fig. 1. The apparent K_m of wild type enzyme for phosphoribosylpyrophosphate was 0.1 mM, of mutant enzyme 1.4 mM. At saturating concentrations of phosphoribosylpyrophosphate, the activity of wild type mutant cell extracts was similar, when both were assayed at 37°C (Fig. 1). At 25°C, however, the mutant enzyme could be maximally stimulated by phosphoribosylpyrophosphate, to only about 50% of the level of the wild type enzyme (data not shown). The mutant enzyme was more thermolabile than the wild type. The kinetics of thermal inactivation of enzyme were determined by heating extracts of wild type and mutant cells at 67°C (Fig. 2). Even at 4°C a difference in stability was apparent; mutant



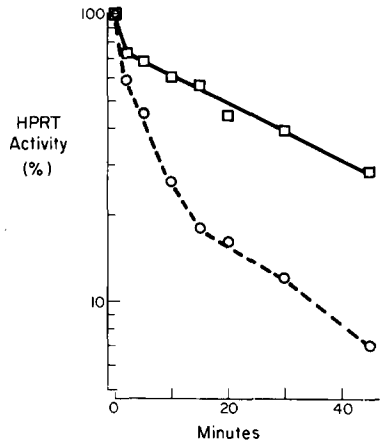


Fig. 1. Dependence of wild type and mutant HPRT on phosphoribosylpyrophosphate (PRPP) concentration. Cell extracts were prepared from cells that had been grown at 33°C for several days. The enzyme assay was performed at 37°C as described in Materials and Methods. □, wild type; ○, U63/16T1.

Fig. 2. Heat inactivation of wild type and mutant HPRT activity at 67°C. The cells extracts (at 0.5 mg protein/ml) were heated in glass tubes under mineral oil. At each time point, an aliquot was removed and chilled in ice water. The enzyme assay was carried out (in triplicate) as described in Materials and Methods □, wild type, ○, U63/16T1.

TABLE IV

PHOSPHORIBOSYLPYROPHOSPHATE CONTENT OF WILD TYPE AND U63/16T1 CELLS WITHOUT OR WITH HAT PRETREATMENT (16.5 h) AT TWO TEMPERATURES

	Phosphoribosylpyrophosphate (nmol/mg protein)			
	33°C		37°C	
	—HAT	+HAT	—HAT	+HAT
Wild type	0.23	0.14	0.34	0.04
U63/16T1	0.58	2.2	1.0	3.2

enzyme activity decayed at a rate of about 60% per day compared to <5% for the wild type.

Phosphoribosylpyrophosphate levels and phosphoribosylpyrophosphate synthetase in mutant and wild type cells. Because the mutant enzyme had a reduced affinity for phosphoribosylpyrophosphate, we tested the hypothesis that phosphoribosylpyrophosphate levels became high enough for enzyme function in the presence of HAT. Wild type and U63/16T1 cells were incubated in the presence and absence of HAT at both 33 and 37°C and the cellular content of phosphoribosylpyrophosphate determined. Although determination of absolute levels varied over two-fold between experiments, the ratios between wild type and mutant levels and between levels of cultures with and without HAT pretreatment were reproducible. Typical results are shown in Table IV. After 16.5 h, HAT had indeed elevated phosphoribosylpyrophosphate content in mutant, but not in wild type cells. After 5 h of treatment with HAT, the phosphoribosylpyrophosphate level of U63/16T1 had increased almost twice as much as after 2.5 h. A longer treatment did not result in still higher levels, suggesting that the concentration reached after 5 h is high enough for function of the mutant enzyme.

To determine whether these observations were the result of a second mutation that affected the activity of phosphoribosylpyrophosphate synthetase, this enzyme activity was measured in wild type of U63/16T1 cells by the method of Green and Martin [12]. The activity of the mutant culture was 20% higher than that of wild type, a difference that lies within the variation among wild type clones. Thus, the higher phosphoribosylpyrophosphate levels in the mutant compared to the wild type in the absence of HAT are likely not due to increased rates of phosphoribosylpyrophosphate synthesis.

Discussion

A mutant obtained by mutagenesis and a two-step selection procedure exhibited an unusual temperature sensitive phenotype: resistance to 6-TG at 33—39°C, resistance to HAT at 33°C and sensitivity to HAT at 37—39°C. We propose that this clone produces a structurally altered HPRT with reduced affinity for the substrate phosphoribosylpyrophosphate and increased thermal lability and that these properties account for the phenotype as follows.

At 33—39°C intracellular phosphoribosylpyrophosphate concentrations are sufficiently below the K_m of HPRT that 6-TG is not converted to a signifi-

cantly toxic degree into nucleotide. Aminopterin prevents endogenous purine synthesis. HPRT is a major path of phosphoribosylpyrophosphate utilization; in the presence of aminopterin, HPRT utilizes a still greater portion of phosphoribosylpyrophosphate pools. Even if the rate of phosphoribosylpyrophosphate synthesis is equal in wild type and mutant cells (as is consistent with direct measurement of phosphoribosylpyrophosphate synthetase activity), steady-state levels of phosphoribosylpyrophosphate become higher in the mutant than in wild type cells when aminopterin is present, because phosphoribosylpyrophosphate synthesis is zero-order and consumption by HPRT follows Michaelis-Menten kinetics. Finally, because the mutant HPRT is thermolabile, less enzyme is present at 37—39°C than at 33°C. At the higher temperatures, although phosphoribosylpyrophosphate levels are elevated, the amount of enzyme activity is not sufficient to sustain cell growth and viability in HAT. This explanation of the phenotype of U63/16T1, based on a lesion affecting only HPRT, is attractive, self consistent and satisfies the principle of parsimony, but is not necessarily correct. Alternatively, mutations in two distinct functions could have occurred, the first presumably in HPRT, rendering the cells resistant to 6-TG, and the second in a function that made the cells resistant to HAT at 33°C.

Cells resistant to both 6-TG and HAT could be isolated by the two-step selection process described here if cultures were first mutagenized with MNNG or ethyl methylsulfonate, but not if they were treated with ICR 191 or X-rays. All four agents effectively generated mutants resistant to 6-TG alone. MNNG and ethyl methylsulfonate cause base substitution mutations in prokaryotes, while ICR 191 and X-rays act predominantly through other mechanisms. The mutagenic specificity of the double resistant cells and the biochemical studies reported here support the hypothesis that MNNG and ethyl methylsulfonate act similarly in animal cells to induce structural mutations that can result in altered enzyme action, without complete loss of enzyme activity [9].

Fenwick and Caskey [2] have described mutants of Chinese hamster cells that had a similar temperature sensitive phenotype, but seemingly with a biochemical lesion different from that of our mutant. Their mutant cells grown at 33°C had 2.6—8.2% of wild type HPRT activity but these values were 0.04—1.2% for cells grown at 39°C. Enzyme assays were done at 1.5 mM phosphoribosylpyrophosphate concentration, hence an alternation in the K_m for phosphoribosylpyrophosphate in the mutant enzyme is unlikely to be responsible for the large differences in activity between mutant and wild type. In contrast, U63/16T1 grown at 33 and 37°C had 100 and 48% respectively of wild type enzyme activity, when assays were done at substrate concentrations saturating for both enzymes. Chasin and Urlaub [13] recently reported a Chinese hamster cell mutant with HPRT exhibiting an increased K_m for phosphoribosylpyrophosphate that was isolated after mutagenesis with ethyl methylsulfonate.

Benke et al. [14] have reported a hyperuricemic patient whose HPRT had a kinetic alteration resembling that described here. Normal control HPRT in extracts from red cells had a K_m for phosphoribosylpyrophosphate of 0.11—0.14 mM while the patients' HPRT had a K_m of 1.0—1.4 mM. When assayed, at saturating concentrations of phosphoribosylpyrophosphate, the specific activity of patients cell extracts (138 μmol/mg per h) exceeded that present in

controls (80 μmol/mg per h). The thermal stability of the enzyme was not described. It is striking that in vitro mutagenesis and selection procedures as described here can be used to obtain from a cloned cell line mutants that appear to reproduce the metabolic defect present in this patient.

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