The Role of Ribonuclease in Regression of Lymphosarcoma P1798

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Summary

RNase activity increases in P1798 lymphoid tumors maintained in BALB/c mice that are undergoing in vitro drug-induced regression. Corticosteroids, 5-fluorouracil, vinblastine, methotrexate, and triethylenemelamine produce regression of appropriate sublines of P1798 lymphosarcoma as well as an increase in RNase activity. Numerous other agents tested had no effect either on tumor regression or on RNase activity. Separation of the different cell types by glass bead chromatography showed that the increased RNase activity was in the purified tumor lymphocytes.

Introduction

P1798 is a transplantable lymphosarcoma developed in BALB/c mice by Lampkin-Hibbard (16-18). Strain I of this tumor showed marked regression when the hosts were treated with glucocorticoids but not when treated with 5-fluorouracil (5-FU). Strain II tumor regressed after treatment with 5-FU but only slightly after treatment with corticosteroids. Glucocorticoid treatment of mice bearing Strain I tumor resulted in increased tumor acid ribonuclease (RNase) activity (22-25). Increase in enzyme activity was not observed in Strain II tumors, following treatment with either 5-FU or corticosteroids (25). This report describes further studies on the relationship between tumor regression and RNase activity. Preliminary results have been presented (1, 2).

Materials and Methods

Methods of inoculation and the source of tumors have been described (25). The BALB/c mice were obtained from Microbiological Associates, generously provided by the Cancer Chemotherapy National Service Center (CCNSC), and from the Roscoe B. Jackson Memorial Laboratories. The CCNSC also provided the steroids used. These were suspended in carboxymethylcellulose medium, prepared by the Armour Chemical Co. for CCNSC, and injected s.c. Other substances in aqueous solutions were administered s.c. With a Kontes conical glass homogenizer, solid tumor was homogenized for 1-1.5 min in 0.25 M sucrose (10% w/v) and centrifuged at 1000 × g for 10 min at 5°C. Enzyme assays were performed on the supernatant fractions. Ascites forms of both strains of P1798 were readily obtained by i.p. inoculation of tumor cells. Cell suspensions from solid tumor were prepared by forcing tissue through a graded series of stainless steel cloth, according to the method of Kaltenbach and Kaltenbach (14). These suspensions consisted of discrete cells when examined by phase contrast microscopy.

Tumor lymphocytes were separated from macrophages by chromatography on siliconized glass beads, a modified procedure of Rabinowitz (30) being used. Glass beads, 177-250 μm (LaPine Scientific Co.), were treated with Desicote (Beckman Instrument Co.), then washed with acetone, water, and Eagle's basal medium. Columns (10 mm in diameter) were filled with the siliconized beads to a height of 200 mm. About 8 ml of cell suspensions were mixed with an equal volume of 10% fetal bovine serum or 20% rat serum in Eagle's medium at room temperature and passed twice through the column. The column was rinsed with about 30 ml of Hanks' balanced salt solution. The combined effluents were free of macrophages. Cell counts were made with a Coulter counter. Aliquots of equal numbers of purified lymphocytes were washed twice with Hanks' balanced salt solution, once with 0.9% saline, and suspended in 0.25 μM sucrose (approximately 5-6 × 107 cells/ml). The cells were completely disrupted by sonication for 5 min in a Raytheon 9-ke sonicator. In some experiments, material that had adhered to the glass beads was eluted by stirring the column contents with water for 15 min at 5°C. The extraction was repeated, and the combined washings were lyophilized and suspended in 1.0 ml of 0.25 M sucrose.

RNase was assayed on appropriate dilutions of the sucrose homogenates by a minor variation in the method of Eichel and Roth (9). One-tenth ml of homogenate, 1.0 ml of 1.0% RNA, and 0.1 ml of 0.2 M acetate buffer, pH 5.7, and containing 0.005 M ethylenediaminetetraacetate (EDTA) were incubated at 37°C for 30 min. The reaction was stopped by addition of 3.0 ml of ice-cold precipitating reagent which was prepared daily by mixing 6 volumes of 0.07% lanthanum acetate at 95°C ethanol with 5 volumes 0.013 M magnesium acetate. The sensitivity of the assay could be increased by reducing the volume of precipitating reagent to 1.7 ml. The reaction vessels were chilled for 15 min and centrifuged at 5°C. The absorbance at 260 μm of the supernatant fractions was used as a measure of RNase activity. Dilution of the supernatant was not necessary under these conditions. The absorbance of an identical reaction mixture precipitated without incubation was determined and an appropriate correction made. An increase of 1.0 absorbance unit/30 min incubation period under the assay conditions was defined as 1 enzyme unit. Substrates used were either whole yeast RNA purified by the method of Crestfield et al. (4) and purchased from Sigma Chemical Co., or whole tumor RNA extracted by the phenol method of Kirby (15). Protein was measured by the biuret reaction or by the method of Lowry et al. (21). Acid phosphatase was determined as described in Sigma Technical Bulletin No. 104.

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Results

Chart 1 illustrates the effect of the administration of 9α-fluoroprednisolone (9-FP) and 5-FU on Strain I tumor growth and on RNase activity. No significant change in tumor size is seen 8 hr following treatment. Definite regression following 9-FP and cessation of tumor growth following 5-FU are evident 24 hr after treatment. Tumor RNase activity is markedly increased 18 hr after treatments. RNase was measured at pH 5.7 because prior studies indicated that at this pH the tumor enzyme exhibited optimal activity and the maximal differences between control and treated tumors. In prior work (25) the increase in RNase activity following 5-FU treatment of Strain I tumor was not considered significant. However, Chart I demonstrates that this strain is not completely resistant to 5-FU, and the definite growth retardation was regularly observed. The effect on retardation of growth and the increased enzyme activity following 5-FU treatment, though less than that observed with corticoid treatment, is in accord with the concept that tumor regression is correlated with RNase activity.

Chart 2 illustrates the effect of the same agents on Strain II tumor. Striking regression followed 5-FU treatment. 9-FP retarded tumor growth. 5-FU caused a 4-fold increase in RNase activity. 9-FP caused a small but significant increase in enzyme activity. The chart illustrates the effect of in vivo treatment with 50 mg/kg. The same results were also obtained when extracted tumor RNA replaced the purified yeast RNA generally used as substrate in the enzyme assay. The RNase increase in Strain II following 5-FU treatment was not observed in prior work (25) for reasons to be discussed later. The increase is again also in accord with the correlation between tumor regression and an increase in RNase activity.

The apparent increase in enzymatic activity shown in Charts 1 and 2 could be a result of nonlinear kinetics in this system or due to an actual increase in catalytic activity. The data illustrated in Chart 3 demonstrate that both factors operate. The time course of the hydrolysis of RNA by freshly prepared tumor homogenates reveals a significant induction period in the reaction mixtures containing homogenates of untreated tumors of both Strain I and Strain II. Following in vivo therapeutic treatments, the induction period is absent or less evident. The chart also illustrates that following the elimination of the induction period, the extent of RNA hydrolysis is proportional to time and is greater in treated tumor homogenates than in control homogenates. When RNase activity is measured only during the linear phase of the in vitro incubation period, the differences between control and treated tumor enzyme activity are clear. In the experiment using Strain I tumors, during the 15 to 45-min incubation interval the control enzyme had an activity of 1.2 O.D. units/gm tissue/min, and the treated tumors an activity of 2.93 O.D. units/gm tissue/min. In the experiment using Strain II tumors, control enzyme had an activity of 1.95 and the 5-FU-treated tumors an activity of 6.2 O.D. units/mg tissue/min. Other experiments showed that during the 10 to 30-min incubation interval, the RNA hydrolysis was proportional to the amount of enzyme used. The induction period observed in the assay of control tumors was greatly reduced and sometimes not detectable in frozen or stored tissue preparations. Thus detection of the lag period in fresh untreated tumor preparations compli-
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1.2
0.9
0.6
0.3
0.1
0
-0.3
-0.6
-0.9
-1.2

ENZYME UNITS/ASSAY

1.0
1.2
0.8
0.6
0.4
0.2
0

INCUBATION PERIOD (min at 37 °C)

10 20 30 40

Chart 3.—The effect of treatment on the rate of RNA hydrolysis by tumor homogenates. RNase assay was performed 18 hr after treatment of mice bearing Strain I or Strain II tumors with materials indicated. The incubation period is shown on the abscissa. △—△, Animals bearing Strain I tumors treated with 25 mg/kg 9-FP; □—□, animals bearing Strain II tumors treated with 25 mg/kg 5-FU; ○—○, control animals treated with suspending medium.

Enhanced RNase activity following effective treatments could result from an increase in enzyme activity in the tumor lymphocytes or to an infiltration of macrophages which has been shown to occur during corticosteroid-induced lymphocyteolysis (8).

Fig. 1 shows that complete separation of tumor cells from macrophages was effected by glass bead chromatography. The column effluents contained about 80% of the applied tumor cells, 60-85% of the RNase activity, and no macrophages. When peritoneal macrophage preparations were chromatographed, the bulk of the acid phosphatase activity adhered to the glass beads of the columns along with the macrophages. It was also demonstrated that RNase is not absorbed by purified tumor cells. Soluble RNase, prepared by freezing the crude tumor homogenates, was incubated with the purified tumor cells for 15 min. A 10-fold excess of soluble over tumor cell enzyme was used. The tumor cells had unchanged specific activity following 3 saline washes.

Table 3 shows the results of RNase assay on purified lymphoid blastine were equally as effective in Strain II as in Strain I tumor. On the other hand, progesterone, testosterone, androsterone, DL-ethionine, deoxy cortisol, estradiol-17β, 6-mercaptopurine, 8-azaguanine, 11-epicortisol, and urethane had no effect on tumor growth or on RNase activity in either strain. Data reported in Table 1 are for a 30-min incubation period in the enzyme assay. Separate experiments using tumors treated with methotrexate, vinblastine, and triethylenemelamine confirmed that the rate of RNA hydrolysis is greater than in controls during the linear phase of the incubation interval, from 10 to 30 min.

Effects of Freezing and of p-Chloromercuribenzoate

Freezing and thawing of postnuclear fractions (1000 x g supernatant fractions) resulted in enhanced enzymatic activity. Chart 4 demonstrates that freeze-thaw treatment released most of the enzyme activity to the high speed supernatant fractions in both control and corticoid-treated tumor samples. However, there was no redistribution of enzyme activity into supernatant fractions resulting from the in vivo corticoid treatment. This last observation confirms a prior report using other methods (25). Table 2 shows that the absolute increase in enzyme activity from freezing was the same in both control and 9-FP-treated tumors. Additional studies showed that freezing reduced the induction period and also increased the rate of reaction after this period when the extent of hydrolysis was strictly proportional to time. The in vivo activation by freezing and the in vivo activation of RNase by corticosteroid treatment were additive. These results show that the in vivo activation of RNase does not resemble the typical in vivo lysosomal activation by various agents which release acid hyrolases from particulate matter into supernatant fractions (15, 38, 39).

Roth (32) and others have demonstrated that incubation of homogenates of numerous tissues with p-chloromercuribenzoate (pCMB) enhances RNase activity. Chart 5 shows that control, treated and frozen homogenates of P1798 had increased RNase activity following pCMB treatment, according to a procedure described by Roth (32). Again, this increase was additive to, and independent of, the increase from in vivo treatment.

Localization of Enzyme Activity to Tumor Cells

Table 1 illustrates further the relationship between tumor regression and increased acid RNase activity. Administration of cortisol, corticosterone, cortisone, vincristine, vinblastine, and triethylenemelamine resulted in increased enzyme activity and regression of Strain I tumor. Methotrexate similarly induced an increase in enzyme activity and regression of Strain II tumor. Not shown in this table are data showing that vincristine and vin-

Table 3 shows the results of RNase assay on purified lymphoid cells but does not invalidate the enzyme assay. It may also be noted from Chart 3 that assays incubated for 30 min (for practical reasons) would alter the figures, but would not change the sense of the results.

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TABLE 1

Effect of Various In Vivo Treatments on Tumor Size and RNase Activity

Experiments 1, 3, 4, and 5 were on BALB/c mice bearing Strain I tumor. Experiment 2 was on BALB/c mice bearing Strain II tumor. Treatment was for 3 daily injections of material in the first 3 experiments, and 2 daily injections in the last 2 experiments. Other materials that had little or no effect on tumor growth or on nuclease activity of either strain under these same conditions were: progesterone, testosterone, androsterone, estradiol, 6-mercaptopurine, 8-azaguanine, 6-azaaridine, 11-epicortisol, DL-ethylene, deoxycorticosterone, all at 50-75 mg/kg, and urethane at 500 mg/kg.

All P values < 0.01.

<table>
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<tr>
<th>EXPERIMENT</th>
<th>SAMPLES</th>
<th>DOSE (mg/kg)</th>
<th>EFFECTS</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Enzyme units</td>
</tr>
<tr>
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<td>Controls (4)*</td>
<td>50</td>
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<td></td>
<td>Cortisol (3)</td>
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<td>281 ± 26</td>
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<td>Controls (5)</td>
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<tr>
<td></td>
<td>Methotrexate (5)</td>
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<td>221 ± 15</td>
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<tr>
<td>3</td>
<td>Controls (3)</td>
<td>100</td>
<td>171 ± 4.5</td>
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<tr>
<td></td>
<td>Corticosterone (4)</td>
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<td></td>
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<tr>
<td></td>
<td>Triethylenemelamine (4)</td>
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<tr>
<td></td>
<td>Vinblastine (3)</td>
<td></td>
<td>509 ± 6</td>
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</table>

* No. of animals.

‡ Mean ± S.E.; P < 0.01. Enzyme activity was proportional to the concentration of tumor homogenates used in the assay.

TABLE 2

Effect of Freezing of Homogenates on RNase Activity

Treatment in vivo was with 9 α-fluoroprednisolone (9-FP), 25 mg/kg, 18 hr before sacrifice. Aliquots of 10% tumor homogenates were frozen (X 5) in Dry Ice and acetone. Fresh and frozen homogenates were simultaneously assayed for RNase activity.

<table>
<thead>
<tr>
<th>SAMPLES</th>
<th>Enzyme units/gm tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
</tr>
<tr>
<td>Controls (3)* 9-FP-treated (3)</td>
<td>111 ± 3b</td>
</tr>
<tr>
<td>Increase from 9-FP</td>
<td>123%</td>
</tr>
</tbody>
</table>

* No. of animals.

‡ Mean ± S.E.; P < 0.01.

tumor cells derived from solid Strain I lymphosarcoma 18 hr after treatment with 9-FP. Corticoid treatment effected a doubling of the specific activity of the lymphoid cells. Similar results were obtained in purified lymphocytes derived from Strain II tumor treated with 5-FU. In lymphocytes purified from ascitic forms of the tumor, the RNase increase was consistent but less impressive, and there was wide variability among different experiments.
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Discussion

Some aspects of the present data suggest that RNase activity may play a causal role in the regression of P1798 lymphosarcoma. Therapeutic agents enhance enzymatic activity, ineffective agents do not. The increase is inherent in the lymphoid cells, and is not due to contamination by phagocytic cells. The increase precedes obvious tumor regression. The variety of effective agents, and the difference in susceptibility between the 2 tumor strains suggest that the initial biochemical events following therapy may have a common path in the activation of RNase. Injected RNase has been used successfully in some systems to induce tumor regression (3, 19, 20, 27) and such experiments indicate that this enzyme can be a causal factor in regression. Further study is needed to exclude the possibility that enhanced nuclease activity is a result rather than a cause of lymphocyte lysis in P1798.

Prior studies had failed to detect an increase in RNase activity following 5-FU treatment of Strain II tumor (25). The present results are due to differences in the substrates used in the enzyme assays. The tumor RNA used as substrate in the earlier studies (25), extracted by the method of Rosenbaum and Brown (31), consists mainly of soluble RNA and other low molecular weight RNA. High molecular weight RNA, insoluble in 1 M NaCl, was discarded in the extraction process (25). In the present studies, the substrate was either yeast RNA purified by the method of Crestfield et al. (4) or total tumor RNA extracted by the phenol method of Kirby (15), and in either case included high molecular weight RNA (insoluble in 1 M NaCl). Work to be reported elsewhere indicates that there are quantitative differences in substrate specificity between control enzyme and the enzyme activity induced by treatment. This difference in substrates used in the enzyme assay could also account for the positive results reported here on the effects of vinblastine on RNase activity that differ from results reported from another laboratory (40).

The kinetic data showing the presence of an induction period in the assay of enzyme from untreated tumors that is absent or reduced in treated tumors suggest the possibility that one mechanism of action of tumor regression involves the in vivo activation of latent RNase or inactivation of an unstable inhibitor following successful therapeutic treatments. Latent RNase and RNase inhibitors have been reported in many other systems (10, 28, 33, 36). This subject is under active study.

The increase in RNase activity in tumor regression is of special interest because it may play a role as an endogenous regulator of protein synthesis and hence of the control of normal and abnormal growth. The enzyme (enzymes) exist with various specificities (7, 28, 34), optimal conditions for activity (26, 32, 35), in different states of activation (32, 33, 35), and in all fractions of the cell (6, 36, 37). Such a diverse enzyme system could constitute a control mechanism for growth in higher organisms. It is possible that the steroids might function via the selective activation of latent RNase. Indirect evidence for this hypothesis is found in recent reports of corticosteroids causing the breakdown of polysomes in thymus lymphocytes (13), the reduced incorporation of amino acids into protein (12, 29), and altered nucleic acid metabolism (11). Recent reports (35) that urea activates RNase I and inhibits RNase II in Escherichia coli could serve as a model for the mechanism of such selective activation. The availability of 2 strains of P1798 lymphosarcoma that react differently to various treatments provides the opportunity to investigate the differences in the genetic components contributing to tumor regression.
Acknowledgment

The authors gratefully acknowledge the technical assistance of Mrs. Annie Mae Miller.

References

Fig. 1.—Suspension of crude P1798 tumor cells. X 800.
Fig. 2.—Purified lymphocytes from same cell suspension after treatment by glass bead column chromatography. X 800.
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