Effect of Chlortetracycline on Regenerating Liver

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SUMMARY

Chlortetracycline, administered intravenously, inhibited the increase in thymidine kinase activity in 24-hr regenerating rat liver. The effect was dose-related and was accompanied by depression of DNA synthesis and thymidylate synthetase activity. The sensitive period for administration of the drug was restricted to the first 12-hr after operation; at 30-hr the enzyme activities escaped inhibition. Nuclear RNA synthesis was not inhibited, nor was ^{14}C-leucine incorporation into acid-precipitable protein when measured at several intervals during this period.

INTRODUCTION

The hepatotoxicity of tetracyclines, which is usually manifested in clinical situations as abnormal lipid metabolism (1) and negative nitrogen balance (2, 3), led us to examine the effect of chlortetracycline on some aspects of nucleic acid metabolism in regenerating rat liver, It was felt that the regenerating tissue, with the characteristics of accelerated growth, might amplify the mammalian toxicity of tetracyclines and permit further definition of the biochemical abnormalities produced by a drug of this type. The studies reported here show that chlortetracycline interferes with the changes in DNA metabolism of regenerating rat liver and arrests the increase of thymidine kinase activity in this tissue.

MATERIALS AND METHODS

Animals and materials. Male Wistar rats were obtained from Simonsen Laboratories and weighed 120–160 g when studied. They were fed Purina laboratory chow and given water ad libitum. Chlortetracycline hydrochloride was supplied by Lederle Laboratories. Tritiated and nonradioactive nucleosides and nucleotides were obtained from Schwarz BioResearch. Phosphoenolpyruvic acid, tricyclohexylamine salt, and crystalline rabbit muscle pyruvate kinase were purchased from Sigma Chemical Company; ^{14}C-leucine was obtained from Calbiochem.

Experimental conditions. Partial hepatectomy was performed under ether anesthesia by the procedure of Higgins and Anderson (4). Chlortetracycline hydrochloride was dissolved in 6% glucose in water or in male Wistar rat serum (pH 7.0) and was administered slowly by the tail vein. Control animals received the suspending medium with no drug. All radioactive compounds were administered by the tail vein. The animals were fasted after surgery, but were given water ad libitum.

Methods. At various times after hepatectomy, the rats were killed by decapitation and bled thoroughly, and the livers were quickly removed and chilled. For enzyme assays, a portion of the right lateral lobe was disrupted in 0.25 M sucrose, 0.012 M Tris-Cl (pH 8.0), and 0.006 M potassium chloride in a Teflon-glass homogenizer. The liver samples were homogenized in 2 or 4

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times their wet weight of medium and then centrifuged at 35,000 × *g* for 45 min. Aliquots of the supernatant fraction were used for enzyme assay and did not vary more than ±2.5% in protein content. For studies on nuclei, the remainder of the liver was disrupted in 0.25 M sucrose–2% citric acid in a Dounce homogenizer. The homogenate was passed through two layers of gauze and centrifuged at 1000 × *g* for 10 min. The pellet was suspended in 10 ml of 2.2 M sucrose and centrifuged for 45 min at 80,000 × *g*. The supernatant fluid was removed, and the pellet was suspended in 7% perchloric acid and centrifuged. This pellet was then dissolved in 1.0 N potassium hydroxide and left overnight at room temperature to hydrolyze the RNA. The solution was then cooled to 0°, and the DNA and protein were precipitated with 7% perchloric acid. The precipitate was collected, washed with cold 5% trichloracetic acid, and extracted for 1 hr in boiling 10% sodium chloride, pH 7.5–8.0 (phenol red), buffered with sodium bicarbonate. The precipitate was discarded, and to the supernatant was added 4 times its volume of 95% ethanol. This mixture was chilled for 10–20 hr at −20°. The resulting precipitate was centrifuged, washed with cold 5% trichloracetic acid, and extracted with boiling 5% trichloracetic acid for 1 hr. DNA determinations were made on the resulting supernatant fraction, and aliquots were taken for measurement of radioactivity.

Thymidine kinase was assayed by the method of Weissman, Smellie, and Paul (5). Each assay tube contained ATP, 1.0 μmole; Tris-chloride, pH 8.0, 10.0 μmoles; phosphoenolpyruvate, 0.5 μmole; magnesium chloride, 1.0 μmole; potassium chloride, 1.0 μmole; thymidine, 0.005 μmole; pyruvate kinase (10 mg/ml), 0.5 μl; tritiated thymidine, 0.25 μCi; and enzyme in a final volume of 0.1 ml. The assay mixture was incubated for 20 min at 37°, and the reaction was stopped by boiling for 2.5 min. Appropriate aliquots were passed over Ecteola cellulose and eluted first with water and then with 0.1 N hydrochloric acid. Aliquots of the eluates were counted to determine remaining thymidine and its phosphorylation products. Thymidylate synthetase was assayed by the method of Roberts (6). The radioactivity of samples dissolved in Bray's solution (7) was measured in a Nuclear-Chicago scintillation spectrometer, model 720. Protein was determined by the biuret method as modified by Gornall *et al.* (8). DNA was measured by the diphenylamine method of Dische (9).

RESULTS

Partial hepatectomy causes pronounced metabolic changes to occur sequentially in the remaining liver. Twenty-four hours

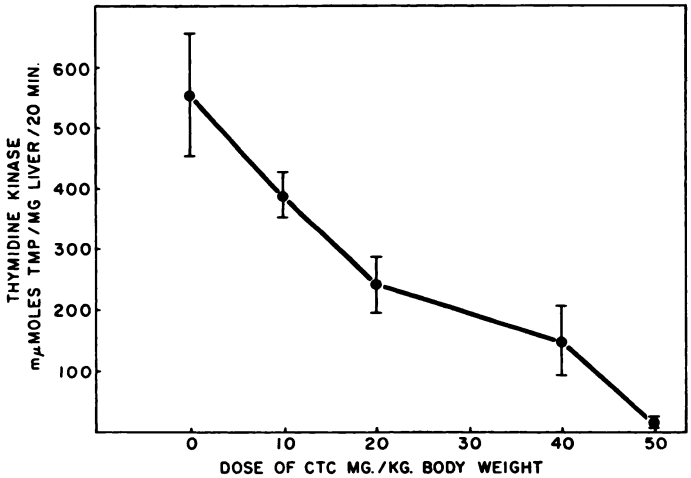

FIG. 1. *Response of thymidine kinase activity in regenerating rat liver to varying doses of chlortetracycline (CTC)*

TABLE 1

Effect of chlortetracycline on protein synthesis and thymidine kinase activity in regenerating liver

Animals were given 6% glucose in water with or without chlortetracycline, 50 mg/kg of body weight intravenously, immediately after partial hepatectomy. Twenty minutes prior to being killed, they were given ^{14}C-leucine, 10 μCi/kg intravenously. ^{14}C-Leucine incorporation into acid-precipitable material was determined on the 35,000 × *g* supernatant fraction of the liver homogenate. No thymidine kinase activity was found in the supernatant fractions from livers which were regenerating for 12 or 16 hr. The enzyme activity in the 24-hr regenerating livers is shown below.

Time of death	Chlortetracycline	^{14}C-Leucine incorporation into protein			Thymidine kinase		
		Rate	Inhibition	*p*	Activity	Inhibition	*p*
hr		*dpm/mg*	%		*mμmoles/mg liver/20 min*	%	
12	−	5390 ± 242[a] (3)[b]					
	+	4139 ± 688 (4)	23	NS[c]			
16	−	5883 ± 334 (4)					
	+	5414 ± 616 (3)	8	NS			
24	−	7709 ± 410 (4)			203 ± 62		
	+	7729 ± 499 (5)	<1	NS	33 ± 9	84	0.02

[a] Standard error of the mean.

[b] Number of animals is given in parentheses.

[c] Not significant; significance was determined by a two-tailed *t*-test.

after removal of two-thirds of the liver, the remaining tissue has markedly elevated levels of many of the enzymes of nucleic metabolism (10), including thymidine kinase. Table 1 shows that the rise in activity of this enzyme at 24 hr after partial hepatectomy was greatly inhibited by a single dose of chlortetracycline, 50 mg/kg, given immediately after surgery. Table 1 also shows that this dose of drug only slightly lowered general hepatic protein synthesis as measured by ^{14}C-leucine incorporation into protein at different times after partial hepatectomy. This depression in protein synthesis was not statistically significant. Figure 1 shows the relation of dose of chlortetracycline to inhibition of thymidine kinase. A single intravenous dose of 50 mg of antibiotic per kilogram immediately after partial hepatectomy completely inhibited the activity of this enzyme when assayed in regenerating liver 24 hr later.

To determine whether nucleic acid synthesis *in vivo* was affected by chlortetracycline, the incorporation of ^{3}H-thymidine

TABLE 2

Effect of chlortetracycline on ^{3}H-thymidine incorporation into DNA in regenerating liver

Animals were given chlortetracycline (50 mg/kg) in rat serum intravenously immediately after partial hepatectomy. Twenty-two hours later, all animals were given ^{3}H-thymidine (10 μCi/kg) intravenously. The rats were killed at 24 hr. DNA and ^{3}H-thymidine incorporation were measured in nuclei, and thymidine kinase was assayed in the 35,000 × *g* supernatant fraction. Each group contained six animals.

Chlortetracycline	Thymidine kinase			^{3}H-Thymidine incorporation into DNA		
	Activity	Inhibition	*p*	Rate	Inhibition	*p*
	mμmoles/mg liver/20 min	%		*dpm/mg*	%	
−	293 ± 62[a]			8850 ± 1715		
+	1 ± 5	99	<0.0005	713 ± 218	91.3	<0.0005

[a] Standard error of the mean.

TABLE 3

Effect of chlortetracycline on ^{3}H-deoxycytidine incorporation into regenerating rat liver and on pyrimidine-metabolizing enzymes

Animals were given chlortetracycline (50 mg/kg) in rat serum intravenously, immediately after partial hepatectomy. Twenty-two hours later, all animals were given ^{3}H-deoxycytidine (40 μCi/kg) intravenously. The rats were killed at 24 hr. DNA and ^{3}H-deoxycytidine incorporation were measured in nuclei. Thymidine kinase and thymidylate synthetase were assayed in the 35,000 × *g* supernatant fraction. Each group contained five animals.

	Thymidine kinase			Thymidylate synthetase			^{3}H-Cytidine incorporation into DNA		
Chlortetracycline	Activity	Inhibition	*p*	Activity	Inhibition	*p*	Rate	Inhibition	*p*
	mμmoles/mg liver/20 min	%		*mμmoles/mg liver/hr*	%		*dpm/mg*	%	
−	274 ± 12[a]			921 ± 29			8210 ± 603		
+	19 ± 6	93.2	<0.0005	317 ± 68	65.6	<0.0005	1440 ± 230	82.5	<0.0005

[a] Standard error of the mean.

into DNA was determined. Chlortetracycline inhibited the incorporation of thymidine into DNA, as shown in Table 2, in a sequence of reactions which require thymidine kinase to form dTMP. Since this enzyme was found to be depressed by chlortetracycline, ^{3}H-deoxycytidine, whose incorporation into DNA is independent of thymidine kinase, was also used as a precursor. Table 3 shows that the formation of DNA from deoxycytidine was depressed over 80% by a single dose of chlortetracycline given 24 hr before the animals were killed.

The activity of thymidylate synthetase rises markedly after partial hepatectomy (11). Table 3 shows that the 24-hr postoperative level of this enzyme was also depressed by chlortetracycline, but that its inhibition was significantly less pronounced than the inhibition of thymidine kinase.

The relationship of time of administration of chlortetracycline to the extent of inhibition of thymidine kinase in 24-hr regenerating liver is shown in Fig. 2. Administration of the drug within the first 12 hr after hepatectomy prevented the appearance of the enzyme. If the drug was given 16 hr after hepatectomy, or later, the enzyme was fully active in the 24-hr regenerating liver.

The inhibition of thymidine kinase by chlortetracycline was not permanent, and by 27–30 hr after surgery in treated animals, the enzyme was elevated to the control level found in the 24-hr regenerating liver. This delayed rise in thymidine kinase activity occurred even if the antibiotic was given 9 hr after partial hepatectomy, a time of injection which completely inhibited the 24-hr rise in thymidine kinase.

Table 4 shows that administration of chlortetracycline also delayed the increase

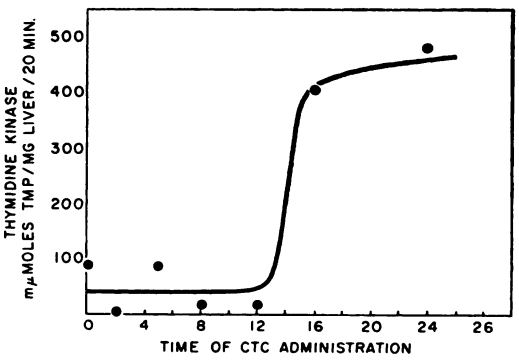

FIG. 2. *Susceptibility at different times of hepatic regeneration to inhibition by chlortetracycline (CTC) of thymidine kinase*

Animals received chlortetracycline (50 mg/kg in 6% glucose in water intravenously) at the times indicated after partial hepatectomy. They were all killed 24 hr after surgery, and their livers were assayed for thymidine kinase. Three to five livers were assayed for each point.

TABLE 4

Escape from inhibition by chlortetracycline of thymidine kinase and DNA synthesis

All animals underwent partial hepatectomy at zero time. At varying times thereafter, they received chlortetracycline (50 mg/kg in 6% glucose in water, intravenously). Controls received 6% glucose in water. Two hours before being killed, the animals in Experiment 2 received ^{3}H-deoxycytidine, 40 μCi/kg in 0.9% NaCl, intravenously. The animals were killed at the times indicated, and the remaining livers were assayed for ^{3}H-deoxycytidine incorporation into DNA and for thymidine kinase activity.

Drug and time of administration	Time of death	Thymidine kinase	^{3}H-Deoxycytidine incorporation into DNA
	hr	*mμmoles/mg liver/20 min*	*dpm/mg*
Experiment 1			
6% glucose	24	824	
Chlortetracycline, zero time	24	0, 69	
Chlortetracycline, 9 hr	24	0, 0	
6% glucose	30	290, 516	
Chlortetracycline, zero time	30	161, 247	
Chlortetracycline, 9 hr	30	325, 343, 510	
Experiment 2			
6% glucose (4)[a]	24	340 ± 22[b]	3297 ± 635
Chlortetracycline, zero time (8)	24	50 ± 13	753 ± 113
6% glucose (8)	30	418 ± 25	2456 ± 300
Chlortetracycline, zero time (8)	30	352 ± 35	4217 ± 650

[a] Number of animals is given in parentheses.
[b] Standard error of the mean.

in DNA synthesis to 30 hr, as measured by ^{3}H-deoxycytidine incorporation.

Several reports (12, 13) have established that following partial hepatectomy, the incorporation of radioactive precursors into the nuclear RNA of liver is increased. We studied the incorporation of ^{14}C-orotic acid into nuclear RNA of chlortetracycline-treated animals. As shown in Table 5, the antibiotic had no effect on the incorporation at 4 or 8 hr postoperatively.

TABLE 5

Effect of chlortetracycline on ^{14}C-orotic acid incorporation into RNA in regenerating liver

Animals were given chlortetracycline (50 mg/kg) in 6% glucose in water intravenously immediately after partial hepatectomy. They were given ^{14}C-orotic acid (10 μCi/kg) in 0.9% NaCl intravenously 20 min prior to the times of death indicated. Counts incorporated into nuclear RNA and the DNA content were determined.

Time of death	^{14}C-Orotic acid incorporation into DNA	
	− Chlortetracycline	+ Chlortetracycline
hr	*dpm/mg*	
4	22,700 ± 2800[a] (4)[b]	25,000 ± 1700 (2)
8	30,500 ± 1190 (4)	33,000 ± 740 (4)

[a] Standard error of the mean.
[b] Number of animals is given in parentheses.

DISCUSSION

The hepatotoxic effects of the tetracyclines have been documented in man and in experimental animals (1, 14). In bacteria, tetracyclines have been shown to interfere with the attachment of aminoacyl-tRNA to ribosomes (15), but since the biological effects of these drugs in bacteria are much greater than in mammals, the role of this mechanism in mammalian toxicity has been disputed. Although the effect of tetracyclines in reducing amino acid incorporation into protein is much less marked in liver than in other tissues (16), the major clinical toxicity of these drugs is hepatic failure. Franklin studied tetracycline effects on tryptophan-induced hepatic tryptophan pyrrolase and found no inhibition by the antibiotic (17), although

he was able to show that the drug inhibited protein synthesis in a cell-free system (18). Because the synthesis of hepatic nucleic acids and protein is selectively increased in regenerating liver, we studied the effects of chlortetracycline on this tissue and were able to demonstrate biochemical alterations at dosage levels within the commonly employed therapeutic range.

After removal of two-thirds of the liver of a rat, there is an immediate rise in the rate of RNA synthesis in the remaining hepatic tissue. Several hours later, the levels of the enzymes related to DNA synthesis exhibit increased activity, and DNA synthesis begins. This is followed by a period of mitotic activity. Bucher and associates have demonstrated that the precise time course of this sequence depends on the age and size of the animals (19), and the effects of diurnal variation are well known (10). Thus, although the sequence of events is generally as described, the precise timing of each event may vary and the reported effects of chemical agents on regeneration may differ.

Thymidine kinase, an enzyme associated with DNA synthesis, increases in the remaining liver after partial hepatectomy (20). As reported here, chlortetracycline inhibited thymidine kinase activity 24 hr after partial hepatectomy, although the incorporation of labeled amino acid into protein was not significantly affected at 12, 16, and 24 hr. Administration of the drug within the first 12 hr after operation suppressed the activity of thymidine kinase at 24 hr. If chlortetracycline was given at 16 hr, a time at which there was still no increase over baseline thymidine kinase activity, the enzyme rose to the same level during the next 8 hr, as it did in regenerating livers of untreated animals. The data suggest that chlortetracycline interfered with the process of regulation of thymidine kinase synthesis to a new rate, but not with the activity of enzyme which was already formed or whose increased rate of synthesis had been programmed. This effect of chlortetracycline is similar to the inhibition by actinomycin D of thymidine kinase (21), and of deoxycytidylate deaminase by puromycin and fluorophenylalanine (21). In each case the drug was only effective in preventing the rise of enzyme activity at 24–28 hr if administered within the first 12–14 hr after hepatectomy. Although chlortetracycline given within the first 12 hr fully inhibited thymidine kinase measured at 24 hr, by 27–30 hr the activity of this enzyme increased. The rate of DNA synthesis, which was inhibited at 24 hr by chlortetracycline, also showed a similar later increase. This delayed escape from inhibition by the drug again resembles the effects reported for actinomycin D (12). A corresponding effect of actinomycin D and chlortetracycline has also been noted in the failure of either drug to block the diet-induced rise in amino acid-metabolizing enzyme (22).

Early in the course of hepatic regeneration, the synthesis of RNA rises markedly. Church and McCarthy (23) were able to demonstrate the appearance of a different species of nuclear RNA within the first hour after partial hepatectomy. A quantitative increase in the rate of RNA synthesis also begins immediately after hepatectomy and continues for the first 5–12 hr (12, 13). We were unable to demonstrate any consistent effect of chlortetracycline on nuclear RNA synthesis. Although an occasional experiment showed inhibition of orotic acid incorporation into nuclear RNA, most experiments failed to substantiate these observations. Furthermore, at no time did isolated nuclei from livers of chlortetracycline-treated animals show any change in their ability to synthesize RNA from radioactive nucleoside triphosphates. Another consideration strongly suggests that chlortetracycline does not interfere with thymidine kinase formation by blocking nuclear RNA synthesis during early regeneration. By 12 hr after partial hepatectomy, most of the increase in nuclear RNA formation has already taken place (12, 13). If the drug had acted mainly by blocking nuclear RNA synthesis, it would not have been effective if given at 12 hr. The fact that chlortetracycline given 12 hr after hepatectomy fully inhibited 24-hr thymidine kinase activity (Fig. 2) suggests that it did not act by inhibition of nuclear RNA formation. The question whether the

antibiotic inhibited the synthesis of a particular species of nuclear RNA or its transformation and transcription is unanswered.

Puromycin and ethionine, known inhibitors of protein synthesis, have been shown to produce fatty livers in rats (24, 25). Both these agents inhibit several of the enzymes that increase in regenerating liver (22). Ethionine has also been shown to block thymidine incorporation into DNA of regenerating liver (26). In regenerating liver, some of the biochemical effects of chlortetracycline resemble changes produced by inhibitors of nucleic acid or protein synthesis. However, one measure of protein synthesis, the incorporation of leucine into cytoplasmic acid-precipitable protein, was not significantly decreased by amounts of chlortetracycline that inhibited thymidine kinase and DNA synthesis; the incorporation of orotic acid into nuclear RNA also was not inhibited under these circumstances. The mechanism of chlortetracycline's hepatotoxicity cannot be deduced by analogy with agents such as actinomycin, an inhibitor of DNA-dependent RNA synthesis, and puromycin, which interferes with ribosomal protein synthesis. Although these drugs act at different sites, many of their net effects on DNA and enzyme synthesis in regenerating liver are similar. Furthermore, actinomycin D has a very high affinity for DNA and binds to nuclei (27, 28); tetracyclines bind to extranuclear structures and cause mitochondrial alterations (29, 30). However, adenosine triphosphate levels are not changed in liver by high levels of tetracyclines, and impaired oxidative phosphorylation is not considered to be an adequate explanation of hepatotoxicity (31). To evaluate the possibility that chlortetracycline inhibited enzyme formation by nonspecific metal binding, its effects were compared with those of EDTA, an agent whose chelating properties are similar to those of chlortetracycline. In unpublished experiments, we found that parenteral administration of amounts of EDTA 2-fold greater than the drug had no effect on thymidine kinase activity.

A more complete description of the molecular effects of chlortetracycline on regenerating liver will require assessment of changes of several species of nuclear and nucleolar RNA and studies of the synthesis of the specific proteins whose activities are depressed by the drug.

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