The Biochemical Basis for the Differential Sensitivity of Intestinal Mucosa and Bone Marrow to 6-Thioguanine*

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SUMMARY

The differential sensitivity of mammalian tissues to 6-thioguanine was studied by measuring the metabolic fate of this agent in the bone marrow and intestinal mucosa of rabbits. After the injection of thioguanine-S<sup>35</sup>, intestinal mucosa contained, per gram of tissue, a sixteen-fold higher level of 6-thiouric acid, but less than one-half the quantity of thioguanine mononucleotide than did bone marrow. Thioguanine nucleotide pyrophosphorylase activity in bone marrow extracts was fivefold greater than in extracts prepared from intestinal mucosa. In contrast, thiouric acid was formed by extracts of intestinal mucosa but not by those of bone marrow. These data suggest that the extreme sensitivity of bone marrow to thioguanine is attributable to the relatively rapid conversion of this agent to the nucleotide level, coupled with an inability to catabolize this molecule to inactive products. The resistance of intestinal mucosa to 6-thioguanine appears to have a twofold basis: (a) a relatively lower nucleotide pyrophosphorylase activity and (b) an active mechanism for conversion of the drug to 6-thiouric acid.

Among the purine analogs used in the treatment of acute leukemia, 6-thioguanine appears to have a unique pattern of toxicity for normal cells. Whereas 6-mercaptopurine and 6-chloropurine damage many tissues, including bone marrow, intestinal mucosa, lymphoid tissue, lung, and liver, the toxicity of thioguanine has been reported to be virtually limited to bone marrow, even when administered in lethal doses (22, 23). Although considerable work has been done on the metabolism of 6-thiopurines in neoplastic tissues and in microorganisms, only one report describes the metabolic fate of these compounds in some drug-susceptible and -resistant normal mammalian cells (19). In neoplastic and bacterial cell populations, the selection of individuals with deleted nucleotide pyrophosphorylase activity has repeatedly been demonstrated to be associated with resistance to these agents (4-6, 13, 18). In addition, other factors appear to be operative in the development of resistance; these include a decreased quantity of analog incorporated into deoxyribonucleic acid (16, 17, 27), an increased rate of catabolism of the inhibitor (10, 12, 27), and a decreased capacity of the drug to permeate cellular membranes (20, 21).

In the present report the metabolism of 6-thioguanine has been compared, both in vivo and in cell-free extracts, in two rapidly proliferating tissues of the rabbit, the intestinal mucosa and bone marrow, to determine the biochemical differences responsible for the differential susceptibility of these tissues to this agent.

MATERIALS AND METHODS

Experiments in vivo.—Albino rabbits weighing 2500-3700 gm. were given injections intraperitoneally of 41-49 mg. of 6-thioguanine (TG) per kg. in volumes of 50 ml. or less. The TG was dissolved by the addition of sodium hydroxide, adjusted to pH 7.5-8.0 with hydrochloric acid, and brought up to volume with isotonic saline or distilled water. Animals were killed by injection of approximately 30 ml. of air into an ear vein, and the entire length of the small intestine was removed, chilled, and cut into sections of approximately 3-cm. length. Feces were expressed from each...
section by light pressure with a spatula, and the mucosa was removed by firm pressure and immediately placed in cold 0.2 M perchloric acid. Histological examination of residual stripped intestine indicated that the tissue removed by this procedure included basal crypt epithelium. The femurs and tibia-fibulas were chilled in ice, cracked open, and 4 ml. (packed cell volume) of red marrow were removed from each animal into 0.2 M perchloric acid. Fractionation of tissues into acid-soluble and nucleic acid components was accomplished by a modification of the method of Schneider et al. (28). The mass of the samples was compared in terms of milliliters of packed wet residue or grams of dried residue remaining after the extraction of nucleic acids.

6-Thiouric acid, TG, and 6-thioguanosine monophosphate (TGMP) present in the acid-soluble fraction were separated on Dowex-1 (formate-form) with increasing concentrations of formic acid and ammonium formate (19). Thioguanosine was collected with TG in the acid-soluble fraction and was not quantified separately. TG associated with the nucleic acid fraction was isolated by chromatography on Dowex-50W (chloride-form). The quantity of TG or its metabolites present in each fraction was determined by measurement of the ultraviolet absorption in 1 N hydrochloric acid at 345 mµ, with the use of the following molar extinction coefficients: 6-thioguanine, 17.0 X 10³; 6-thiouric acid, 22.5 X 10³; and 6-thioguanine mononucleotide, 20.6 X 10³.

When TG-S36 (Isotopes Specialties Co., Burbank, Calif.) (3.8 mc/mn mole) was employed, the concentration of metabolites was determined by evaporating column eluates and taking up the residue in 0.01 N NaOH. The samples were plated on aluminum planchets and counted in a Nuclear-Chicago gas-flow counter equipped with a “micromil” window. Counts were corrected for self-absorption and the number of µmoles of TG-equivalents was calculated.

Histology.—Pieces of small intestine from untreated and TG-treated rabbits were fixed in Bouin’s solution and stained with hematoxylin and eosin. Smears were made from marrow of the femur and tibia-fibula and were fixed and stained with Wright-Giemsa.

Experiments in vitro.—Extracts of intestinal mucosa and bone marrow were prepared by suspending the cells in sodium phosphate buffer (0.1 M, pH 7.5) and sonicating in a Branson sonifier at 8 amp. with two 30-second bursts; tubes containing cell suspensions were immersed in an ethanol: ice bath during sonication. The sonicates were centrifuged at 80,000 X g for 3 hours, and the supernatant fraction was collected. The protein content of the extracts was determined by the biuret method (15).

Nucleotide pyrophosphorylase activity was assayed by incubating the following mixture at 37°C for periods up to 1 hour: TG, 0.75 µmole; phosphoribosylpyrophosphate, 1.5 µmoles; magnesium chloride, 5.0 µmoles; phosphate buffer (pH 7.5), 90 µmoles; and enzyme extract, 0.1-0.4 ml. (0.78-3.12 mg. of protein); total volume, 0.85-1.15 ml. The reaction was terminated by the addition of 0.5 ml. of 1 M perchloric acid, precipitated protein was removed by centrifugation, and the neutralized supernatant fluid was chromatographed on Dowex-1 (formate-form) (19). The concentrations of TG and TGMP were measured spectrophotometrically at 345 mµ, and the results are expressed as µmoles of TGMP formed per mg. of protein.

Thioguanase activity was measured by incubating the following mixture at 37°C for 3 hours: TG-S36, 8.1 X 10⁻⁵ µmole; phosphate buffer (pH 6.2-7.6), 80-100 µmoles; and enzyme, 0.2 ml. (1.56 mg. of protein); total volume, 1.1-1.3 ml. An aliquot of each reaction mixture was chromatographed on Whatman No. 1 filter paper for 25-39 hours in a mixture (3:7) of 1 N ammonium acetate (pH 7.5) and 95 per cent ethanol. Known samples of TG-S36, 6-thioxanthine, and 6-thiouric acid were run simultaneously. Metabolites on the chromatograms were localized by measuring radioactivity with a Vanguard Autoscaner 880 and with a long-wave ultraviolet lamp (3660 A). Radioactive spots were eluted quantitatively from the chromatograms with 0.1 N NaOH. Total radioactivity present in each spot was determined by a Packard Tri-Carb liquid scintillation spectrometer. The conversion of guanine to uric acid was determined by a spectrophotometric assay (29).

RESULTS

Histopathologic changes produced by 6-thioguanine.—Bone marrow from the femur and tibia-fibula of a normal rabbit showed active erythropoiesis. A rabbit of the same age and weight was given 150 mg. of TG (41 mg/kg) every 6 hours for a total of three injections and killed 42 hours after the last injection; the femur and tibia-fibula were almost entirely depleted of nucleated erythroid cells and contained mainly mature polymorphonuclear leukocytes and lymphocytes. Differential counting of bone marrow from the control and treated animals (Table 1) showed that the percentage of nucleated erythroid cells present in the marrow decreased from 41.5 to 2.1 after the drug treatment. In contrast, the small intestine
was relatively resistant to TG; the architecture of the tissue remained intact, the epithelium of the villi showed no cytologic change, and neither inflammatory cellular infiltration nor hemorrhage was observed. Some intestinal lesions were produced, however, since, within the glandular crypts, hyaline bodies containing deeply basophilic inclusions were found in moderate numbers in the intestinal preparations from TG-treated animals (Fig. 1). It should be stressed that the figure selected was a region showing a maximum number of areas of nuclear atypia. Similar bodies have been reported in the small intestinal crypts of man after intravenous treatment with amethopterin (30).

The metabolism of 6-thioguanine in vivo.—Chart 1 shows the metabolic fate of TG in the intestinal mucosa and bone marrow of a rabbit given a single intraperitoneal injection of TG-S35 (49 mg/kg). The total quantity of TG and its metabolites in the intestinal mucosa was about twice the quantity present in an equivalent amount of bone marrow; in addition, the metabolic disposition of this drug was different in the two tissues. Thus, the quantity of unreacted TG and 6-thioguanosine was fivefold greater in intestinal mucosa than in bone marrow. The catabolic product, 6-thiouric acid, which was present in the intestine, was essentially absent from bone marrow. In contrast, the concentration of TG mononucleotide was twice as great in bone marrow, whereas the amount of radioactivity from TG associated with the combined nucleic acid fraction was the same in both tissues.

The metabolism of 6-thioguanine in vitro.—Since the smaller quantity of TGMP found in the intestinal mucosa could be the result either of increased catabolism of thioguanine or of decreased nucleotide pyrophosphorylase activity, the capacity of the two tissues to carry out these processes was compared in cell-free extracts. A representative experiment, which measured the TG nucleotide-forming ability of extracts from each tissue, is shown in Chart 2. TG nucleotide pyrophosphorylase activity of bone marrow extracts was fivefold greater than that of intestinal mucosa. The lesser activity of mucosal extracts presumably is not to be accounted for by the presence of an inhibitor, since a fourfold increase in the enzyme concentration resulted in a comparable increase in the quantity of TGMP formed.

Since 6-thiouric acid is probably not accumulated intracellularly but is excreted as it is synthesized, determination of the 6-thiouric acid content of the cells at any given time is not an accurate measure of the catabolic capacity of the tissue. For this reason, the deamination of TG to 6-thioxanthine and its subsequent oxidation to 6-thiouric acid by cell-free extracts of intestinal mucosa and bone marrow were measured. Incubation of TG-S35 with extracts of intestinal mucosa...
at pH 6.2 and 6.6, followed by chromatography on paper, yielded two radioactive peaks: one corresponded in location to TG (R_6 0.52) and the other to 6-thiouric acid (R_6 0.33). The 6-thiouric acid contained about 12 per cent of the radioactivity found on the strip (Table 2). In contrast, only TG appeared on the chromatograms in the assays that employed bone marrow extract; thus, under the conditions of this assay procedure, extracts of intestinal mucosa were capable of catabolizing TG to 6-thiouric acid at a rate of $0.07 \times 10^{-2}$ μmoles/hour/mg of protein, whereas

**DISCUSSION**

The present study shows that large doses of TG deplete rabbit bone marrow of nucleated erythroid precursors but cause relatively little damage to the mucosa of the small intestine. These results are in accord with the histological findings of Philips et al. (23) in other rodents and in dogs. The metabolic disposition of TG in bone marrow and intestinal mucosa, after its administration in vivo, suggested that resistance of intestinal mucosa might be accounted for by two processes: (a) a relatively low capacity for nucleotide formation and (b) an active catabolic mechanism. The quantity of radioactivity from TG-S^{35} associated with the nucleic acid fraction was similar in both tissues. This is in contrast to the findings of LePage (16) with other rodents, in which it was found that the bone marrow system incorporated twice as much TG into the nucleic acid fraction as did the whole intestine; this difference may reflect either a higher rate of incorporation of TG into intestinal mucosa, as compared with the entire intestinal structure, or the fact that the dosage of TG used in the present studies was four- to fivefold higher.

Measurement of the nucleotide-forming capacity and catabolic activity of cell-free extracts prepared from these tissues confirmed the results obtained in vivo, because nucleotide pyrophosphorylase activity was fivefold greater in bone marrow than in intestinal mucosa and 6-thiouric acid formation could be demonstrated only in extracts prepared from mucosa.

**TABLE 2**

<table>
<thead>
<tr>
<th>Tissue extract</th>
<th>pH</th>
<th>6-Thiouric acid (μmoles×10^{-9})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal mucosa</td>
<td>6.6</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>0.26</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>6.6</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>6.2</td>
<td>0.26</td>
</tr>
</tbody>
</table>

The following mixture was incubated at 37°C for 3 hours: thioguanine-S^{35}, $8.1 \times 10^{-2}$ μmoles ($37.0 \times 10^3$ counts/min/μmole); phosphate buffer (pH 7.5), 80 μmoles; enzyme protein, 1.56 mg; final volume, 1.1 ml.

Conversion of TG to 6-thiouric acid in extracts of intestinal mucosa proceeded at a rate only 4 per cent that of the conversion of guanine to uric acid. The extensive conversion in vivo of TG to 6-thiouric acid by intestine, which has been demonstrated here and by Moore and LePage (19), suggests that the enzyme may have been altered during preparation of the extracts in such manner that a lowered affinity for TG resulted, or the conditions in vitro were not optimal for the formation of 6-thiouric acid. It is of interest that 6-thiouric acid was formed by extracts of intestinal mucosa and that no 6-thioxanthine was detected. This reflects the marked activity of xanthine oxidase in this tissue (24, 31). In this regard, 6-thioxanthine is converted to 6-thiouric acid by purified milk xanthine oxidase at one-half the rate of the conversion of xanthine to uric acid (3). Bone marrow, on the other hand, formed neither 6-thioxanthine nor 6-thiouric acid from TG; these
Fig. 1.—Glandular crypt of the small intestine from a rabbit treated intraperitoneally with 41 mg. of 6-thioguanine/kg every 6 hours for a total of three injections and killed 42 hours after the last dose. Abnormal basophilic bodies are shown. Hematoxylin and eosin stain, ×1900. E = epithelial cell nucleus; U = lumen; ← = abnormal body.
results are in accord with the reported absence in rabbit bone marrow of xanthine oxidase activity (1). The inability to form 6-thioxanthine also would suggest that guanase activity is absent from this tissue.

Assuming that TG and guanine are metabolized by the same enzymes, it would appear that bone marrow cells have an effective guanine-conserving mechanism (i.e., a relatively active nucleotide-forming system and low catabolic activity). Since bone marrow appears to be unable to synthesize purine nucleotides de novo and must rely on a mechanism (i.e., a relatively active nucleotide-containing compounds to cells of the bone marrow. In this regard, Henderson and LePage (11) have shown that adenine is transported between tissues by red blood cells.

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