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BIOCHEMICAL MECHANISM OF RESISTANCE OF CULTURED SARCOMA 180 CELLS TO 6-THIOGUANINE

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Abstract—A 6-thioguanine (6TG) resistant clone (S180/TG) of the murine neoplastic cell line Sarcoma 180 (S180) synthesized significantly less 6-thioguanosine 5'-monophosphate (6TGMP) than parent sensitive S180 cells when exposed to 6TG in culture. The decrease in the intracellular level of 6TGMP in S180/TG cells did not appear to be due to a change in the (a) rate of uptake of 6TG (or guanine), (b) synthesis and accumulation of 5-phosphoribosyl 1-pyrophosphate (PRPP) from glucose, (c) capacity to enzymatically convert 6TG (or guanine) to its corresponding 5'-mononucleotide in the presence of exogenous PRPP, or (d) rate of hydrolysis of phosphate groups from newly synthesized 6TGMP. Contrasting with the decreased conversion of 6TG to 6TGMP by S180/TG cells was the finding of significantly more guanine conversion to guanine nucleotides by intact S180/TG cells, a phenomenon that resulted in approximately 4- and 2-fold greater incorporation of [8-^{14}C]guanine into RNA and DNA, respectively, in resistant cells than in the parent sensitive subline. The findings suggest that the mechanism by which S180/TG cells achieve insensitivity to the 6-thiopurine was through decreased synthesis of analog nucleotide; this phenomenon appears to be the result of altered purine metabolism in resistant cells which selectively permits the conversion of guanine to guanine nucleotides, while suppressing the anabolism of 6TG to 6TGMP.

The 6-thiopurines [i.e. 6-thioguanine (6TG) and 6-mercaptopurine] are useful agents in the treatment of the acute leukemias of man. To express their anticancer activity, conversion to the corresponding 5'-nucleotide level by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is required [1]. A major limitation to the effective use of these purine analogs to control human leukemia is the relative ease with which neoplastic cells acquire resistance to these antimetabolites. The most prevalent biochemical alteration responsible for resistance to these agents in transplanted animal tumors and microorganisms is loss or marked decrease in the activity of HGPRT [1]; however, in human leukemic leukocytes this mechanism appears to be expressed infrequently [2–5]. An alternate mechanism described by this laboratory and confirmed by others for both murine S180/TG cells [6–8] and human acute leukemia cells [3, 5] involved a marked increase in alkaline phosphatase activity which appeared to function to degrade 6-thioguanosine 5'-monophosphate (6TGMP) in resistant cells at an increased rate.

In this report, we describe the spontaneous loss of the high levels of alkaline phosphatase activity of S180/TG cells to a basal level lower than that of the sensitive parent subline following long-term passage in culture without loss of resistance to the cytotoxic action of 6TG. The uptake and metabolism of guanine and 6TG by S180 and S180/TG cells were compared in an effort to elucidate the mechanism by which cultured S180/TG cells achieve their insensitivity to 6TG. S180/TG cells synthesized less 6TGMP from 6TG than the corresponding drug-sensitive cells; in contrast, S180/TG cells converted guanine to guanine nucleotides in much greater amounts than did S180. Since uptake of guanine and 6TG and their enzymatic conversion by HGPRT to the corresponding 5'-mononucleotide were similar in S180 and S180/TG, possible mechanisms of resistance include differences in (a) the intracellular location of HGPRT in sensitive and resistant cells which influence the availability of substrate, or (b) processes regulating the conversion of these purines to their nucleotide levels in the two cell lines.

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MATERIALS AND METHODS

S180 and S180/TG ascites cells were maintained in CD-1 female mice (Charles River Breeding Laboratories, Portage, MI) by weekly intraperitoneal inoculation of approximately 10^6 cells/mouse. The derivation of S180/TG has been described by our laboratory [8, 9]. Clones were established in culture from S180 and S180/TG ascites cells collected from mice, and exponentially growing cells derived therefrom were maintained in Fischer's medium (Grand Island Biological Co., Grand Island, NY) containing 100 μg streptomycin/ml, 100 units penicillin/ml, and 10% horse serum and were grown at 37° in an atmosphere containing 5% CO_2.

The effects of 6TG on cellular proliferation were measured with stock solutions of 6TG in PBS (8.0 g NaCl, 0.2 g KCl, 2.16 g $Na_2HPO_4 \cdot 7H_2O$ and 0.2 g KH_2PO_4 per liter of H_2O, pH 7.4) sterilized by filtration through 0.20 μm Nalgene filters (Nalge Sybron Co., Rochester, NY). Five hundred microliters of drug solution was added to 4.5 ml of cell suspension in Fischer's medium; control groups

received an equal volume of PBS. The rates of cell proliferation were measured at various times thereafter by determination of cell numbers using a Coulter model ZBI particle counter.

Alkaline phosphatase activity was measured at 37° by determining the initial rate of hydrolysis of *p*-nitrophenylphosphate (PNPP; Sigma Chemical Co., St. Louis, MO) as the change in absorbance at 410 nm [10]. Reaction mixtures consisted of 0.7 M Tris–HCl buffer (pH 9.4) containing 10^{-2} M $MgCl_2 \cdot 6H_2O$, 10^{-3} M PNPP and an appropriate amount of enzyme (0.6 mg protein of a cell suspension sonicated three times for 15 sec each at 20% output of a Branson sonicator) in a total volume of 1.0 ml. One unit of activity is expressed as the hydrolysis of 1 nmole of PNPP per min based on a molar absorbance of *p*-nitrophenol in the buffer used of 1.7×10^4 moles $\cdot l^{-1} \cdot cm^{-1}$. Protein concentration was determined by the method of Bradford [11] using Bio-Rad dye reagent (Bio-Rad, Richmond, CA).

Measurement of purine nucleotide formation by S180 and S180/TG was conducted with exponentially growing cells suspended at a concentration of 5% in Fischer's medium containing 10% horse serum. [^{35}S]6TG was synthesized as previously described [12]. 6TG was dried at 110° *in vacuo* over P_2O_5 for several hours. To a suspension of 500 mg of 6TG in 200 ml of dry pyridine which had been refluxing for 3 hr was added 10 mCi of elemental ^{35}S (New England Nuclear Corp., Boston, MA). The suspension was refluxed for 3.5 hr and the pyridine was removed *in vacuo* with the addition of toluene. The dry residue was recrystallized from 480 ml of warm water. The yield was 110 mg of [^{35}S]6TG (6.5×10^5 cpm/μmole).

The formation of nucleotides was measured by incubating cells at 37° with 0.1 mM [^{35}S]6TG (6.5×10^5 cpm/μmole) or 0.1 mM [8-^{14}C]guanine (28 mCi/mmole) (New England Nuclear Corp.). At various times thereafter, cells were collected by centrifugation at 12,000 *g*, extracted with ice-cold 0.2 N perchloric acid, the supernatant solution was neutralized with KOH, clarified by centrifugation and subjected to chromatographic analysis. A Varian model 5000 high-pressure liquid chromatograph equipped with a Whatman anion-exchanger (Partisil PXS, SAX, 4.6 mm inside diameter × 25 cm) was used for the separation of nucleotides. A linear gradient from 10 mM to 1.0 M NaH_2PO_4 (pH 3.5) was used; the gradient was formed by varying the output of two pumps over a 30-min period. The flow rate was 1.0 ml/min and the column temperature was 25°. The radioactivity in the collected fractions (1 ml each) was measured by liquid scintillation spectrometry.

HGPRT activity was measured by sonicating 0.5 g of cells as a 3% suspension in ice-cold water three times for 15 sec each at 20% of the output of a Branson sonicator. The cell sonicates were centrifuged at 12,000 *g* for 10 min at 4°, and the supernatant solution was dialyzed for 20 hr against 4 liters of 50 mM Tris–HCl (pH 8.0). The standard assay contained 50 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2 \cdot 6H_2O$, 1 mM 5-phosphoribosyl 1-pyrophosphate (PRPP; Sigma Chemical Co.), and 10 μM of either [8-^{14}C]guanine (28 mCi/mmole) or [^{35}S]6TG (6.5×10^5 cpm/μmole) in a total volume of 100 μl; control samples were incubated in the absence of PRPP. The reaction was initiated by the addition of enzyme (approximately 50 μg protein) and terminated by the addition of 50 μl of 1.2 N $HClO_4$. Separation of radioactive nucleotide product from the substrate was accomplished using high-pressure liquid chromatography, and the radioactivity was measured as described above.

The rate of PRPP accumulation from glucose in tumor cells was measured by the method of Henderson and Khoo [13]. Exponentially growing tumor cells (200 mg wet weight) suspended in 10 ml of calcium-free Krebs–Ringer phosphate medium (pH 7.4) were incubated at 37° in the presence of 5.5 mM glucose. At intervals, portions of the cell suspension were removed and PRPP therein was extracted by boiling the samples in glass tubes for 30 sec. The concentration of PRPP was determined by measuring the radioactive adenosine 5′-monophosphate obtained after incubation of extracts with 200 nmoles [8-^{14}C]adenine (40 mCi/mmole) (New England Nuclear Corp.) and separation of the product by high-pressure liquid chromatography.

The uptake of radioactive purines by tumor cells was carried out with exponentially growing cells incubated in Fischer's medium containing 10% horse serum and either 10^{-6} M [8-^{14}C]guanine (28 mCi/mmole) or [^{35}S]6TG (6.5×10^5 cpm/μmole) at 4°. At various times, 10^6 cells (0.2 ml) were added to Eppendorf microcentrifuge tubes containing 0.3 ml of oil mixture [84 parts silicone oil (Aldrich Chemical Co., Milwaukee, WI) and 16 parts paraffin oil (Fisher Scientific Co., Fair Lawn, NJ) by weight (final density = 1.03 μg/ml)] layered above 0.1 ml of 0.6 N $HClO_4$ in 10% (w/v) sucrose solution [14]. The tubes were subjected to centrifugation in an Eppendorf microcentrifuge for 15 sec (12,000 *g*), and the radioactivity present in the acid layer was measured.

Measurement of [8-^{14}C]guanine incorporation into the nucleic acids was conducted in exponentially growing cells suspended in Fischer's medium containing 10% horse serum at a concentration of 10^7 cells/ml. [8-^{14}C]Guanine (10^{-6} M; 28 mCi/mmole) was added and the suspension was incubated at 37°. Cells were collected at various times thereafter by centrifugation at 12,000 *g* for 15 sec and were washed three times with ice-cold 0.2 N $HClO_4$. Cell pellets were hydrolyzed with 0.3 M KOH for 30 min at 60° and the alkali-soluble fraction was collected after addition of 1.0 ml of chilled 0.2 N $HClO_4$. The residue was washed two more times with chilled 0.2 N $HClO_4$, and the radioactivity present in separated RNA and DNA was determined as described above.

RESULTS

The alkaline phosphatase activities of S180 and S180/TG cells maintained by continuous passage of ascites cells in mice, and of 6-thiopurine-sensitive and -resistant cells derived from these neoplasms, but passaged in culture in Fischer's medium for approximately 3 years, were measured, and the results are shown in Table 1. The enzyme activity of sonicates of S180/TG ascites cells propagated in

Table 1. Alkaline phosphatase activities of S180 and S180/TG cells

Cell line	Specific activity* (units/mg)
S180 (mouse ascites cells)	0.46 ± 0.01†
S180/TG (mouse ascites cells)	6.25 ± 0.01
S180 (cultured cells)	2.50 ± 0.06
S180/TG (cultured cells)	0.89 ± 0.08

* Enzyme activities were measured by determination of the absorbance of *p*-nitrophenol at 410 nm.

† Average values from three separate assays ± standard errors.

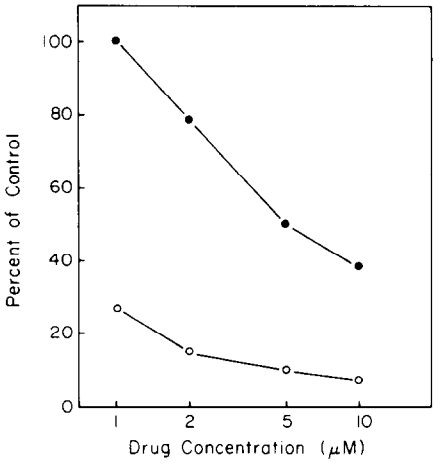

Fig. 1. Effects of various concentrations of 6TG on the replication of S180 and S180/TG cells. Cells were incubated in Fischer's medium containing 10% horse serum at 37° in the presence and absence of 6TG; their numbers were determined with a Coulter model ZBI particle counter 48 hr later. Key: S180 (○) and S180/TG (●). Untreated cells of both lines grew logarithmically from an initial concentration of 1.8×10^4 cells/ml to 1.1×10^5 cells/ml in 48 hr. The values are the averages of two separate experiments of two to three samples each; the S.E. was 8 percent of the value or less.

mice was 14-fold greater than that of the drug-sensitive parent S180. Following long-term maintenance in culture, the population of S180 selected by these conditions showed slightly greater particulate-bound alkaline phosphatase activity than the parent tumor maintained *in vivo*; in contrast, the cultured line of S180/TG had markedly less enzyme. The loss of the high levels of alkaline phosphatase activity by S180/TG cells grown in culture did not result in a reversion of this subline to a state of sensitivity to 6TG. Thus, while the rate of proliferation of S180 was inhibited by more than 70 percent following exposure to 1.0 μM 6TG for 48 hr, this concentration of the 6-thiopurine was noninhibitory to S180/TG (Fig. 1). To inhibit the replication of S180/TG cells in culture by 50 percent required a level of 5 μM 6TG. This contrasts with the finding that about 0.2 μM 6TG is required to cause a comparable degree of inhibition of the replication of the parent S180 subline under these conditions.

To determine the relationship between the cytotoxicity of 6TG to cultured S180 and S180/TG cells and their capacity to accumulate 6TGMP, 6-thiopurine-sensitive and -resistant cells were incubated with [^{35}S]6TG and the acid-soluble concentration of 6TGMP was measured (Fig. 2); at the earliest time of incubation measured, the rate of phosphorylation of 6TG by S180/TG cells was approximately 50 percent less than that of the parent sensitive subline, and the ratio of 6TGMP present in S180 cells to that of S180/TG was 3.6 at 60 min. In an effort to ascertain the mechanism responsible for the decreased level of 6TGMP in resistant cells, the HGPRT activity of dialyzed cell-free extracts of S180 and S180/TG was measured using both [^{35}S]6TG and [8-^{14}C]guanine as substrates. Under assay conditions in which linear conversion of these purines to the corresponding 5′-mononucleotide was obtained as a function of time and protein concentration, no significant difference was observed between sensitive and resistant cell-free extracts in the initial rate of phosphorylation and in total enzyme activity; the average initial rates of conversion were approximately 820 and 1500 pmoles·(mg

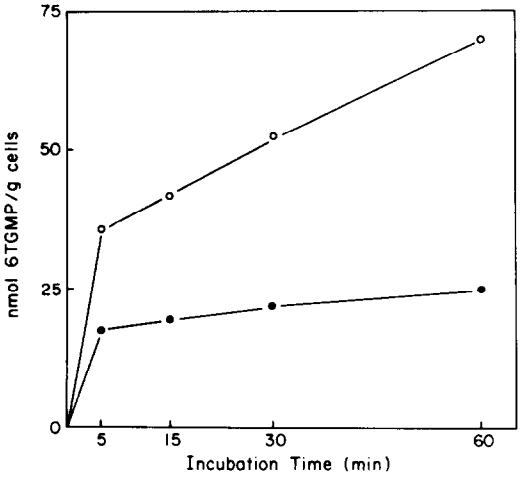

Fig. 2. Rate of formation of 6TGMP by S180 and S180/TG cells. Five percent cell suspensions of S180 and S180/TG in Fischer's medium containing 10% horse serum were incubated with 0.1 mM [^{35}S]6TG. At various times thereafter, the intracellular concentration of 6TGMP was measured as described. Key: S180 (○) and S180/TG (●). Each value is the average of results from two experiments of two to three samples each; the S.E. was 7 percent of the value or less.

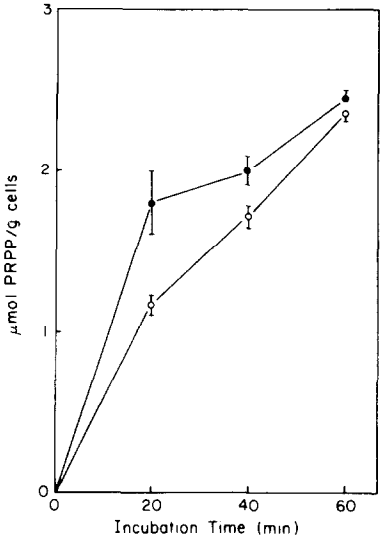

Fig. 3. Rate of PRPP synthesis by S180 and S180/TG cells. Tumor cells (200 mg wet weight) were incubated at 37° in 10 ml of calcium-free Krebs–Ringer phosphate medium containing 5.5 mM glucose. At various times thereafter, the concentration of PRPP was measured as described in Materials and Methods. Key: S180 (○) and S180/TG (●). Each value is the average ± S.E. of results from three experiments.

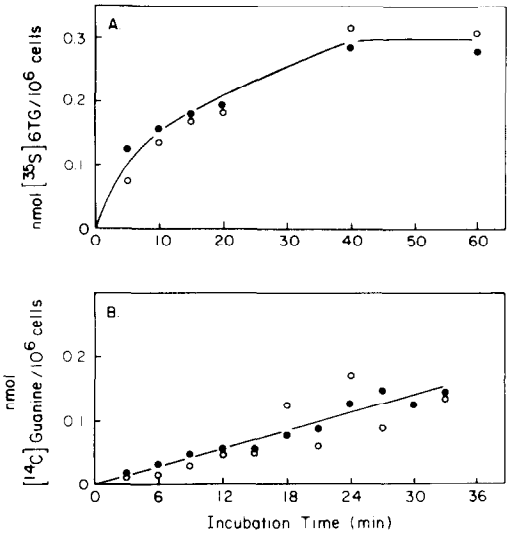

Fig. 4. Rate of uptake of guanine and 6TG into S180 and S180/TG cells. A suspension of tumor cells (1×10^7 cells/ml) was incubated with either 10^{-6} M [^{35}S]6TG (A) or [8-^{14}C]guanine (B) at 4°. At various times thereafter, cells were centrifuged through an oil layer, and the radioactivity present in the cellular pellet was determined as described in Materials and Methods. Key: S180 (○) and S180/TG (●). The data are from a representative experiment which was consistently reproducible.

protein)$^{-1}$ · min^{-1} at 37° for 6TG and guanine, respectively, for both sensitive and resistant lines.

The PRPP synthetic activities of the two cultured lines were also compared to determine whether inadequate resources of PRPP in 6-thiopurine-resistant cells might account for their diminished ability to form 6TGMP. The rate of accumulation of PRPP from glucose was measured by the method of Henderson and Khoo [13]; although resistant cells synthesized slightly more PRPP than the parent sensitive subline at early times, by 60 min both cell lines contained approximately 40 nmoles PRPP · min^{-1} · (g tumor cells)$^{-1}$ (Fig. 3).

The inability to detect biochemical changes in cell-free systems which would explain the decreased conversion of 6TG to 6TGMP by intact S180/TG cells led us to compare the abilities of S180 and S180/TG to transport both the 6-thiopurine and guanine across the cell membrane. The results of these studies, shown in Fig. 4, indicated that no major differences in purine uptake exist between S180 and S180/TG cells.

Since no difference in 6TG metabolism by resistant S180/TG cells was obtained that would explain the lower levels of 6TGMP present in these cells compared to S180, guanine metabolism was measured in S180 and S180/TG cells to determine whether the lower rate of conversion of 6TG to 6TGMP by the 6-thiopurine-resistant subline was a characteristic of S180/TG cells towards guanine and its analogs in general. The metabolic utilization of guanine by intact cells was examined, and Fig. 5 shows the chromatographic profile of radioactive guanine nucleotides in S180 and S180/TG following 30 min of incubation at 37°; the amounts of radioactive GMP, GDP and GTP in S180/TG cells were found

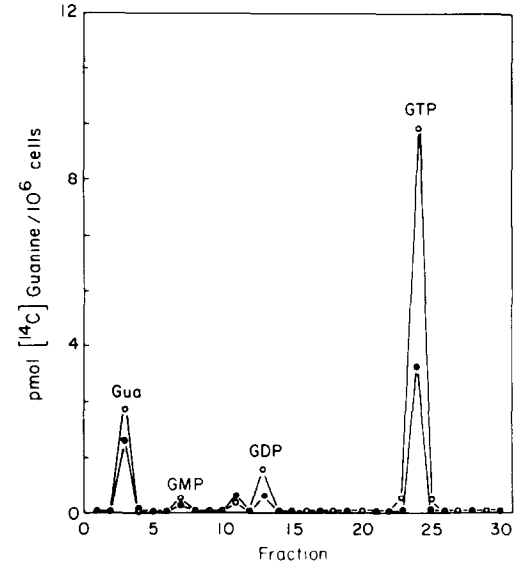

Fig. 5. Incorporation of [8-^{14}C]guanine into acid-soluble nucleotides by S180 and S180/TG. Exponentially growing cells suspended in Fischer's medium containing 10% horse serum were incubated with 10^{-6} M [8-^{14}C]guanine for 30 min at 37°. Cells were collected, the acid-soluble fraction was prepared, and radioactivity in guanine nucleotides was determined as described in Materials and Methods. Key: S180 (●) and S180/TG (○). The profile represents the results from a representative experiment which was consistently reproducible.

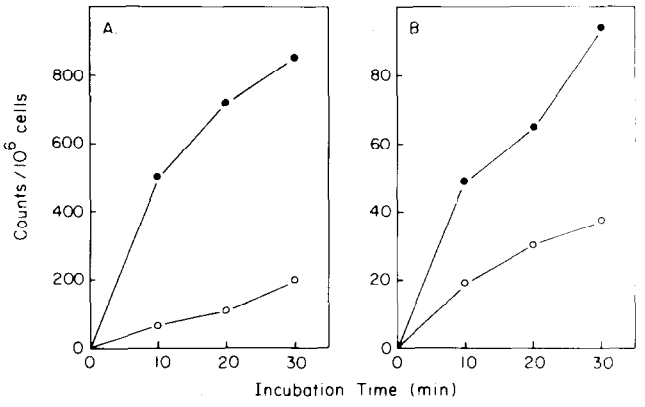

Fig. 6. Rate of incorporation of [8-^{14}C]guanine into RNA (panel A) and DNA (panel B) of S180 (○) and S180/TG (●). Exponentially growing cells suspended in Fischer's medium containing 10% horse serum were incubated with 10^{-6} M [8-^{14}C]guanine at 37°. At various times thereafter, radioactivity in RNA and DNA was determined as described in Materials and Methods. The values are the averages of two separate experiments of two to three samples each; the S.E. was 5 percent of the value or less.

to be 2-, 2.5- and 3.8-fold greater, respectively, than those present in 6-thiopurine-sensitive cells. The greater ability of the resistant cells to convert [8-^{14}C]guanine into nucleotides was confirmed by measuring the incorporation of radioactive guanine into the nucleic acids (Fig. 6); the results of this experiment demonstrated that S180/TG cells utilized 4- and 2-fold more [^{14}C]guanine for RNA and DNA synthesis, respectively, than drug-sensitive S180 cells.

DISCUSSION

The 6-thiopurine-resistant subline S180/TG has been shown to have markedly increased levels of particulate-bound alkaline phosphatase activity and no apparent alterations in HGPRT activity compared to its parent drug-sensitive tumor, prompting the suggestion by this laboratory that the low intracellular levels of 6TGMP, the active nucleotide form of 6TG, present in S180/TG cells following exposure to 6TG were the result of hydrolysis of 6TGMP to the nucleoside level by alkaline phosphatase and the subsequent loss of 6-thioguanosine from resistant cells [7]. Although the high alkaline phosphatase activity of S180/TG cells has been stable since its development in 1964 when passaged weekly in mice in ascites cell form [9], we now report that a subline of this tumor transferred to culture and maintained *in vitro* for about a 3-year period has spontaneously lost the high alkaline phosphatase activity without loss of resistance to the 6-thiopurine. These findings indicate that either high particulate-bound alkaline phosphatase is not involved in the mechanism by which S180/TG cells exert their resistance to 6TG *in vivo*, or that catabolism of 6TGMP by alkaline phosphatase is important for the expression of drug insensitivity, but that this cell line is a double mutant and more than one mechanism is involved in the expression of drug resistance.

The cultured line of S180/TG produced less 6TGMP when treated with 6TG than did the parent neoplasm. The mechanism by which the drug-resistant cells form smaller amounts of 6TGMP is not clear, for S180 and S180/TG appear to be similar in (a) their capacities to transport both 6TG and guanine across cell membranes, (b) their abilities to synthesize and accumulate PRPP, and (c) their levels and properties of HGPRT. In contrast to the lower conversion of 6TG to its nucleotide form (which exists primarily at the level of 6TGMP) by S180/TG cells, the metabolic utilization of guanine for nucleotide synthesis was markedly enhanced in this neoplastic cell line, as compared to parent drug-sensitive S180 cells. Such a phenomenon (i.e. increased conversion of purines to nucleotides), was reported previously by others [15] in a 6-mercaptopurine-resistant line of Ehrlich ascites cells, although little significance was attributed to this property.

These findings suggest that an alteration has occurred in S180/TG cells which permits them to distinguish between 6TG and guanine as substrates. Although no differences in the kinetic properties of HGPRT of S180 and S180/TG cells have been obtained, it is conceivable that the enzyme exists in a different state *in situ* and the assay of its activity in cell-free preparations results in changes in its properties. That such a mechanism is possible is supported by the finding that HGPRT may exist in two molecular sizes which differ in heat stability, the configuration of the enzyme being dependent upon the presence of PRPP [16, 17]. Alternatively, it is also conceivable that the intracellular location of HGPRT is different in the two sublines, and that distributional differences between guanine and 6TG are responsible for the observed differential in nucleotide forming ability. Whether either mechanism is involved in the expression of the insensitivity of S180/TG to 6TG requires further investigation.

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