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Abstract

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BIOCHEMICAL AND MORPHOLOGICAL STUDY ON HEPATOTOXICITY OF AZATHIOPRINE IN RAT

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Abstract. Sprague-Dawley rats given azathioprine in the diet for 3 to 4 weeks developed severe liver damage. Elevations of serum alkaline phosphatase and γ-glutamyl transpeptidase activities were associated with increased hepatic glucose 6-phosphate dehydrogenase levels and decreased liver glucose 6-phosphatase activities, i. e., conditions which were commonly observed in various hepatotoxin-induced liver injuries. Light and electron microscopic observations revealed centrolobular necrosis with large scars and the proliferation of the mitochondria and rough endoplasmic reticulum. This model could be used to study the mechanisms of azathioprine-induced liver damage and its prevention.

Key words: azathioprine, liver injury, mechanisms of hepatotoxicity, phenobarbital, microsomal enzymes.

Azathioprine is used as an immunosuppressive agent in the treatment of chronic liver diseases, especially active chronic hepatitis (1), although it has been suggested to be a hepatotoxic agent (2). Whelan and Sherlock (3) have already reported that azathioprine does not improve liver functions in various chronic liver diseases and some cases cause damage. There have been many reports on hepatic lesions and changes in hepatic functions in dogs and rats given azathioprine (4–6). It was difficult, however, to understand from previous results what the biochemical mechanisms of azathioprine-induced liver damage are and how the liver participates the efficacy of this drug as an immunosuppressant, since little is known about the pharmacodynamics of azathioprine and the detection of its immunosuppressive activity. Azathioprine has to be metabolized to terminal active compounds before acting an antimetabolite. This metabolic transformation seems to be dependent on adequate hepatic functions (7). In patients with severe liver diseases, therefore, the metabolisms of azathioprine may be altered, resulting in decreased levels of circulating biologically active metabolites of the drug. It is not well known which particular metabolites of azathioprine are primarily essential for the pathogenesis of hepatic injury.

In order to prevent azathioprine-induced hepatic damages without decreasing immunosuppressive activity of this drug, the mechanisms of hepatotoxicity should be evaluated in detail from a study of its pharmacokinetics. This study is concerned with the biochemical and pathological development of hepatic injury during chronic administration of azathioprine to rats.

MATERIALS AND METHODS

Treatments of animals. Male Sprague-Dawley rats weighing 180 g were used in this study. Animals were fed powdered Laboratory Chow *ad libitum* with free access to water. Azathioprine was added to the diets as 0.1 or 0.05% unless otherwise indicated. In most of the experiment, rats were fed the "high" azathioprine oral dose for the first 9 days and thereafter the "low" for 12 to 26 days. A half dose of azathioprine was similarly given to a group of rats as described in table 1, in which the drug was added to the diet as 0.05 or 0.025%. The administration of phenobarbital to the experimental animals was by addition to drinking water at 0.025% for 7 days before starting oral doses of azathioprine diets and then continued until sacrifice.

Biochemical analyses of liver injury. In an attempt to determine the extent and type of liver damage induced by azathioprine the following biochemical analyses were performed. Serum glutamic pyruvic transaminase (GPT), glutamic oxalacetic transaminase (GOT), alkaline phosphatase (Al-Pase), cholinesterase, γ-glutamyl transpeptidase (γ-GTP) activities and bilirubin concentrations were determined by the routine laboratory methods (8). Liver triglyceride content and activities of glucose 6-phosphate dehydrogenase (G6PD) in liver supernatants and glucose 6-phosphatase (G6Pase) in homogenates were measured as described previously (8). Protein content was determined by the method of Lowry *et al.* (9). Preparation of liver microsomes and determination of microsomal drug-metabolizing enzymes such as aniline hydroxylase and cytochrome P-450 were performe as reported previously (10). All the results were expressed as mean ± standard error of the mean. Significant differences between the mean values were determined by the Student's t-test after analysis of variance.

Morphological observations of the liver. A piece of the left lobe of the liver was used for histological examinations after fixation in buffered 4% formalin. Paraffin sections were stained with hematoxylin-eosin and azan. Some sections of the liver were specially stained for glycogen, bilirubin and reticulum. For electron microscopic examinations, livers were carefully cut into 1-mm blocks and fixed in buffered 2.5% glutaraldehyde at 4°C for 2 h. The tissues were then postfixed in buffered 1% osmium tetraoxide for 2 h followed by dehydration with graded ethanol and embedded in Epon. Thin sections were cut with a glass knife on a Portor-Blum MT IIB Ultramicrotome and were examined with a JEM-7 electron microscope.

RESULTS

The body weights of azathioprine-treated animals decreased gradually to 85% of the initial weight during feeding of the "high" azathioprine diet and

thereafter remained almost constant, although body weights of control rats treated with phenobarbital alone continued to increase until sacrifice. No death occurred in either group during 4 week observation (Fig. 1). Serum GPT,

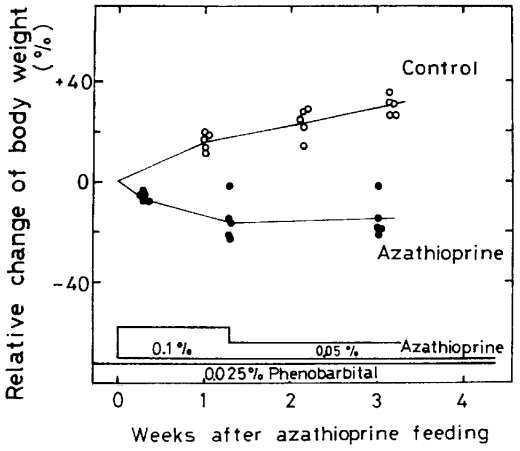

Fig. 1. Body weight of azathioprine-treated and control rats during the experiments. The relative changes of the body weight (%) were calculated from the initial body weight immediately before the administration of azathioprine in Laboratory Chow (●) or Laboratory Chow alone (○). The number of rats was 5 for both experiments. All rats were treated with phenobarbital one week before starting the feeding of azathioprine as described under Materials and Methods. The solid lines show the average values at the indicated times.

GOT and Al-Pase activities were determined serially during the experiment by collecting small quantities of blood from the tail vein. The enzyme activities seem to increase to varing degrees between the 2nd and 3rd week of the drug-feeding (Fig. 2). The macroscopic appearance of the liver in azathioprine-treated rats was characterized by enlargement with increased consistency and fine granular surface, while control rats had normal liver surface even in the 4th week of the feeding (Fig. 3).

The liver from rats sacrificed in the 4th week of the feeding revealed remarkable histological changes in that portions of centrolobular hepatic necrosis were replaced by large scars in which proliferating bile ductules were observed. The acidphilic body and usual arrangement of liver cells were also observed in the midzonal region of the liver lobules (Fig. 4). The liver cells in the periportal areas were relatively unchanged with no polymorphonuclear leucocytic infiltrations. Bleeding from near necrotic portions was observed in the 3rd week rather than in the 4th week of the experiment. No morphological changes were observed in the liver of control animals. Electron microscopic observations of the liver in the 4th week of feeding revealed the characteristic ultrastructural changes

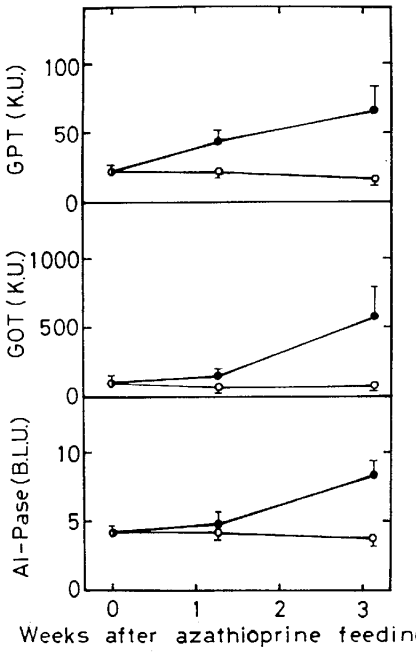

Fig. 2. Changes in serum GPT, GOT and Al-Pase activities during the experiments with and without azathioprine feeding. Small quantities of blood were collected from the teil vein at the indicated times. The vertical lines indicate the standard error of the mean. Other details were described in Fig. 1.

Fig. 3. Macroscopic appearance of the liver in an azathioprine-treated rat. A rat was treated with azathioprine for 3 weeks as described under Materials and Methods, percent liver weight being 4.7 at sacrifice. The biochemical parameters of this rat revealed serum GOT, 375 K. U.; GPT, 70 K. U.; γ-GTP, 4 mU.; Al-Pase, 7.0 B. L. U.; bilirubin, 0.44 mg/dl; liver triglyceride, 4.3 mg/g liver; hepatic G6Pase, 32 mU. /mg protein and liver G6PD, 70.3 mU. /mg protein.

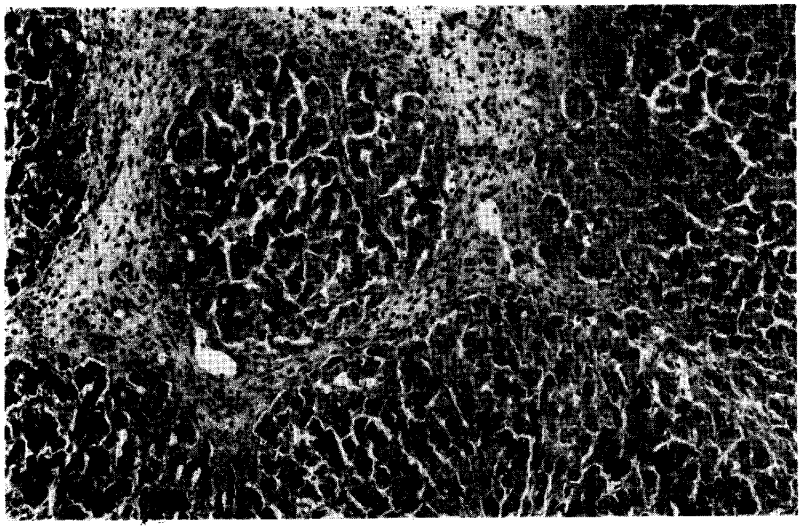

Fig. 4. Histological findings of the liver in an azathioprine-treated rat. A rat was fed with azathioprine for 4 weeks as described earlier. H & E stain, ×100. The biochemical data of this rat are shown below: Serum GPT, 35 K.U.; GOT, 131 K.U.; bilirubin, 0.14 mg/dl; γ-GTP, 9 mU.; Al-Pase, 7.7 B.L.U.; cholinesterase, 0.11 △pH U.; liver triglyceride, 4.1mg/g liver; liver G6PD, 74 mU./mg protein and hepatic G6Pase, 36 mU./mg protein.

(Fig. 5). The diffuse proliferation of the smooth endoplasmic reticulum, which would be expected following chronic administration of phenobarbital, was not observed in azathioprine-treated rats. In the peripheral and midzonal regions of the liver lobules, cells showing proliferation of the rough endoplasmic reticulum with the enlargement of its cisternae and marked proliferation of the mitochondria with atypia were detected. Large vacuoles of lipid and distruption of plasma membranes with release of the mitochondria were observed in the parenchymal cells remaining in the centrolobular areas. The control rat liver showed typical proliferation of the smooth endoplasmic reticulum but no other specific changes.

Total serum bilirubin concentration and GPT activities increased in the 3rd week of the azathioprine feeding. Elevation of serum Al-Pase and γ-GTP activities and decreased activities of cholinesterase were also observed at this period. In rats treated with a half dose of azathioprine, these parameters were only slightly abnormal or within normal ranges as compared with those treated with phenobarbital alone (Table 1). Slightly increased levels of hepatic triglyceride were observed upon the treatment with azathioprine and phenobarbital. Increased G6PD and decreased G6Pase activities in the liver, which were commonly observed in various hepatotoxin-treated animals (8), were also found in azathioprine-treated rats. Phenobarbital-treated control rats showed similar results but to a lesse extent. The difference in G6PD and G6Pase activities between these

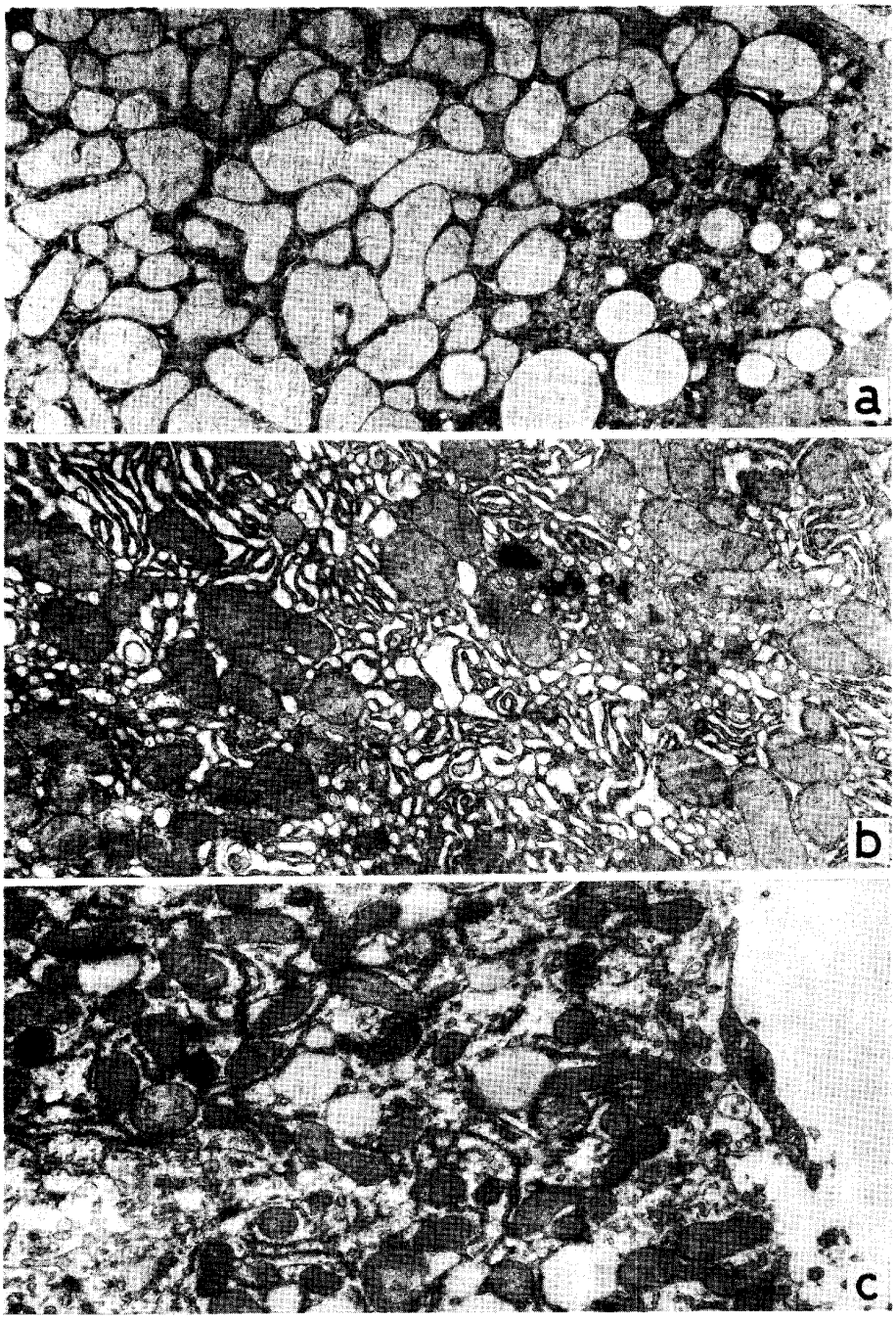

Fig. 5. Ultrastructural findings of liver cells in the peripheral (a), midzonal (b) and centrolobular areas (c) of the liver lobule. ×125,000. A rat was fed with azathioprine for 25 days as described in the text. The biochemical data of the rat examined are as follows: Serum GPT, 800 K. U.; GOT, 2020 K. U.; cholinesterase, 0.18 △ pH U.; Al-Pase, 2.5 B. L. U.; liver triglyceride, 9.2 mg/g liver; hepatic G6PD, 56 mU. /mg protien and liver G6Pase, 42.7 mU. /mg protein.

TABLE 1. EFFECT OF CHRONIC ADMINISTRATION OF AZATHIOPRINE ON BIOCHEMICAL PARAMETERS IN SERUM AND LIVER EXTRACT

Treatment (No. of rats)	Day of treatment	Total bilirubin (mg/dl)	GPT (K. U.)	Al-Pase (B. L. U.)	γ-GTP (mU.)	Cholinesterase (△pH U.)
None (3)	—	0.33±0.02	29±3	4.1±0.4	1.0±0.6	0.33±0.05
Phenobarbital (3)	26	0.36±0.02	17±2	3.0±0.5	0.7±0.3	0.28±0.06
Azathioprine + Phenobarbital (5)	22	0.64±0.15	65±19	8.3±0.9*	14.6±6.1	0.14±0.02*
Azathioprine[a] + Phenobarbital (3)	28	0.36±0.03	14±1	3.0±0.5	4.7±1.2	0.25±0.03

a Azathioprine was given at an half dose as described under Materials and Methods.
* $P<0.05$

TABLE 1 (Continued)

Treatment	Percent liver weight	Triglyceride (mg/g liver)	G6Pase (mU. /mg protein)	G6PD (mU. /mg protein)
None	3.1±0.2	2.8±0.5	84±3	29±3
Phenobarbital	3.6±0.3	6.3±1.3	53±5*	44±4*
Azathioprine + Phenobarbital	5.6±0.2*	9.0±2.7	32±3*	82±15*
Azathioprine[a] + Phenobarbital	4.0±0.2	5.3±1.1	47±2*	82±17*

two groups were found to be statistically significant. Increases in percent liver weight and decreased activities of G6Pase were observed even in rats treated with a half dose of azathioprine.

Microsomal enzyme activities were increased as expected in phenobarbital-treated control rats. Moreover, in azathioprine-treated rats no increase in aniline hydroxylase activities and cytochrome P-450 contents were found as judged from electron microscopic observations (Table 2).

TABLE 2. EFFECT OF CHRONIC ADMINISTRATION OF AZATHIOPRINE ON ENZYME ACTIVITIES IN LIVER MICROSOMES

Treatment (No. of rats)	Day of treatment	Aniline hydroxylase (mU. /mg protein)	Cytochrome P-450 (nmoles/mg protein)
None (3)	—	0.80±0.07	1.03±0.07
Phenobarbital (3)	26	1.26±0.10*	2.33±0.20*
Azathioprine + Phenobarbital (4)	26	0.92±0.18	1.20±0.30

* $P<0.05$

DISCUSSION

Azathioprine is now widely used in the treatment of pathological conditions that seem to have an autoimmune basis (11). This drug has been already reported to be less toxic and more effective than 6-mercaptopurine in preventing rejection of renal homografts in humans and laboratory animals (12). Azathioprine is non-enzymatically split *in vivo* and *in vitro* to 6-mercaptopurine by sulfhydryl compounds (13). Pre-treatment by the oral administration of cysteine or glutathione lowers the anti-tumor and anti-immune effects of azathioprine but not those of 6-mercaptopurine (14). This may be due to more rapid conversion of azathioprine into 6-mercaptopurine before azathioprine penetrates the immunologically active cells, azathioprine being more effective than 6-mercaptopurine in passing the permeability barrier of the target cells (12). However, the exact reasons for the chemotherapeutic advantages of azathioprine over 6-mercaptopurine are still not known, since the biological action of azathioprine is not simply due to its prior conversion into 6-mercaptopurine (12).

The liver contains enzymatic systems involved in the anabolic pathway such as hypoxanthine-guanine phosphoribosyl transferase as well as the catabolic pathway including xanthine oxidase (15). The anabolic pathway is important, since the particular metabolites are considered to be primarily responsible for the action of immunosuppressive drugs. 6-Thioinosine monophosphate, 6-thioguanine monophosphate and 6-methylthioinosine monophosphate can inhibit several enzymes in the purine biosynthetic pathway (15). The levels of biologically active metabolites of azathioprine such as 6-thioinosine 5′-monophosphate, 6-thioguanine 5′-monophosphate and 6-methyl 6-thioinosine monophosphate in the circulating blood and liver following the oral administration have not been accurately measured. The relationship between the metabolite levels of these compounds in blood and the effectiveness of azathioprine is now being studied using high pressure liquid chromatography in our laboratory.

Among the side effects of azathioprine treatment, liver cell necrosis (16), impaired liver cell regeneration (17) and increased fibrosis in the liver (6) have been observed already in experimental animals such as dogs and rats. However, Ranek *et al.* (14) have reported that decreased fibrosis and enhanced regenerative activity in CCl_4-induced cirrhosis were obtained by feeding a lower dose of azathioprine (6 mg/kg body weight for 6 months). These data support the clinical use of azathioprine in chronic active liver diseases. The doses used in the present study and the previous experiments (6, 16, 17) have been higher as compared with the doses used in Ranek's experiment (14). The administration of azathioprine simultaneously with administration of phenobarbital was tolerated by rats at least for 4 weeks in the present experiment and severe hepatic damages

could be successfully prepared in short time after starting azathioprine feeding. Similar hepatic injuries occurred upon the administration of azathioprine alone without giving phenobarbital (unpublished observations). Although the exact relationship between metabolism of 6-mercaptopurine and hepatic microsomal enzymes was not certain at the start of this experiment, we thought that more remarkable azathioprine-induced hepatic injury could be obtained by simultaneous giving phenobarbital as described in CCl_4 and other hepatotoxin treatments (18). However, Yoshimura (19) has recently reported that 6-mercaptopurine can be desulfurated *in vitro* into a biologically inactive substance, hypoxanthine, by hepatic microsomal enzymes from rats. Furthermore, mice treated with phenobarbital were resistent to the lethal effect of 6-mercaptopurine, because of the stimulation of catabolism of this drug (20). These observations are consistent with our findings reported in this communication.

It is very important to know which metabolites of azathioprine formed in the liver are responsible for the pathogenesis of hepatic injury and what biochemical mechanisms are. Liver damage produced by the administration of azathioprine, 6-mercaptopurine and thioinosine riboside with and without an inhibitor of xanthine oxidase, 4-hydroxy-pyrazolo (3, 4)- pyrimidine, are now being compared biochemically.

Characteristic features of the liver in azathioprine-treated rats were severe centrolobular necrosis of liver cells in the early stage of the 4th week of the feeding, and necrotic areas were thereafter replaced by scars. The bleeding which was observed in the 3rd week disappeared in the 4th week.

But the mechanisms of bleeding in the hepatic lobule and its relationship to necrosis of liver cells are not well known. Six Mongrel dogs were fed with azathioprine at a dose of 2–4 mg/kg body weight for 40 days ; in all six dogs the centrizonal area was most affected with either necrosis or pallor of the hepatocyte without mononuclear cell infiltration (21). In rat experiments with 0.02% azathioprine diet for 12 weeks, 2 out of 11 rats had the disturbed architecture of hepatic cords and 2 mild necrosis of parenchymal cells adjacent to central veins (6). The proliferation of the mitochondria observed by electron microscopy may be due to thiouric acid-related metabolites formed by xanthine oxidase.

Simultaneous administrations of phenobarbital with azathioprine did not produce the proliferation of the smooth endoplasmic reticulum as well as the increase of drug-metabolizing enzyme activities, although treatments with azathioprine alone significantly reduced drug-metabolizing enzyme activities in liver microsomes (unpublished data).

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