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Role of ribonuclease in the inhibition of Ehrlich Ascites tumor growth

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THE ROLE OF RIBONUCLEASE IN THE INHIBITION OF EHRLICH ASCITES TUMOR GROWTH

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Introduction

Several investigators have reported that ribonuclease (RNase) is effective in prolonging the survival time of mice with Ehrlich ascites tumor (EAT).[1-3] Rapidly growing normal tissue has been shown to have increased amounts of ribonuclease, compared to neoplastic growth where decreased levels of RNase have been found.[4-11] However, de Lamirande found RNase to be ineffective in prolonging the survival time of mice with EAT.[12] Therefore, this study was undertaken to determine the effect of RNase *in vivo* and *in vitro* on EAT growth in mice and their subsequent survival time.

Ribonuclease is an enzyme whose amino acid sequence is known.[13] However, its mechanisms of action on ribonucleic acid (RNA) is still in dispute. While RNase degrades RNA by a phosphate transfer reaction and hydrolysis, it has been shown to promote the formation of a different RNA molecule.[14, 15] There are probably two different ribonucleases - one active in an acid pH and the other in an alkaline pH.[16, 17] Moreover, a ribonuclease inhibitor has been isolated, but the interaction between RNase and the inhibitor *in vivo* is not clearly understood as yet.[18, 19] Thus, the understanding of the action of ribonuclease in neoplasia must await further elucidation of its physiological role.

Review of the Literature

The relationship between ribonuclease and neoplasia is a tenuous one, as illustrated by the references cited below which contain conflicting ideas in this regard, and which are summarized by Roth in a comprehensive review of the literature.[20] Presently the biochemical and physiological role of this enzyme is unclear, as is that of a ribonuclease inhibitor more recently discovered.

Some of the early work with RNase and its effect on tumors was reported by Ledoux in which he found that daily injections of RNase significantly prolonged the survival time of mice with Ehrlich ascites carcinoma, Krebs carcinoma, and Crocker sarcoma.[1, 2] An *in vitro* study on Landschutz ascites cells showed a marked effect on cellular metabolism, which Ledoux felt could explain the anti-tumor effects observed above.[21] Podolsky, Wase, and Cardenas used RNase in mice with EAT and observed that the growth of the Ehrlich tumor was inhibited.[3] Smears of the treated tumor cells showed marked cytological changes in cell size as well as structure, along with a greatly reduced ribonucleic acid content as compared to control smears.

Consistent with the findings that RNase inhibits tumor growth has been the work of Daoust, Amano, and Cantero.[10, 22] Using histochemical analysis of nucleases of tumor cells, they found low or negligible ribonuclease and deoxribonuclease (DNase) in the tumor cells, but they did find RNase and DNase activity in normal cells. Necrotic areas of tissues examined showed intense RNase activity. They emphasize that nuclease

determinations done on tumor tissue homogenates by biochemical methods might include necrotic and connective tissue areas, and thus, give a misleading value of concentrations.

In 1957, Brody undertook a study concerning RNase activity and the growth rate of tissue (human placenta).[4] He found that during the period of logarithmic growth, when there was an increased cellular turnover rate, there was high RNase activity. These results were expanded in 1958 when Brody and Balis found that RNase and DNase activities show completely different patterns in normal and neoplastic growth.[5] The normal tissue response to a growth stimulus involves a sharp increase in the activities of these enzymes; however, neoplastic tissues do not seem to respond in this way. Significantly enough, they found no detectable RNase in Ehrlich ascites carcinoma. In two cases of cancer of the stomach, it was found that when the neoplastic tissue was compared to the same patient's normal gastric mucosa, the cancer tissue had increased RNA, increased DNA, but decreased ribonuclease and decreased deoxyribonuclease.

Using an <u>in vitro</u> biochemical method, Ledoux and Brandli determined RNase activity and ribonucleic acid content in normal and cancerous human uteri.[6] They found that the tumor cells contain more RNA per unit than normal cells, and that the tumor cells have less ribonuclease activity than the corresponding normal control cells. Silber recently reported that more RNase was found in normal leukocytes and lymphocytes than in neoplastic cells from human cases of chronic granulocytic leukemia, chronic lymphocytic leukemia, polycythemia

rubra vera, and agnogenic myeloid metaplasia.[7] Shapot has also found insignificant RNase and DNase activity in neoplastic tissue.[11]

Indirect evidence of the inhibitory action of RNase on neoplastic growth has been described by Ambellan and Hollander.[23] Their work showed that more effective drug therapy of lymphosarcoma P-1798 was associated with a rise of tumor ribonuclease in contrast to the ineffective therapy. They demonstrated that strain I of this tumor, which is more sensitive to corticosteroids, had increased ribonuclease activity in the tumor in vitro after treatment with corticosteroids. This strain is not as sensitive to 5-Fluorouracil (5-FU) and 5-FU treatment caused less of an increase in ribonuclease activity. In contrast, strain II of this tumor is more sensitive to 5-FU and following treatment with this agent, they demonstrated increased tumor ribonuclease activity, while corticosteroids caused a less striking increase in RNase activity.

On the other hand, de Lamirande found that after inoculation of Ehrlich carcinoma cells into mice, daily injections of RNase did not increase the survival time of the treated group.[12] These results do not agree with the work of Ledoux or Podolsky as cited above. Moreover, de Lamirande demonstrated that the ineffectiveness of RNase was not due to lack of penetration of the enzyme into the ascitic cancer cells by determining RNase activity of the ascitic cells and of the ascitic fluid from control and treated mice.

The effect of this enzyme on mitosis and cytological abnormalities in a regenerating liver were studied by

de Lamirande in an earlier paper.[24] While xanthine oxidase and deoxyribonuclease treatment were associated with an increased incidence of mitotic figures in these liver cells, and while cytological abnormalities were seen in the regenerating livers in mice treated with these enzymes, ribonuclease treatment did not significantly affect this aspect. Also, cytological abnormalities were not seen in association with ribonuclease treatment.

Different types of ribonuclease have been identified depending on tissue of origin and pH. Roth and de Lamirande in 1954 showed that RNase from the liver had two primary areas of activity -- one in the acid pH range and the other in the alkaline pH range.[25, 26] Bergel reports that most of the crystalline preparations, if sufficiently purified, contain an enzyme with a molecular weight of about 13,000 with a known amino acid sequence.[13, 14] This substance has a complex enzymatic action on RNA involving catalysis as follows:

> The transfer of the 3' phosphate of a pyrimidine ribotide residue from the 5' position of the adjoining nucleotide to the 2' position of the pyrimidine ribotide itself whereby a cyclic phosphate is formed; it activates the latter to react in the 2' position with water, amounting to a hydrolytic breakdown, or to react with alcohols or other nucleotides, representing a synthesizing process.[14]

Deavin et al extend this work in a detailed biochemical study of the mechanism of action of RNase.[27] Thus, it is seen that its biochemical action is complex and involving anabolic as well as catabolic functions.

The anabolic function of ribonuclease was studied further by Ledoux and Vanderhaeghe who demonstrated that in Landschutz ascites tumors, the metabolism of pyrimidines is greatly accelerated in the presence of RNase and thus there is seen a rapid appearance of a different ribonucleic acid.[15] Sherif has proposed the idea that there is a different structure for cytoplasmic RNA in malignant material and that this deranged RNA may propagate itself.[28]

Another study by de Lamirande was made to elucidate the behavior of intracellular ribonucleases in various tissues.[16] He found variations in concentration and distributions of acid and alkaline RNase depending on the tissue studied. He presents evidence consistent with the idea that rat liver acid and alkaline RNase probably have different functions as Ledoux had previously suggested.[17]

Furthermore, Ledoux studied the action of RNase on rat bone marrow and found that RNase taken up by bone marrow cells does not seemingly modify cellular ribonucleic acid metabolism.[29] This is another example of the variability of this enzyme system depending on tissues used as well as different techniques. Another complicating point is that in 1964, Roth pointed out that many RNA preparations contain impurities which he felt may be metal ions that interfere with determination of RNA and RNase activity.[30]

The study of ribonuclease is further complicated by the discovery of a ribonuclease inhibitor. Roth has done much of the work with this substance.[18, 19] He has shown

that it appears to have widespread occurrence although he emphasizes that its physiological role is unknown. He postulates that this inhibitor may be one of the factors affecting changes which take place during cell division. Activity of the inhibitor is found in the supernatant fraction of homogenized rat livers. While it is known that heparin is an inhibitor of RNase, Roth has shown that the material in the supernatant fraction is not heparin, but the possibility of a heparin-lipoprotein complex has not been excluded.

Shortman has studied this RNase inhibitor in rats and found that levels of RNase inhibitors shortly after partial hepatectomy are increased.[31] He concludes:

> It is conceivable that the early rise in inhibitor is part of a tooling-up process for hyperactive protein synthesis and in particular is one of the changes leading to accumulation of RNA by the cell.[31]

Following the rise of inhibitors of RNase in his experimental system, he found a later rise in acid RNase and postulates that this phenomenon may be concerned with a "switching-of" mechanism in RNA synthesis.

The ribonuclease inhibitor system has been studied in neoplastic tissue (rat hepatoma) by Roth[30] and by Chakravorty and Busch.[32] Roth found that, in general, RNase inhibitor activity was lower in the tumors than in normal tissues. However, in tumors grown intraperitoneally, there was a marked increase in RNase inhibitor activity. On the other hand, Chakravorty found that the ratio of inhibitor to free RNase was much greater in neoplastic livers. Here again is

a conflict of data as Roth found increase RNase inhibitor activity in only two of the tumors studied.

Colter, Kuhn, and Ellem have studied ribonuclease and RNase inhibitor in mouse ascites tumors.[8, 9] They found that Ehrlich ascites tumor cells had little RNase activity at physiological pH's, but had pH optima at 4.8 and 8.4. In the presence of 4×10^{-4} p-chloromercuribenzoate, (p-CMB) ribonuclease activity was found to be optimal in the pH range of 6.5 - 7.2, correlating with the disappearance of acid and alkaline peaks. P-chloromercuribenzoate is a sulfhydryl inhibitor which has been shown to reverse the action of RNase inhibitor by Roth.[18] Colter et al studied several other mouse ascites tumors and found similar acid and alkaline pH optima for RNase which were reduced to a single peak in the physiological pH range by p-CMB. They present data compatible with the theory that p-CMB releases RNase from an inactive enzyme inhibitor complex.

In summary, most investigators have found RNase in neoplastic tissue and, in general, the administration of exogenous RNase slows the neoplastic process. On the other hand, some have found no effect of RNase on mitosis or neoplasia <u>in vivo</u>. The proposal has been made that an abnormal (malignant) RNA is found in cancer cells, and some authors have shown that RNase is associated with the formation of a different RNA. Also, the study of RNase is complicated by the proposal that acid and alkaline RNase may have different functions. Ledoux has shown that RNase is taken up by bone

marrow, but apparently does not influence RNA metabolism, showing that the effects of RNase may depend upon the target organ and that this enzyme does not appear to act similarly on RNA metabolism in every tissue. The problem of impurities in RNA presents difficulties in finding an accurate assay system. Finally, the role of RNase inhibitor has not been clearly elucidated.

The study of RNase and neoplasia has evolved from Ledoux's early observations that mice with tumors had a prolonged survival time to the more sophisticated studies of RNase activities in neoplastic and normal tissue and the elucidation of an inhibitor system. The physiological as well as the neoplastic role of these factors is as yet unclear, but could eventually lead to a more complete understanding of basic cellular metabolism and its relationship to neoplasia.

Materials and Methods

Animals: White Swiss HA/IER mice from the Charles River Mouse Farms, Brookline, Massachusetts, were used, fed autoclaved Purina chow, and kept in an air-conditioned environment. Male and female mice 8-10 weeks of age, weighing 20-35 grams were employed in all of the experiments.

Ehrlich ascites tumor: Ehrlich ascites tumor cells were obtained from Dr. T. Hauschka, Roswell Park Memorial Institute and maintained by serial passage of 6×10^6 cells every 10-14 days in the White Swiss mouse. The inoculum used in all experiments was measured in normal saline by direct hemocytometer count.

Ribonuclease: 5X recrystallized bovine pancreatic ribonuclease was obtained from Mann Research Laboratories, Inc. This was stored in a dessicated container at 4° C. Weighed aliquots of RNase were dissolved in sterile saline just prior to use.

Procedure: The mice were divided into three groups. All the mice in group A received 6×10^6 Ehrlich ascites tumor cells by intraperitoneal (IP) injections. Starting on the day following tumor inoculation, daily injections of 3.0 mgm. RNase were administered for 7 days to 9 tumor hosts. Another test group received 5.0 mgm. of RNase in a similar manner. Eleven control mice received daily injections of 0.1 cc isotonic sterile saline for 7 days. (See Table I)

In group B, varying doses of RNase were incubated with 6×10^6 EAT cells at 37° C. for 30 minutes (See Table II). One group received a mixture of 0.1 mgm. RNase incubated with 6×10^6 tumor cells. One mgm. RNase per 6×10^6 cells was injected IP into another group. Three mgm. and 5 mgm. of RNase were injected in a similar manner after incubation in the next two test groups respectively. The control mice received 6×10^6 EAT cells incubated with sterile saline at 37° C. for 30 minutes.

In group C, varying doses of tumor cells were incubated with 5 mgm. RNase at 37° C. for 30 minutes prior to IP injection (See Table III). In the first test group, 1×10^6 EAT cells were used. In subsequent test groups, 500,000 cells, 50,000 cells and 5,000 EAT cells were used.

The control groups in this experiment received 1 X 10^6, 500,000; 50,000; and 5,000 tumor cells respectively, incubated in the same manner with sterile saline.

Results

As can be seen from Table I, there is no significant difference between mean survival times (MST) of the treated and the untreated mice in Group A. Increasing the dose of RNase to 5 mgm. per day did not affect the survival time.

There was no significant difference between the mean survival times in Group B when test animals received 6 X 10^6 EAT incubated with varying doses of RNase. One mouse in the group receiving 5 mgm. RNase (marked with a cross in Table II) lived 42 days. However, the mean survival times (MST) of group B are similar to those of Group A (all mice received 6 X 10^6 EAT cells). The manner of administering ribonuclease nor the total dose used in Groups A and B had any affect in prolonging the life span.

As illustrated in Table III, the test animals in Group C who received varying amounts of EAT incubated with 5.0 mgm. RNase had a significantly longer mean survival time as compared to the controls. The difference between survival times is statistically significant ($p<.0005$). The mice who received 5,000 EAT cells and 5.0 mgm. RNase were all living with no signs of tumor growth after 50 days. In contrast, control mice receiving the same amount of tumor cells had died at 39.7 days. While this is the most dramatic example of the effect of RNase incubated with tumor cells *in vitro*, all mice in this group demonstrated a significant effect of RNase.

Discussion

Group A: Daily injections of RNase did not affect the survival time of treated mice. These results differ from those reported by Ledoux and Podolsky, who found significant extension of survival time of mice treated with RNase.[1-3] de Lamirande, however, found that RNase had no effect on survival time when massive doses of tumor inoculum (65 million cells) were used.[12] Yet, Podolsky found that 4-5 mgm. RNase injected IP for 32 consecutive days prolonged the MST in treated mice.[3] He does not include the number of tumor cells used in the initial inoculum. In this experiment only seven daily injections of RNase were administered; Ledoux does not report how many consecutive days he gave RNase.[1, 2] Furthermore, Podolsky has shown that when he stopped giving RNase, tumor growth which had previously been inhibited started to proliferate again.[3]

However, the failure to increase the survival time with daily injections of RNase for seven days does not necessarily show that ribonuclease has no effect on tumor cells. Due to the dynamics of tumor growth and the lack of treatment after the first seven days, effects of RNase may not be detectable by measuring only the survival time of mice. The mitotic doubling time of EAT is approximately 21 hours.[33] In seven days, the tumor would double about 8 times; in 22 days, the tumor doubles 25 times. If the initial inoculum were 6×10^6 cells, at the end of seven days (about 8 mitotic cycles), the host would have about 1.53×10^9 tumor cells; and at the end of twenty-two days (25 mitotic cycles), which is the mean survival time of control mice, the host animal would have 1.99

X 10^{14} tumor cells. We can thus assume that 1.99 X 10^{14} tumor cells is lethal. If one were to assume that the tumor was inhibited for two mitotic cycles in the first seven days of RNase treatment, then the treated animals would have about 3.84 X 10^{8} cells (6 mitotic cycles) at the end of seven days. Then if one assumes that there was no longer any effect of RNase on the tumors in the next 15 days, these treated animals at the end of twenty-two days would have undergone another 17 mitotic divisions and would have about 4.96 X 10^{13} tumor cells. This is only 2 mitotic cycles away from the lethal dose (1.99 X 10^{14}) and it would take only 1.5 days (36 hours) to reach this level. But as can be seen from Table I, 1.5 days is within one standard deviation from the mean and thus, if the RNase had any effect, it could not be determined by this experimental system.

Of course, the above is an abstract example helping to explain the observed results. There are many variables not taken into account. For example, although it has been shown that the mitotic doubling time is 18 hours, this figure is certainly not constant due to the logarithmic growth curve of this tumor. Also the use of a rigid mathematical figure (18 hours) in a complex biological system is fraught with errors. For example, as the mouse grew weaker, perhaps whatever host defenses which may have been operating in the early days of tumor inoculation may be destroyed and the tumor may multiply even more rapidly. But on the other hand, necessary tumor nutrients may be metabolized in the first few days of growth. Then in the last few days prior to death, the tumor would

conceivably multiply less rapidly.

Group B: Incubation of EAT with varying doses of RNase in vitro and subsequent injection into mice did not significantly prolong the survival time. No report of the use of this experimental system has been encountered in the literature to date. However, the results obtained here would tend to support the results seen in Group A as well as those of de Lamirande who could not demonstrate any significant effect of RNase on tumor cells.[12] Yet, there is the possibility that RNase may affect these cells, but that the dose of tumor (6×10^6 cells) was too massive for all concentrations of ribonuclease used. Perhaps ribonuclease inhibitor, which Roth has shown to be present in great quantities in intraperitoneal tumors,[30] prevents the effect that exogenous RNase may have. Although it is not known where RNase inhibitor works in this system, experiments are being carried out in this laboratory with p-CMB (which is thought to release RNase from an enzyme inhibitor complex -- thus effectively inhibiting the action of RNase inhibitor).

Group C: In this group with varying amounts of tumor, keeping the dose of RNase constant, there is significant prolongation of survival time in the test animals. This experiment supports the concept that ribonuclease does have an inhibitory action on Ehrlich ascites tumor cells. It appears that the action of ribonuclease is dose-related and that massive amounts of tumor, as used by de Lamirande and as used in this experiment, are not affected significantly by the doses of ribonuclease used. This result may depend on an insufficient

concentration of ribonuclease per cell, or it could be due to the action of ribonuclease inhibitor. Although 6 X 10^6 EAT cells incubated with 5.0 mgm. RNase did not prolong the MST (Table II), 1 X 10^6 EAT cells incubated with 5.0 mgm. RNase in a similar manner did (Table III). This suggests a dose-response relationship between EAT and RNase. In vitro mixing of RNase and EAT with subsequent incubation avoids the multiple biological factors that may be present when a tumor bearing animal is treated with daily injections of ribonuclease. Before the mechanism of action of ribonuclease on neoplastic cells is clearly elucidated, these in vivo factors must be more clearly understood.

* * * *

The value of this study is best appreciated by placing these findings into the broad picture of oncological research. By in large, effective chemotherapy for cancer is still a future dream of the medical world. A key to cancer chemotherapy is to understand the basic pathological defects involved in the neoplastic transformation. Inhibition of tumor growth by ribonuclease suggests an abnormality of the RNA-RNase system in the neoplastic cell. Conceivably, the uncontrolled proliferation of neoplasia could be related to defects in the system which is so intimately involved in the process of cellular replication. Rather than giving the final answers, this paper helps demonstrate many of the still unknown factors involved in the search for more complete understanding of the neoplastic process.

Summary

Some of the current literature concerning the role of ribonuclease in neoplasia is reviewed.

An unsuccessful attempt was made to duplicate the finding previously reported, that daily injections of ribonuclease could prolong the survival of mice with Ehrlich ascites tumor.

Therefore, an experimental system was devised involving the *in vitro* incubation of ribonuclease with Ehrlich ascites tumor prior to injection into mice. Varying doses of RNase incubated with 6×10^6 EAT cells had no effect on survival times of treated animals, but varying numbers of EAT cells incubated with 5.0 mgm. RNase did significantly prolong the mean survival time in all test groups.

TABLE I (Group A)

	6×10^6 EAT Injected IP		
	Number of mice	Dose of ribonuclease (mgm/day X 7 days)	Mean survival time (days)
(1)	9	3	20.7 ± 1.12*
(2)	5	5	22.3 ± 1.53*
(3)	11 (Control)	0	22.5 ± 2.94*

* One standard deviation

TABLE II (Group B)

	Ribonuclease Incubated With 6×10^6 EAT and Injected IP		
	Number of mice	Dose of ribonuclease (mgm in incubation misture)	Mean survival time (days)
(1)	5	0.1	22 ± 3.46*
(2)	5	1.0	20.2 ± 0.63*
(3)	6	3.0	21.6 ± 3.21*
(4)	18	5.0	22.6 ± 5.74*+
(5)	7	0	$19.8 \pm .761$*

\+ One mouse in this group lived 42 days
* One standard deviation

Table III (Group C)

	Number of mice	Number of EAT cells	Mean survival time (days)	p-value
		5 mgm. RNase Incubated with EAT and Injected IP		
(1)	9	1×10^6	37.6 ± 9.6*	<0.0005
(2)	10	5×10^5	49.3 ± 2.2*	<0.0005
(3)	10	5×10^4	48.3 ± 3.6*	<0.0005
(4)	10	5×10^3	50 +	
		Saline Incubated with EAT (control)		
(1)	6	1×10^6	23.3 ± 5.1*	
(2)	6	5×10^5	26.1 ± 2.3*	
(3)	6	5×10^4	29.5 ± 4.4*	
(4)	6	5×10^3	39.7 ± 4.3*	

* One standard deviation
\+ All mice alive at 50 days

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