METABOLIC ALTERATIONS OF LIVER REGENERATION. XI.*

MODULATION OF THE UPTAKE OF OROTIC ACID INTO RIBONUCLEIC ACIDS IN REGENERATING RAT LIVER

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During the course of liver regeneration the utilization of orotic acid for the synthesis of ribonucleic acids in the liver increases. The enhanced synthesis of RNA during early stages of liver regeneration is not associated with a concomitant increase of hepatic orotate phosphoribosyltransferase and orotidine 5'-phosphate decarboxylase. The administration of 5-azacytidine at different time intervals before and after partial hepatectomy results in the enhanced or depressed uptake of orotic acid into liver RNA. Whereas the short-term effect of the drug blocks RNA synthesis, the incorporation of orotic acid was extensively increased when 5-azacytidine was given prior to partial hepatectomy. In fasting animals the incorporation of orotic acid into liver RNA was enhanced similarly. The effect of 5-azacytidine on the gastrointestinal tract and its ability to mimic fasting are discussed.

When the liver is induced to regenerate by the stimulus given by partial hepatectomy the cells are diverted to a new role and this transition begins immediately after the hepatectomy[1,2]. The initial phases of liver regeneration are characterized by the enhanced synthesis of ribonucleic acids[1,3,4] and the first observable response to partial hepatectomy is the production of rapidly labelled high-molecular-weight RNA in liver nuclei of sequences not produced by normal liver[5].

Recently we have found in intact rats that immediately following the administration of 5-azacytidine the decreased utilization of orotic acid for the labelling of liver RNA occurs; at later stages, however, a considerable enhancement of the uptake of orotic acid into RNA has been observed [6]. The present work was aimed to enhance by 5-azacytidine, a potent anticancer agent[7-9], the labelling of RNA in e. rly stages of liver regeneration.

MATERIAL AND METHODS

Chemicals. 5-Azacytidine and 5-azaorotate (diammonium salt) were prepared in this Institute. Orotidine 5'-phosphate, adenosine 5'-triphosphate and 5-phosphoribosyl-1-pyrophosphate were delivered by Calbiochem (Luzern, Switzerland). Orotic-[2-^{14}C] acid (48 μCi/μmol) and thymi-

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dine-[2-^{14}C] (44 μCi/μmol) were obtained from the Institute for Research, Production and Uses of Radioisotopes (Prague, Czechoslovakia).

Treatment of animals. Groups of 4—6 female Wistar rats (165—180 g) kept under standard conditions were used for the experiments. Partial hepatectomy (66%) was performed under light ether narcosis[10]. Drugs were administered *i.p.* in a maximal volume of 0·3 ml and the experiments were started between 8—10 A.M. The animals were killed by cervical dislocation during different phases of liver regeneration, usually 1 or 3 hours after the administration of the label.

Metabolic conversion of orotic acid in vitro. Cell-free extracts were prepared from livers removed immediately after killing and homogenized under cooling in a glass homogenizer with a tight-fitting Teflon pestle with 3 volumes of 25 mM Tris-HCl buffer (pH 7·5) with 5 mM Mg^{2+}-ions and 25 mM-KCl. Homogenates were centrifuged (10 000 *g*, 20 min, 2°C) and defatted supernatant fractions were used as a source of enzyme activity. Incubation was carried out for 5 min at 37°C in a reaction mixture (0·5 ml) containing 0·1 mM orotic-[2-^{14}C] acid, 40 mM Tris-HCl buffer (pH 7·5), 0·4 mM 5-phosphoribosyl-1-pyrophosphate and equimolar Mg^{2+}-ions with 0·1 ml of enzyme fraction corresponding to 25 mg of the liver.[6] Orotidine 5'-phosphate decarboxylase was measured under similar conditions using 0·1 mM orotidine-[2-^{14}C] 5'-phosphate. Analysis of aliquots of the incubation mixture was performed chromatographically on Whatman No 1 paper in a solvent system composed of isobutyric acid–ammonium hydroxide–water (66 : 1·5 : 33) and after drying in a solvent system composed of isopropyl alcohol–ammonium hydroxide–water (7 : 1 : 2) with appropriate standards.

Utilization of orotic acid for hepatic RNA synthesis in vivo. Orotic-[2-^{14}C] acid (usually 0·5 μCi/0·2 μmol per animal) was administered *i.p.* to groups of 4—6 rats 1 or 2 h before killing. The removed liver was homogenized and repeatedly extracted under cooling with 0·2M-$HClO_4$. Isolation of spectroscopically pure uridine and cytidine 2'(3')-phosphates following an alkaline hydrolysis of RNA (1M-KOH, 18 h, 20°C) has been carried out chromatographically as described[6]. The radioactivity of samples was assayed in a Packard liquid scintillation spectrometer and the UV absorbance was measured using Unicam SP 700 spectrophotometer. The rate of RNA synthesis was expressed as the specific radioactivity of isolated nucleotide in dpm/μmol.

RESULTS

Modulation of the Uptake of Orotic Acid into RNA *in Regenerating Rat Liver*

Previously we studied[11] the effect of different inhibitors and specially of 5-azacytidine on the incorporation of orotic and 5-fluoroorotic acid into rat liver RNA. The short-term effect of 5-azacytidine led to the inhibition of the synthesis of liver RNA's; at longer time intervals following the administration the labelling of liver RNA's by orotate-[6-^{14}C] was considerably enhanced while the incorporation of 5-fluoro-orotic-[2-^{14}C] acid remained unchanged.

Table I indicates that analogous changes in the utilization of orotic acid following the administration of 5-azacytidine occur also in regenerating liver although the enhancement is less remarkable than in intact animals. Furthermore it has been observed that the dose of the analogue required for the maximum increase of the utilization of orotic acid is different in intact (10—12 μmol/100 g) and in partially hepa-

TABLE I

Incorporation of Orotic Acid into RNA in Intact and Regenerating Rat Liver Following 5-Azacytidine Treatment

Groups of 4—6 female rats (174—180 g) were given *i.p.* 5-azacytidine (8 μmol/100 g) or 0·9% NaCl as indicated, and 2 hours before killing orotic-[2-^{14}C] acid (0·5 μCi/0·2 μmol per rat) was injected. Specific radioactivity of isolated nucleotides from the liver RNA of animals without 5-azacytidine = 100%.

5-Azacytidine treatment h	Incorporation, dpm/μmol ± S.E.			
	uridine 2′(3′)-phosphate	%	cytidine 2′(3′)-phosphate	%
Intact				
Control	2 450 ± 173	100	378 ± 17	100
2	670 ± 57	27·5	91 ± 8	24·1
24	7 625 ± 805	311	1 187 ± 86	314
Regenerating				
Control	9 608 ± 710	100	1 524 ± 131	100
2	2 757 ± 275	28·7	489 ± 45	32·1
24	16 341 ± 1 740	170	2 668 ± 212	175

TABLE II

Enhanced Utilization of Orotic Acid for Liver RNA Synthesis Following 5-Azacytidine Administration or Starvation of Animals

Groups of 3 female intact and partially hepatectomized rats (160 g) were given *i.p.* 5-azacytidine (10 μmol/100 g) immediately after partial hepatectomy (24 h before killing) or were starved for 24 h with water *ad libitum*. The animals were killed 2 h after the labelling with orotic-[2-^{14}C] acid (1 μCi/0·2 μmol per animal), and the rate of RNA synthesis is expressed as specific radioactivity of isolated uridine 2′(3′)-phosphate in dpm/μmol.

Animals	Intact		Partially hepatectomized	
	stomach weight g ± S.E.	RNA synthesis dpm/μmol ± S.E.	stomach weight g ± S.E.	RNA synthesis dpm/μmol ± S.E.
Control	3·45 ± 0·42	3 320 ± 228	2·46 ± 0·29	14 622 ± 1 527
5-Azacytidine-treated	8·67 ± 1·58	14 865 ± 1 035	4·85 ± 0·75	21 815 ± 1 760
Starved	1·65 ± 0·20	12 660 ± 1 427	1·62 ± 0·15	24 060 ± 762

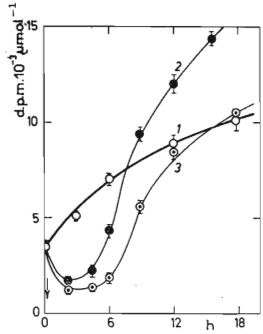

FIG. 1

Uptake of Orotic Acid for RNA Synthesis in Regenerating Liver of Rats Treated with 5-Azacytidine (10 μmol/100 g) and 5-Azaorotate (10 μmol/100 g)

The drugs were injected *i.p.* immediately after partial hepatectomy to groups of 5—6 female rats (175 g) and the animals were killed at different time intervals thereafter. Orotic-[2-^{14}C] acid (0·5 μCi/0·2 μmol per animal) was given to the animals 2 h before killing. *1* Control, *2* 5-azacytidine, *3* 5-azaorotate. h, Time of regeneration. Incorporation is given in d.p.m. . 10^{-3} /μmol.

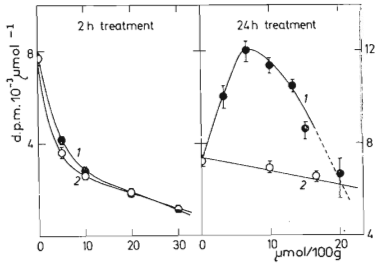

FIG. 2

Different Effect of 5-Azacytidine and 5-Azaorotate on Orotic Acid Utilization for RNA Synthesis during Short and Long-Term Treatment

Compounds were administered *i.p.* to groups of 5—6 rats (170 g) at different dose levels 2 and 24 h before killing, respectively. Orotic-[2-^{14}C] acid (0·5 μCi/0·2 μmol per animal) was given to the animals *i.p.* 2 h before killing. RNA synthesis is expressed as specific radioactivity of isolated uridine 2′(3′)-phosphate. *1* 5-Azacytidine, *2* 5-azaorotate. Incorporation is given in d.p.m. . . 10^{-3}/μmol; dose level of drugs in μmol/100 g.

tectomized (5–6 μmol/100 g) animals. In both instances a considerable fatty degeneration of liver is observed and the body weight of the animals decreases. In adrenalectomized animals the inhibition or the stimulation of the uptake of orotic acid following 5-azacytidine is hormone- independent and is similar to that of control sham-operated animals (unpublished).

The increased utilization of orotic acid for the synthesis of RNA during liver regeneration is given in Fig. 1. A 2 hour pulse of orotic-[2-^{14}C] acid led to the labelling of nuclear and cytoplasmic liver RNA's which have been subjected to further analysis as a total liver RNA. The administration of 5-azacytidine which inhibits after corresponding metabolic transformation orotidine 5'-phosphate decarboxylase[12] results initially in the block of RNA synthesis *de novo*; at later stages the enhanced uptake of orotic acid into RNA is observed. 5-Azaorotate is equally inhibitory to orotidine 5'-phosphate decarboxylase[13,14] but its administration under identical conditions does not lead to the increased incorporation of orotic acid at later stages.

The different effect of both analogues is clearly shown in Fig. 2. Inhibition and stimulation of the incorporation of orotic acid depends on the dosage of the drugs.

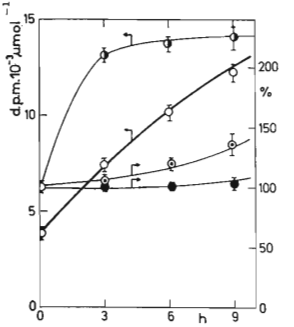

FIG. 3

RNA Synthesis and the Activity of Orotate Phosphoribosyltransferase (●) and Orotidine 5'-Phosphate Decarboxylase (⊙) in Regenerating Rat Liver

RNA synthesis is expressed as specific radioactivity of isolated uridine 2'(3')-phosphate from the total liver RNA after 2 h of labelling with orotic-[2-^{14}C] acid (0·5 μCi/0·2 μmol per animal). O, Control regenerating liver; ◑, after 20 h pre-treatment of animals (4–5 in each group) with 5-azacytidine (10 μmol/100 g). The activity of enzymes in cell-free liver extracts was assayed as described and is expressed as per cent of sham-operated controls (100%). h, Time of regeneration; incorporation is given in d.p.m. . 10^{-3}/μmol.

Whereas the inhibition at 2 hours following their administration is similar in both cases, no stimulation occurs at 24 hours following 5-azaorotate. The enhanced utilization of orotic acid that is observed after its initial inhibition is thus not due to the changed level of intracellular metabolites which occurs at that time.

Increased Incorporation of Orotic Acid into Hepatic RNA *without Simultaneous Enhancement of Orotate Phosphoribosyltransferase and Orotidine 5'-phosphate Decarboxylase*

The activity of different enzymes is often higher than required in a given situation. Evidently such is the case of hepatic orotate phosphoribosyltransferase and orotidine 5'-phosphate decarboxylase. Remarkable changes in RNA synthesis during early stages of liver regeneration are not associated with the corresponding enhancement of the activity of both enzymes; the increased activity appears at late phases of regeneration[15] when the majority of newly formed RNA has already been synthesized[16]. The enhanced uptake of orotic acid following 5-azacytidine is not accompanied by an increased activity of the enzymes (Fig. 3) responsible for its metabolic trans-

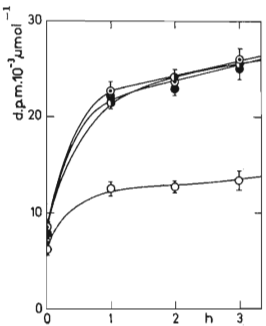

FIG. 4

Similar Enhancement of Orotic Acid Uptake into RNA during Early Stages of Liver Regeneration in Starved and 5-Azacytidine Pretreated Animals

Groups of 4—5 female rats (170—175 g) were given *i.p.* 5-azacytidine (●; 10 μmol/100 g), starved (○), and finally pretreated with 5-azacytidine and starved (◑). 22 hours later the animals-including controls receiving food *ad libitum* (○) were subjected to partial hepatectomy and 1 hour before killing orotic-[2-^{14}C] acid (1 μCi/0·2 μmol per animal) was administered. h, Time of regeneration; incorporation is given in d.p.m. . 10^{-3}/ μmol.

formation. It seems that both enzymes do not play an essential role in the process of liver regeneration, and that their enhancement at later stages is merely a sign of the increased metabolic activity in regenerating liver.

Simulation of Starvation in 5-Azacytidine-Treated Rats as Revealed by the Enhanced Utilization of Orotic Acid for Hepatic RNA *Synthesis*

In analogy to the late effect of 5-azacytidine the enhanced incorporation of orotic acid into RNA of intact and regenerating rat liver has been observed also in 24 h fasting rats. The increased stomach weight and the lowered body weight indicate the impairment of gastrointestinal function in rats following the administration of 5-azacytidine (Table II). The enhanced incorporation of orotic-[2-^{14}C] acid during initial stages of liver regeneration following 5-azacytidine administration is similar to the enhancement observed in fasting rats (Fig. 4). In all cases the uptake of orotic acid was increased to the same extent.

DISCUSSION

Our data seem to indicate that the poor transport from the stomach following 5-azacytidine results in the increased utilization of injected orotic acid for the synthesis of liver RNA. Since RNA molecules formed during the early stages of regeneration are of vital importance for the further course of liver regeneration[5] the increased utilization of orotic acid may be used also for the experimental purpose. Discrepancy between the uptake of orotic acid which is increased and of 5-fluoroorotic acid which remains unchanged following 5-azacytidine[11] indicates that in case of starvation simulated by the administration of 5-azacytidine the diminution of free nucleotide pool occurs. The enhanced synthesis of desoxyribonucleic acids in the liver of 5-azacytidine-treated rats subjected to partial hepatectomy is based on a different mechanism[17].

The increased labelling of RNA in fasting animals has been described previously[18,19]. During the course of starvation progressive reduction in the total RNA content of the liver takes place and a rapid preferential loss of free ribosomes occurs during first 24 hours of fasting. Re-feeding of fasted rats causes rapid increase in the RNA content of the liver and the enhancement of RNA polymerase activity[18]. The maximum of RNA synthesis is characterized by the daily rhythm and was reported to occur at 11 P.M. The periodic increase in template activity of mouse liver chromatin was observed but the extent of the nocturnal rise in its activity was not affected by starvation of animals[20]. Oscillations in the metabolism of orotic acid in rats adapted to a controlled feeding schedule were studied by Whittle and Potter[21] who found that the conversion of orotic acid to uridine 5'-monophosphate did not vary with the nutritional state of the animals throughout the cycle. However, the

further metabolism of uridine 5'-monophosphate and its incorporation into liver RNA did show cyclic variations, corresponding with the cyclic variation of the total liver RNA content.

The uptake of orotic acid into liver RNA may be controlled by the administration of 5-azacytidine during different phases preceding partial hepatectomy (Fig. 1). The changes in the enhancement of the enzymes responsible for the conversion of orotic acid to uridine 5'-monophosphate (Fig. 3) have been excluded. On the other hand, the role played by the interference of 5-azacytidine with the changes in the synthesis of liver RNA on account of the daily rhythm has not been elucidated. Equally the effect of 5-azacytidine on the transport and absorption from the gastrointestinal tract will require further investigation.

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