Ribonuclease Inhibitor in Lymphosarcoma P1798: Effects of Steroid and 5-FU (32720)

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Lymphosarcoma P1798 maintained in BALB/c mice occurs in two strains that regress following treatment with different therapeutic agents. Strain I is corticoid sensitive, but relatively resistant to 5-fluorouracil (5FU). Strain II responds to 5FU treatment but is relatively resistant to glucocorticoids (1). Some materials affect both strains. With successful treatments in either strain, tumor regression evident within about 24 hours is preceded by an increase in acid ribonuclease activity evident about 6 hours following treatment (2). Further evidence for the participation of ribonuclease in tumor regression is the observation that a variety of cytostatic agents active against ascites tumors in rats also lead to a large increase (300–900%) in ribonuclease activity in thymus and spleen (3), although there was no effect on rat liver ribonuclease.

Certain aspects of the data reported on lymphosarcoma (2) suggested that, rather than new enzyme synthesis, activation of latent enzyme or inactivation of an unstable ribonuclease inhibitor is occurring after *in vivo* drug treatments. Ribonuclease activity in high speed supernatant fractions from tumors of untreated hosts shows a lag period of 5–10 min before a final linear rate of hydrolysis of added substrate is attained. In similar preparations from tumors of treated animals the lag period was reduced or absent. Enzyme activity is increased in controls and the induction period is eliminated by freeze–thawing, or treatment with acid, urea, or *p*-chloromercuribenzoate, all of which have been shown to inactivate ribonuclease inhibitors in other systems (4, 5). Indirect evidence was reported that the increase in alkaline ribonuclease activity, which accompanies thymus regression after glucocorticoid treatments in rats, may in turn be responding to an earlier decrease in the activity of an endogenous ribonuclease inhibitor (6). When thymus homogenates were treated with acid, a further increase in ribonuclease activity was observed and differences between control and the steroid-treated thymus homogenates were abolished.

This study presents data on direct measurements of ribonuclease inhibitor activity in two strains of lymphosarcoma P1798 following effective therapeutic treatments.

Materials and Methods. Lymphosarcoma P1798 was maintained in BALB/c mice obtained from Microbiological Associates through Cancer Chemotherapy National Service Center (CCNSC) as previously described (7). Mice bearing Strain I tumor, corticoid sensitive, were treated with single injections of 9α-fluoroprednisolone (9FP), 50 mg/kg. Mice bearing Strain II, the corticoid resistant tumor, were treated with 5FU, 50 mg/kg. Tumors were homogenized in 0.25 *M* sucrose and the supernatant from centrifugation at 105,000*g* for 60 min was used as the crude inhibitor preparation.

Ribonuclease inhibitor was assayed by an adaptation of the procedure of Roth (4). The incubation mixture contained 1 mμg of bovine pancreatic ribonuclease (Worthington Biochemical Corp.), 0.05 mg of RNA (Mann Chemical Co.) purified by the method of Crestfield, Smith, and Allen (8), 0.025 *M* sodium phosphate buffer, pH 7.6, and 0.05 ml of high speed supernatant fractions of varying dilutions in a total volume of 0.2 ml. The RNA was added last. After 20 min of incubation at 37°C reactions were stopped by addition of 1.4 ml of the lanthanum–magnesium–ethanol precipitating reagent recently described (9). Mixtures were chilled, centrifuged, and the absorbance of the supernatant fractions was measured at 260 mμ. Appropriate tissue and substrate blanks were run, and experimental values were corrected for the blanks. An inhibitor unit is defined according to the method of Roth (4) or Short-

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TABLE I. The Effect of 18-Hour Therapeutic Treatments on Ribonuclease Inhibitor Activity in Lymphosarcoma P1798.[a]

Inhibitor source	mg of protein/assay	Inhibition of *p*-RNase (%)		
		Control	5FU-treated	9FP-treated
Strain II	0.230	82	47	82
(steroid resistant)	0.115	59	20	58
	0.075	26	5	19
Strain I	0.46	86	77	80
(steroid sensitive)	0.26	60	60	50
	0.13	47	41	41

[a] Animals bearing Strain I or Strain II tumors were given single injections of treatment materials (50 mg/kg) in suspending medium, as specified. Controls were given similar injections of medium alone.

man (5), as the amount of activity that produces 50% inhibition of pancreatic ribonuclease under given experimental conditions.

Results. Ribonuclease inhibitor activity 18 hours after a single injection of therapeutic agents is shown in Table I. In Strain II, the steroid-resistant (FU sensitive) strain, there was a noticeable decrease in inhibitor activity following 5FU treatments. In six separate experiments the extent of the decrease in inhibitor activity following treatments varied widely, depending upon handling of the preparations, but the nature of the effect was constant. No such effect on inhibitor occurred following administration of 9FP to animals. In Strain I tumor, which is corticoid sensitive and 5 FU resistant, there was no effect on inhibitor activity with either 9FP or 5FU treatments.

Table II shows that the decrease in inhibitor activity occurs within 4 hours of administration of 5FU to animals bearing Strain II tumor. At this time there was no change in acid ribonuclease activity in these same fractions. These results are consistent with prior reports that the earliest detectable increase in acid ribonuclease is observed at about 6 hours following treatments (2,7). The large variance shown in Table II is again due to different handling of the preparations. Inhibitor assay of freshly prepared high speed supernatant fractions showed decreased activity of 2- to 3-fold following treatments, but in 1-day-old preparations (4°) the decrease was 30–50%. After 3- to 4-days inhibitor activity is greatly reduced and there are no differences between control and treated tumor preparations. Treatment of Strain I tumor with 9FP did not result in any change in inhibitor activity. Controls of Strain I tumor had about half as much inhibitor activity as controls of Strain II tumor.

TABLE II. The Effect of 4 Hour Therapeutic Treatments on Ribonuclease Inhibitor Activity in Lymphosarcoma P1798.[a]

Inhibitor source	Inhibitor units/mg of protein	
	Control	Treated
Strain II	.60 (±.09)	.31 (±.05)[b]
Strain I	.37	.34

[a] Animals bearing Strain I tumor were treated with 50 mg/kg of 9FP. Animals bearing Strain II tumor were treated with 50 mg/kg of 5FU, 4 hours before sacrifice.

[b] SEM; $n = 3$; $t < 0.05$.

The ribonuclease inhibitor was sensitive to acid and to heat. When supernatant fractions were brought to pH 5.9 by addition of dilute H_2SO_4, inhibitor activity of controls was reduced about 50% and differences between control and treated tumors of Strain II were eliminated. Treatment of supernatant fractions at 55° for 15 min abolished all inhibitor activity.

Discussion. The increase in acid ribonuclease activity following therapeutic treatments of tumor previously reported was common to both strains (2). However, the variety of effective agents, the difference in

susceptibility to treatment between the two strains, and the 6-hour delay in increase of the enzyme activity, all emphasize that the initial biochemical events following treatments are still undetected, and probably differ between the two strains. Now it appears that one of these prior events that differ between the strains is the effect of treatment on a ribonuclease inhibitor which is reduced in Strain II, but shows no effect on Strain I tumor. There is at present no direct connection between the inhibitor factor in tumors active against alkaline bovine pancreatic ribonuclease and the observed increase in acid ribonuclease activity observed following treatments. A connection cannot be excluded until the inhibitor can be tested for activity against the endogenous tumor ribonuclease after further purification and characterization. A fairly purified enzyme is essential for inhibitor assay. Characterization of the increased enzyme activity in lymphosarcoma regression is esssential since it has been found that during cell transformation of plants, following bacterial infection, completely new types of ribonucleases are formed (10).

This report presents evidence for one of the differences in biochemical responses noted between Strain I and Strain II tumors following effective treatments. A difference in binding capacity of steroids between the two strains has also be recently reported (11). The role of ribonuclease inhibitor may be important in tumor regression since many recent experiments in other systems suggest that the inhibitor can inactivate endogenous ribonucleases (12,13,14) which in turn control RNA, protein synthesis, and cell proliferation.

Summary. Administration of 5-fluorouracil (5FU) to animals bearing Strain II lymphosarcoma P1798 results in a decrease in activity of a ribonuclease inhibitor at 4 hours. This precedes by about 2 hours the increase in acid ribonuclease activity, and precedes by about 20 hours any measurable tumor regression. Treatment of this strain with 9α-fluoroprednisolone (9FP) had no effect on inhibitor activity. Treatment of Strain I tumors with 9FP which also leads to increased ribonuclease activity and tumor regression had no effect on the ribonuclease inhibitor, nor did treatment with 5FU in this strain. There was about half as much inhibitor activity present in controls of Strain I as in controls of Strain II.

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