ELEVATED RIBONUCLEASE ACTIVITY IN THE THYMUS AND WHITE BLOOD CELLS OF GENETICALLY CANcer PRONE MICE

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Several inbred mouse strains are characterized by a high incidence of spontaneous neoplastic transformation which begins generally after 5 mo of age and results in death by 13 mo. The presence of oncogenic virus in a number of these strains has made these popular models for the in vivo study of virus-induced tumor development (1, 2). Because of our interest in determining why certain strains of mice develop tumor at a late age even though the virus is present at birth, as well as our interest in detecting biochemical anomalies in such mice before the onset of disease, we have examined ribonuclease activity in lymphoid cells. Ribonucleases are a class of enzymes which degrade RNA to oligonucleotides and which can play a role in both the restriction of the RNA virus and the synthesis of virus RNA resulting in eventual transformation of infected cells. Our results demonstrate that of 10 mouse strains tested, those six characterized by a high incidence of spontaneous tumor at an early age have elevated ribonuclease activity associated with the thymus and/or peripheral white blood cells.

Materials and Methods

Description of Mice Chosen for Study. 8-wk old, virgin female mice of the following strains were obtained from the Jackson Laboratories, Bar Harbor, Maine: AKR, BALB/c, C3H/He, C57BL/6, DBA/2, C58, NZB, PL, RF, SJL. Six of these strains (AKR, C3H/He, C58, PL, RF, and SJL) are characterized by a high incidence of spontaneous tumors and were therefore considered "high risk" or "high incidence" strains for purpose of this study (Table I).

The strains C57BL/6, DBA/2, and BALB/c were considered to be "low incidence controls" because they represent classic examples of strains which have low incidences of spontaneous neoplasia, and for which the major cause of death is the sum of various vectors relating to old age (Table I). Although some neoplastic lesions may be found in these animals at autopsy, such tumors are not the primary cause of death. Rather, foci of neoplastic transformation are observed only after extensive microscopic evaluation of serially sectioned tissue prepared at autopsy. These three strains, in which neoplastic lesions are coincidental with old age, are thus distinguished from those strains in which such lesions become invasive and result in premature death.

The NZB strain serves as an additional control because although such mice are infected with the same C-type RNA virus associated with leukemia in the AKR strain, NZB mice effectively restrict neoplastic development and die instead of glomerulonephritis resulting from an autoimmune response to the tumor virus (3).

Pathological Findings at Autopsy of Sacrificed Mice. Postmortem examination revealed the...
While microscopic foci of neoplastic transformation were present in the indicated percentage of aged animals at autopsy, in general, the tumors did not cause death. Absence of tumor on gross examination and this was confirmed on microscopic examination of various tissues including the spleen and thymus. Although the size of the thymus varies from strain to strain, all thymus homogenates were adjusted to equal protein concentration before the assay of ribonuclease activity in order to equalize such disparities.

Assay of Ribonuclease Activity. The ability of various samples to degrade synthetic polynucleotides to acid-soluble mono- and oligonucleotides was determined by the method of Levy et al. (4). The polynucleotide substrate, polyuridylic acid (poly U), was obtained from P-L Biochemicals, Milwaukee, Wis.

For the assay of thymus-associated ribonuclease activity, thymuses from five mice were homogenized at 4°C in teflon pestle homogenizers containing 3 ml saline. Cell debris was removed by centrifugation for 15 min at 10,000 g and supernatant solutions were collected. The protein concentration of the samples was measured by ultraviolet absorption (5) which compared favorably to values determined by the fluorescamine assay (6). All samples were adjusted to 7.5 mg protein/ml. A typical incubation mixture contained 0.1 ml sample, 0.1 ml poly U (2.5 mg/ml), 0.3 ml H2O, and 0.5 ml 0.2 M acetate buffer, pH 5.0. Controls consisted of a sample blank (containing no nucleic acid) and a substrate blank (containing no sample). These were handled identically to experimental mixtures. After incubation of the mixtures at 37°C, the reactions were terminated by 2.0 ml cold 12% perchloric acid containing 20 mM lanthanum nitrate. After cooling for 15 min at 4°C, the reaction mixtures were centrifuged 15 min at 3,000 g, and the amount of degradation to acid-soluble nucleotides was determined at 260 nm with a Beckman DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) using a 1 cm path length cuvet. Control values were subtracted from experimental values and although Fig. 1 presents the amount of degradation observed at various times, only the linear range of enzyme activity was used to determine units of activity. A unit of ribonuclease activity is defined as an increase of 0.1 unit absorbance at 260 nm in 5 min under assay conditions.

For the assay of white blood cell (WBC)-associated activity, blood (5 ml) from each strain was collected in 40-ml heparinized saline. Cell debris was removed by centrifugation for 15 min at 10,000 g and supernatant solutions were collected. The protein concentration of the samples was measured by ultraviolet absorption (5) which compared favorably to values determined by the fluorescamine assay (6). All samples were adjusted to 7.5 mg protein/ml. A typical incubation mixture contained 0.1 ml sample, 0.1 ml poly U (2.5 mg/ml), 0.3 ml H2O, and 0.5 ml 0.2 M acetate buffer, pH 5.0. Controls consisted of a sample blank (containing no nucleic acid) and a substrate blank (containing no sample). These were handled identically to experimental mixtures. After incubation of the mixtures at 37°C, the reactions were terminated by 2.0 ml cold 12% perchloric acid containing 20 mM lanthanum nitrate. After cooling for 15 min at 4°C, the reaction mixtures were centrifuged 15 min at 3,000 g, and the amount of degradation to acid-soluble nucleotides was determined at 260 nm with a Beckman DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) using a 1 cm path length cuvet. Control values were subtracted from experimental values and although Fig. 1 presents the amount of degradation observed at various times, only the linear range of enzyme activity was used to determine units of activity. A unit of ribonuclease activity is defined as an increase of 0.1 unit absorbance at 260 nm in 5 min under assay conditions.

| Strain     | Sex | Neoplasm resulting in death | Incidence | Age at onset of disease | Mean lifespan |
|------------|-----|----------------------------|-----------|-------------------------|---------------|
| AKR        | F   | Lymphocytic leukemia       | % mo mo %| 91 8 90 13             | 91 90         |
| SJL        | F   | Reticulum cell neoplasm, type B (Hodgkin's) lesion | 62 | — 14 1/2 | |
| RP         | F   | Leukemia, primarily reticulum cell sarcoma | 90 10 11 1/2 | 14 1/2 | |
| CS8        | F   | Lymphocytic leukemia      | 71 — 14 1/2 | 14 1/2 | |
| PL         | F   | All tumors               | 50 8.8 11 | 11 | |
| C3H/He     | F   | Mammary carcinoma        | 60-100 4 10-12 | 10-12 | |
| NZB        | F   | Death due to autoimmune glomerulonephritis, although reticulum cell neoplasm observed in some at autopsy | — | 4 | 10-12|
| C57BL/6    | F   | Nonspecific, occurring late | 14* Late 22 | 22 | |
| Balb/c     | F   | Nonspecific, occurring late | 29* Late 19 | 19 | |
| DBA/2      | F   | Mammary tumors, occurring late | 39* 14 | 23 1/2 | |

* While microscopic foci of neoplastic transformation were present in the indicated percentage of aged animals at autopsy, in general, the tumors did not cause death.

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1 Both the thymus and WBC ribonuclease activity directed against poly U were optimal at pH 5.0.
reproducibility of these studies. After decantation, the cells were resuspended in 2 ml saline and then counted. A 50% recovery of cells having 100% viability was achieved, and after adjusting the cell concentration to $10 \times 10^6$/ml, an equal volume of 0.2% Triton-X (Sigma Chemical Co., St. Louis, Mo.) was added. The cell preparation was placed at 37°C for 10 min with intermittent mixing. The WBC-lysate was immediately centrifuged (3,000 g, 10 min), and 0.1 ml supernatant solution was used in the assay described above. One unit of ribonuclease activity was defined as a change in optical density of 0.1 unit absorbance at 260 nm in 20 min under assay conditions.

**Results**

*Ribonuclease Activity Associated with the Thymus of 8-Wk Old Female Mice.* Fig. 1 presents a typical assay of the ribonuclease activity for cell-free thymus homogenates. The degradation of poly U to acid soluble mono and oligonucleotides was followed by the increased optical density observed at 260 nm after centrifugation of acid-precipitable macromolecules. The figure shows that the slopes of the curves (and hence the enzyme activities) were greater for all five strains possessing a high incidence of hematogenous malignancies - leukemia and reticulum cell neoplasms: AKR (●), C58 (○), PL (▲), RF (△), SJL (○); C3H/He mice (■) develop a spontaneous mammary carcinoma and the nuclease activity for NZB mice (■) was similar. The following were used as controls: C57BL/6, BALB/c, and DBA/2, all represented by (X).

![Graph](image)

Fig. 1. Thymus-associated ribonuclease activity directed against poly U. Cell-free thymus homogenates were prepared and adjusted to 7.5 mg/ml using five thymuses from each strain of mouse. Each point represents the degradation of polyuridylic acid to acid-soluble nucleotides when the enzyme reactions were stopped with perchloric acid. The following are characterized by a high incidence of spontaneous lymphatic tumors (leukemia and reticulum cell neoplasms: AKR (●), C58 (○), PL (▲), RF (△), SJL (○); CeH/He mice (■) develop a spontaneous mammary carcinoma and the nuclease activity for NZB mice (■) was similar. The following were used as controls: C57BL/6, BALB/c, and DBA/2, all represented by (X).

The following are characterized by a high incidence of lymphatic malignancies resulting in premature mortality, were found to have a statistically significant elevation in thymus-associated RNase

Table II presents a statistical analysis of five such experiments. The AKR, SJL, RF, C58, and PL strains of mice, all of which are characterized by a high incidence of lymphatic malignancies resulting in premature mortality, were found to have a statistically significant elevation in thymus-associated RNase.
Thymus-Associated and White Blood Cell-Associated Ribonuclease Activity

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Table II
Thymus-Associated and White Blood Cell-Associated Ribonuclease Activity

| Strain               | Thymus Rnase activity | WBC Rnase activity |
|----------------------|-----------------------|--------------------|
|                      | Mean µ/ml              | SD µ/ml            |
|                      | P value §              | Mean µ/ml          |
|                      |                       | SD µ/ml            |
|                      |                       | P value §          |
| Low incidence control| 2.8 ± 1.2             | 2.4 ± 0.5          | —                  |
| C57BL/6              | 3.5 ± 0.9             | 2.5 ± 0.4          | —                  |
| BALB/c               | 2.3 ± 0.9             | 3.0 ± 1.2          | —                  |
| NZB                  | 3.1 ± 1.5             | 2.6 ± 0.4          | —                  |
| High Incidence       |                       |                    |
| hematologic malignancy|                      |                    |
| AKR                  | 15.8 ± 1.8            | 5.0 ± 1.0          | <0.001             |
| SJL                  | 6.7 ± 1.8             | 2.9 ± 0.7          | NS                 |
| RF                   | 17.9 ± 1.7            | 4.8 ± 0.7          | <0.001             |
| C3H                  | 9.8 ± 1.2             | 2.5 ± 0.6          | NS                 |
| PL                   | 5.1 ± 0.7             | 5.9 ± 0.8          | <0.001             |
| High incidence       |                       |                    |
| mammary carcinomas   | 3.2 ± 1.4             | 5.2 ± 0.7          | <0.001             |

* Cell free thymus homogenates were assayed for ribonuclease activity against poly U. One unit of activity is defined as a change in optical density of 0.1 at 260 nm in 5 min under assay conditions. For the assay of WBC-associated activity, the white cells isolated from 5 ml blood of each strain were adjusted to a concentration of 10 x 10^6/ml before lysis with an equal volume of 0.2% Triton X. After centrifugation, the supernatant solution was analyzed for ribonuclease activity directed against poly U. One unit of activity is defined as a change in optical density of 0.1 at 260 nm in 20 min under assay conditions.

$P$ value was determined by the Student's t test in which the activity for the high incidence tumor strains was compared to the total combined data of the control strains, C57BL/6, BALB/c, DBA/2, and NZB. NS indicates not significantly different from controls.

activity directed against poly U. Based upon a chi square test, the probability is greater than 99.9% that such elevated thymus-associated activity relates to the predisposition of these strains toward development of spontaneous tumor.

Similarly, the ribonuclease activity of WBC lysates was determined. The four strains, AKR, RF, PL, and C3H/HeJ had abnormally elevated WBC-associated RNase activity directed against poly U (Table II). The C3H/HeJ strain, 60% of which may develop a mammary carcinoma, had this increased activity, although thymus-associated activity was normal. Again, activity observed for the NZB strain was normal. In addition, the analysis of this data by the chi square test shows that the probability is greater than 99.9% that the elevated WBR activity relates to the high incidence of spontaneous tumor in these four strains.

Discussion

This study demonstrates that ribonuclease activity is a useful biochemical marker which distinguishes those strains of mice that are prone to develop spontaneous neoplasia from those strains in which cancer occurs less frequently and is not the major cause of death. Mice of all five strains characterized by a high propensity to develop lymphatic tumors had elevated thymus-associated ribonuclease activity and three of these had elevated WBC activity as well (Table II). The C3H/HeJ strain, which tends toward spontaneous mammary carcinoma, exhibited only elevated white cell activity. In short, every strain which is predisposed toward the development of a specific spontaneous tumor had
abnormally elevated ribonuclease activity in cell-free thymus and/or white cell preparations. This abnormal activity was observed in young mice, free of clinically detectable disease. Although there is no way of knowing which of the mice sacrificed might have failed to develop spontaneous tumor, the point to be made is that the genetic susceptibility of a strain toward neoplastic disease can be identified before the onset of neoplasia.

The abnormal enzyme activity observed in the thymus may reflect the variations in the type, number, or function of the various subpopulations of thymus cells (8). However, there is no apparent correlation of elevated enzyme activity with morphological differences in the blood. Indeed, few if any such morphological differences exist for the strains tested (9).

Whether elevated thymus ribonuclease activity relates to a protective mechanism which prevents the development of lymphatic tumors at an early age, or whether such levels represent the partial transformation of thymocytes by C-type virus is unclear. A partial transformation would allow for the production of virus progeny concomitant with the maintenance of specialized cell functions, as observed for example in the AKR strain (10). Mice of the NZB strain are infected by Gross virus, but unlike the AKR strain, die of glomerulonephritis rather than of leukemia. The observation that the NZB possesses normal thymus activity while the AKR has elevated activity indicates that increased ribonuclease levels in the thymus represent an increased risk of eventual leukemia rather than simply the infection by an oncogenic agent. The data suggest the importance of maintaining some required balance in nucleic acid metabolism which allows cells to function normally in the presence of virus at an early age although spontaneous tumor develops at some later time. Although the full physiologic significance of the data presented is yet unclear, the marked association of elevated ribonuclease activity with the propensity to develop neoplastic disease suggests that such assays may be a useful biochemical probe of the genetics of cancer.

Summary

Ribonuclease activity in cell-free thymus homogenates was elevated for five strains of mice genetically predisposed toward leukemia or reticulum cell neoplasms (AKR, C58, PL, RF, and SJL). Such increased activity was directed against polyuridylic acid and was observed in 8-wk old mice, well before the onset of neoplastic transformation. Similarly, white blood cell ribonuclease activity was elevated in mice of the strains AKR, C3H/He, PL, and RF. Statistical analysis indicated that such elevated activity in these strains related to their high incidence of spontaneous neoplastic disease. Elevated ribonuclease activity thus represents a new biochemical marker relating to the genetic propensity of some strains of mice to die prematurely of spontaneous neoplasia.

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