 Differences in the Properties of Cytoplasmic Particles and in the Expression of Virus-specific Information in the Thymus of Normal and Lymphomatous AKR Mice

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ABSTRACT

The properties of virus-like cytoplasmic particles in thymic tissue from normal and lymphomatous AKR mice and the expression of leukemia-related sequences in these tissues have been investigated. The retrovirus nature of the particles was indicated by the presence of RNA-dependent DNA polymerase as judged by the ability of the associated enzymatic activity to carry out endogenous RNase-sensitive DNA synthesis or utilize polycytidylicate and poly(2'-O-methylcytidylate) and by the chromatographic properties of the purified enzyme. Equilibrium centrifugation studies of the particles extracted from the two types of tissue revealed a difference in the respective buoyant densities. A density of 1.157 g/ml was estimated for particles isolated from lymphomatous thymic tissue and for the Gross passage A virus, a leukemogenic strain of AKR murine leukemia virus. In contrast, a peak value of reverse transcriptase activity was observed at 1.145 g/ml in the gradient analysis of cytoplasmic particles prepared from the thymus of 1- and 2-month-old mice. Molecular hybridization studies aimed at the detection of differences in the expression of viralspecific information in RNA extracted from the normal and lymphomatous thymic tissues were carried out with a Moloney MuLV complementary DNA probe. Forty to 50% of the probe hybridized with the RNA from normal thymic tissue with an equivalent value of the product of the time (sec) of hybridization and the RNA concentration (moles of nucleotides per liter) at which 50% hybridization is reached; C_{t,m} product of the time (sec) of hybridization and the RNA concentration (moles of nucleotides per liter) reached; C_{t,m} product of the time (sec) of hybridization and the RNA concentration (moles of nucleotides per liter) is hybridized at a rate suggestive of approximately 100 genome equivalents per cell. About 80% of the probe hybridized with the RNA from lymphomatous tissue.

INTRODUCTION

Several literature reports have suggested that the leukemia that occurs as a thymic lymphoma in the AKR mouse strain, which has a high incidence of leukemia, is preceded by an extended preleukemic period during which some as yet undefined event(s) takes place that leads to the overt expression of the disease. Histological studies of the thymus (21) revealed abnormal changes in the morphology of the target organ preceding the development of the disease at a time when no transplantable leukemia cells could be detected. A leukemogenic factor able to accelerate the appearance of leukemia when injected into newborn AKR mice has been found in the thymus but not in the spleen of mice as young as 3 months of age, and this factor was shown to increase as a function of age (15, 22, 23). However, studies examining the tissue distribution and time course of appearance of the MuLV known to infect chronically the AKR mouse strain indicated that the thymic level of virus quantitated by the XC plaque test for infectious virus was low and constant until the overt expression of the disease (26). Recent reports have shown that an increase in expression of MuLV antigens on thymic cell surfaces occurs in preleukemic AKR mice (17) and further, that 2 additional classes of virus, xenotropic and N-tropic-amphotropic, appear in the thymus but not in other lymphoid organs (14, 16). It has been suggested that a recombinant between the ecotropic and xenotropic viruses might be the etiological agent in AKR leukemia (8, 14). In this communication, we report (a) biochemical evidence of differences between virus-like particles found in the thymus of lymphomatous
AKR mice and particles found in the thymus of normal, healthy mice and (b) evidence from molecular hybridization studies that additional viral information homologous to M-MuLV is expressed in the RNA of lymphomatous thymus as compared to the RNA of normal thymic tissue. The relevance of such findings to the development of the disease is discussed.

MATERIALS AND METHODS

Mice. Female AKR mice used throughout this study were obtained primarily from a colony established by Dr. Ralph Graff at The Jewish Hospital of St. Louis (St. Louis, Mo.); mice obtained from The Jackson Laboratory, Bar Harbor, Maine, were also used.

Virus. Lyophilized Gross passage A virus was purchased from the American Type Culture Collection, Rockville, Md. It was reconstituted in 1 ml distilled H2O to a protein concentration of 0.5 mg/ml and was stored at -70°. The virus was banded on sucrose gradients as described below. M-MuLV was purified from the growth medium of infected normal rat kidney cells, as described (12).

Preparation of Cytoplasmic Particles. Comparable amounts of thymic tissue, approximately 5 g, were obtained from 60 to 65 mice of a particular age (1 or 2 months) or from 8 to 9 lymphomatous retired breeders. The mice were sacrificed by cervical dislocation, and the thymuses were removed and Dounce-homogenized at 4° in 5 ml of TNE buffer per g of tissue. Nuclei and cell debris were sedimented by centrifugation at 2000 rpm in an International refrigerated centrifuge; the pellet was resuspended in the original volume of buffer and homogenized a second time; the suspension was centrifuged again at 2000 rpm. The supernatants from these centrifugations were combined and centrifuged for 20 min at 10,000 rpm in an SS-34 rotor of a Sorvall RC-2 centrifuge. The resulting postmitochondrial supernatants were layered on 2-mL cushions of 20% glycerol in TNE buffer and centrifuged for 60 min at 27,000 rpm in a SW-41 rotor; this and all subsequent centrifugations were carried out in a Spinco L-2 centrifuge. The pellets (27K) were carefully resuspended in 3 ml of TNE buffer, layered on 33 ml 15 to 65% sucrose gradients in TNE buffer, and spun to equilibrium by centrifugation for 16 hr at 25,000 rpm in an SW-27 rotor. The refractive index, protein concentration, and DNA polymerase activity were determined for fractions across the gradients. Fractions with densities between 1.10 and 1.11 g/ml were then pooled, diluted with buffer, and centrifuged for 90 min in a Ti-60 rotor at 45,000 rpm. The pellets (45K) were resuspended and layered on 33 ml 15 to 65% sucrose gradients and recentrifuged to equilibrium.

Enzyme Purification. Purification of DNA polymerase activities was carried out according to the method of Lewis et al. (20). Briefly, after the final centrifugation described previously, sucrose gradient fractions with densities between 1.10 and 1.11 g/ml were pooled and brought to 0.35 M KCl and 0.5% Triton. For removal of nucleic acid, the pool was then passed through a DEAE-cellulose column (2.5 x 3.5 cm) equilibrated with Buffer A in 0.35 M KCl. The material not retained by the DEAE-cellulose was dialyzed overnight against Buffer A and then was passed over a second DEAE-cellulose column in Buffer A. The column was washed, and the sample was eluted with Buffer A in 0.075 M KCl. This eluate was passed directly onto a phosphocellulose column (0.9 x 12 cm), equilibrated with Buffer A, and pretreated with bovine serum albumin (28). The column was washed with Buffer A, and a 180-ml gradient, 0.1 to 0.6 M KCl in Buffer A containing 0.02% Triton and bovine serum albumin, 100 µg/ml, was applied. Fractions were collected, and DNA polymerase activities were measured with different template primers as indicated.

DNA Polymerase Assays. DNA polymerase activity associated with cytoplasmonic particles was measured at 37° in the following reaction mixture (0.1 ml): 20 mM Tris-Cl, pH 8; 10 mM NaCl; 10 mM dithiothreitol; 1 mM MnCl2; 0.1% NP-40; labeled triphosphate at 1.6 x 10⁻³ M and 15 µCi/assay; template at 20 µg/ml; and primer at 5 µg/ml. [³H]dGTP was the labeled substrate when poly(C) and oligo(dG) were used as template and primer, and [³H]dTTTP was the labeled substrate with poly(A) or poly(dA) and oligo(dT). When poly(C)m was used, the concentration of MnCl2 was 0.2 mM (10). NP-40 and NaCl were omitted from the assay mixture when the activity of purified DNA polymerases was measured. The reaction was terminated by the addition of 10 µl of 0.2 M EDTA and 1 mM ATP, and the amount of polymer product was determined by the radioactivity retained on DEAE-cellulose paper discs (2).

The assay mixture for endogenous DNA polymerase activity (RNase-sensitive DNA polymerase activity measured in the absence of added template) contained: 40 mM Tris-Cl, pH 8; 30 mM NaCl; 10 mM dithiothreitol; 1 mM MnCl2; 0.1% NP-40; and 0.1 mM dATP, dCTP, and TTP with [³H]dGTP (9.3 Ci/mmol) at 1.6 to 3.2 x 10⁻⁵ M and 15 to 30 µCi/assay or 0.1 mM dATP, dCTP, and dGTP with [³H]dTPP (51 to 60 Ci/ mmol) at 1.4 x 10⁻⁵ M and 75 µCi/assay. The reaction was terminated by the addition of 50 µl of a mixture containing 40 mM EDTA, 1 mM ATP, and calf thymus DNA, 400 µg/ml. Cold 1 N perchloric acid was then added, and after 10 min at 4° the precipitate was collected by centrifugation. The precipitate was repeatedly (3 times) dissolved in 0.2 M NaOH, reprecipitated with 1 N perchloric acid, and centrifuged. After the final centrifugation, the pellet was suspended in cold 5% TCA, collected on a nitrocellulose membrane, and washed with cold 5% TCA followed by 0.1% HCl. The filters were dried, and the incorporated radioactivity was measured in a liquid scintillation counter.

RNA Extraction. Three g of thymic tissue were Dounce-homogenized in 20 ml of 0.05 M sodium acetate, pH 5.1:0.002 M EDTA. One % sodium dodecyl sulfate and 500 µg/ml of preincubated pronase were then added, and the mixture was incubated at 37° for 30 min. Nucleic acid was extracted according to the procedure described by Scherrer (27) with water-saturated phenol containing 0.1% hydroxyquinoline at 55°, followed by 3 extractions of the aqueous phase with an equal volume containing 1 part of ethanol and 1 part of chloroform:isoamyl alcohol (24:1) at 22-23°. Potassium acetate was then added to a final concentration of 0.3 M, and nucleic acid was precipitated with 2.5 volumes of ethanol at -20°. The precipitate was collected by centrifugation, resuspended in 0.05 M sodium acetate, pH 5.1:0.01 M Mg²⁺, and incubated at 37° for 60 min with ribonuclease-treated DNase, 50 µg/ml, followed by a
then combined, and the RNA was precipitated with ethanol. The precipitate was resuspended in 0.01 M Tris-Cl, pH 7.4:0.001 M EDTA to a final concentration of approximately 30 mg/ml. The RNA content was determined by a microquantitative modification of the colorimetric reaction with orcinol (24) suggested by Zamenhof (32). The M-MuLV RNA was prepared as described by Bondurant et al. (4).

Preparation of [3H]DNA Complementary to the M-MuLV Genome. Five mg of M-MuLV were incubated at 37° for 24 hr in a 5 ml reaction mixture containing 2 x 10⁻³ M ATP, GTP, and CTP and 1 mCi of [3H]TTP (specific activity, 50 Ci/mmol); 50 mM Tris-Cl, pH 8.2; 25 mM NaCl; 9 mM magnesium acetate; 6 mM dithiothreitol; 0.01% Triton; and actinomycin D, 50 µg/ml. The reaction was terminated by the addition of 0.02 M EDTA. The mixture was then incubated at 37° for 30 min with proteinase K, 400 µg/ml (EM Laboratories, Inc., Elmhford, N. Y.) in the presence of 0.5% sodium dodecyl sulfate. Yeast tRNA, 50 µg/ml, was added, and the mixture was deproteinized by 3 successive extractions with an equal volume of phenol:chloroform:alcohol. The aqueous phases were deproteinized by 3 successive extractions with an equal volume of phenol:chloroform:alcohol. The aqueous phases were combined, and potassium acetate was added to 0.3 M. Nucleic acid was precipitated with ethanol, redissolved in 0.3 M NaOH, and hydrolyzed at 37° for 18 hr, followed by neutralization with HCl. Yeast tRNA, 50 µg/ml, was then added, and the mixture was loaded onto a Sephadex G-50 column equilibrated with 0.02 M ammonium bicarbonate to remove unreacted triphosphates. The eluted cDNA was lyophilized and subsequently resolubilized in 0.01 M Tris-Cl, pH 7.4:0.001 M EDTA buffer. The [3H]cDNA was at least 99% acid soluble upon treatment with S1 nuclease.

DNA:RNA Hybridization. The reactions of hybridization between the Moloney cDNA probe and viral or cellular RNA were carried out in a 32-µl volume containing 50 µg of yeast tRNA; 0.7 M NaCl; 50 mM Tris-Cl, pH 7.5; 2 mM EDTA; 0.1 ng of cDNA (2000 cpm), and an appropriate amount of RNA. The hybridization mixtures were sealed in siliconized microcapillaries, heated for 2 min at 100°, and then incubated at 68° for the indicated periods of time. After incubation the capillaries were rapidly cooled, and the contents were incubated with S1 nuclease to determine the percentage of hybridization (11). The assay mixture for S1 nuclease digestion contained: an empirically determined amount of nuclease; 0.03 M sodium acetate, pH 4.6; 0.05 M NaCl; 0.001 M ZnSO₄; 5% glycerol; and 100 µg of heat-denatured calf thymus DNA, in a final volume of 1 ml. After 2 hr of incubation at 44°, 50 µg of undenatured calf thymus DNA were added to each assay tube, and the reaction was terminated by the addition of 10% TCA at 4°. The acid-insoluble cpm were collected on B6 nitrocellulose filters that were washed with 10% TCA, dried, and counted in organic scintillation fluid.

Reagents. RNase A was from Worthington Biochemical Corp., Freehold, N. J. [3H]TTP and [3H]dGTP were obtained from New England Nuclear, Boston, Mass.; unlabeled deoxyribonucleotides were obtained from P-L Biochemicals, Milwaukee, Wis.; oligo(dT)₁₂₋₁₈ and oligo(dG)₁₂₋₁₈ were obtained from Collaborative Research, Waltham, Mass. Poly(Cm) was kindly provided by Dr. G. Gerard, Institute for Molecular Virology, St. Louis University School of Medicine, St. Louis, Mo. DEAE-cellulose (DE-52), phosphocellulose (P-11), and DEAE-cellulose paper discs were from H. Reeve Angel & Co., Inc., Clifton, N. J. Nitrocellulose filters (B6) were from Schleicher & Schuell, Keene, N. H. S1 nuclease was purified from α-amylase as described by Sutton (29).

RESULTS

Presence of Virus-like Particles in Normal and Lymphomatous Thymic Tissue. In this work the presence of virus-like particles was demonstrated in the cytoplasmic fraction of thymic cells from normal and lymphomatous mice with RNA-dependent DNA polymerase activity and buoyant density measurements as the criteria for detection. Poly(C), a template utilized by the RNA-dependent DNA polymerase of retroviruses and under appropriate assay conditions by cellular DNA-dependent DNA polymerase γ (18), was used as a probe for the viral enzyme; where possible, endogenous activity, defined as RNase-sensitive DNA polymerization in the absence of added template, was also measured to confirm the viral origin of the enzymatic activity.

As indicated in Table 1, poly(C)-directed enzymatic activity was associated with cytoplasmic particles of both normal and lymphomatous tissue. Poly(Cm), a template that is not copied by cellular DNA-dependent DNA polymerases and is thus highly specific for the RNA-dependent DNA polymerase (9), was also utilized. A ratio of poly(C)- to poly(Cm)-directed incorporation of about 3:1 was observed when the respective optimal concentrations of Mn²⁺ ions, 1 and 0.2 mM were used (Table 1). When Mg²⁺, 5 to 10 mM, was used with poly(C) instead of Mn²⁺, both templates were equally effective, as reported for purified MuLV RNA-dependent DNA polymerase (10). Therefore DNA polymerase γ if present does not contribute to the poly(C) response. The possibility that the measured [3H]dGTP incorporation was due to cellular terminal transferase was examined since this enzyme, which is specific for thymic tissue (6), has been

<table>
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<th>Template primer</th>
<th>2 mos. old</th>
<th>Lymphomatous</th>
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<tr>
<td>Poly(C):oligo(dG)</td>
<td>12,000</td>
<td>87,000</td>
</tr>
<tr>
<td>Poly(Cm):oligo(dG)</td>
<td>3,000</td>
<td>32,000</td>
</tr>
<tr>
<td>Oligo(dG)</td>
<td>900</td>
<td>800</td>
</tr>
<tr>
<td>Background</td>
<td>400</td>
<td>400</td>
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a Terminal transferase activity, with the standard DNA polymerase assay mixture minus template.
b cpm incorporated at time 0.

Table 1

RNA-dependent DNA polymerase in thymic particles from 2-month-old and lymphomatous mice

Fifty-eight 2-month-old mice and 7 lymphomatous retired breeders were sacrificed to give 6.2 and 4 g of thymic tissue, respectively. Cytoplasmic particles were isolated by means of 2 successive equilibrium gradient centrifugations. Aliquots were taken from the resuspended 45K pellet for assay of RNA-dependent DNA polymerase activity; 78 and 65 µg/ml of protein from 2-month-old and lymphomatous sources, respectively, were used. All assays were done in duplicate, and the average values are reported without background correction.
shown to elongate oligo- and polydeoxyribonucleotides at the 3'-hydroxyl end by the addition of deoxyribonucleotides. No significant incorporation above background levels was observed when template was omitted from the assay mixture.

For further characterization of the thymic particles, the postmitochondrial pellets were subjected to successive equilibrium centrifugations on 15 to 65% sucrose density gradients, and poly(C)-directed and endogenous activities were measured across the gradients. Chart 1 illustrates the density distribution patterns of RNA-dependent DNA polymerase-containing particles from normal and lymphomatous thymus after 2 centrifugations on 15 to 65% sucrose gradients. With particles from lymphomatous thymus, a peak of poly(C)-directed activity was observed in the density region characteristic of C-type virus at about 1.156 g/ml with a shoulder at 1.136. In a parallel gradient with particles from 2-month-old mice, the peak sedimented at a buoyant density of 1.136 g/ml. An average density difference of 0.0125 was calculated from several paired determinations measuring the position of peak reverse transcriptase activity in thymic particles from normal (1- to 2-month-old) and lymphomatous mice (Table 2); the mean density values were 1.1456 and 1.1574 g/ml, respectively. A mean value of 1.1571 g/ml was observed with the AKR Gross passage A virus.

With the particles from lymphomatous thymus, the peak of endogenous DNA polymerase activity sedimented at the same position as did the poly(C)-directed activity. No endogenous activity was detectable in the gradient containing particles from the thymus of 2-month-old mice. When dGTP polymerization was measured with poly(C) as template and oligo(dT) instead of oligo(dG), no incorporation was observed, thus ruling out the presence of terminal transferase or oligo(dT) instead of oligo(dG), no incorporation was observed, thus ruling out the presence of terminal transferase activity. No significant incorporation above background levels was observed when template was omitted from the assay mixture.

Table 2

<table>
<thead>
<tr>
<th>Source</th>
<th>Buoyant density (ρ) (g/ml)</th>
</tr>
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<tbody>
<tr>
<td>1- to 2-month-old mice</td>
<td>1.1456 ± 0.0077* (7)*</td>
</tr>
<tr>
<td>Lymphomatous mice (L)</td>
<td>1.1574 ± 0.0064 (8)</td>
</tr>
<tr>
<td>Passage A virus</td>
<td>1.1571 ± 0.0012 (2)</td>
</tr>
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*Mean ± S.D.
*Numbers in parentheses, number of determinations.
*Mean of difference in density observed in paired determinations.
*With particles from lymphomatous and 1- to 2-month-old mice.

ase and is also utilized by the nuclear DNA polymerase y. It is not accepted by the cellular DNA-dependent DNA polymerase α and only under particular assay conditions is it copied by the nuclear DNA-dependent DNA polymerase β. Poly(dA) is copied by the DNA polymerase β and less effectively by y and α in order of decreasing efficiency; it is a poor template for the RNA-dependent DNA polymerase (see Ref. 31 for a review). Our results showed that the profile of poly(A)-directed activity paralleled that observed with poly(C), both for particles isolated from thymic tissue from 1- and 2-month-old as well as from lymphomatous mice (Charts 1 and 2).

With particles from normal thymic tissue, the pattern of poly(dA)-directed activity paralleled that of poly(A), and peak values were found at the same low density position (Chart 2A). In gradients examining particles from lymphomatous tissue, the peak of poly(dA)-directed activity was associated with the particles of low density (Chart 2B).

**Chromatographic Separation of DNA Polymerase Activities.** Enzyme purification was carried out to substantiate the presence of RNA-dependent DNA polymerase in particles from normal and lymphomatous thymic tissue and to separate and characterize the DNA polymerase activity with poly(dA). The activities were solubilized from rebanded thymic particles and were subjected to a 2-step purification procedure involving DEAE-cellulose and phosphocellulose chromatography. In the DEAE-cellulose step, the enzymatic activity was eluted with 75 mm KCl, a salt concentration that allows elution of RNA-dependent DNA polymerase and DNA polymerase β. DNA polymerases α and γ, if present, would be retained (20). Following the subsequent phosphocellulose step, alternate fractions from the column were tested.
for the ability to carry out DNA synthesis directed by several template primer systems. The results of the analysis (Chart 3) demonstrated that the DNA polymerase activity that utilizes poly(A) as template was eluted at 0.2 M KCl, characteristic of RNA-dependent DNA polymerase. It was completely separated from the activity that utilized poly(dA), which was eluted at 0.42 M KCl, characteristic of cellular DNA polymerase β. The profile of poly(C)-directed activity paralleled that of poly(A) in the material purified from lymphomatous tissue (Chart 3B), thus confirming the RNA-dependent DNA polymerase nature of the enzymatic activity eluted at 0.2 M KCl. Poly(C)-directed activity was also observed in the peak fraction of the poly(A)-directed activity when the enzymatic activity purified from normal thymic tissue was examined identically (see legend to Chart 3A).

Additional Expression of Viral Information Related to M-MuLV Genome in the Thymus of Lymphomatous AKR Mice. For detection of any change in the expression of viral RNA that might lead to a better understanding of the age-dependent onset of leukemia, molecular hybridization experiments were designed to detect leukemia-related sequences in the RNA extracted from the thymus of 2-month-old and lymphomatous AKR mice. A [3H]cDNA probe was synthesized with M-MuLV. The Moloney virus was chosen because (a) it has sequence homology with the endogenous, but nonleukemogenic AKR virus [50 to 70% when the extent of cDNA:RNA hybridization was detected by chromatography on hydroxyapatite, S. Chattopadhyay, personal communication; 50% by S, nuclease digestion (30)], and (b) it induces the same type of thymic lymphoma as the AKR passage A virus (13), which suggests the presence of leukemia-specific information in its genome. The [3H]cDNA probe was hybridized with excess viral or cellular RNA to ensure that the virus-specific RNA concentration remained unchanged during the hybridization reaction. Under these conditions, hybridization of the cDNA is a pseudo-first-order reaction (1) with the measured C{l/s} values for the RNA preparations reflecting the relative fractions of the RNA that are virus specific (19).

The labeled probe was first hybridized to excess 70S RNA from M-MuLV; the final extent of hybridization was 80 to 85% (Chart 4). The symmetry of the curve and the C{l/s} value of 0.03 indicate that the probe is representative of the viral genome. In contrast, only 40 to 50% of the hybridizable sequences in the probe were homologous to RNA sequences in cells from the thymus of normal mice; an approximate C{l/s} of 1 x 10\(^{-3}\) mol × sec/liter was observed for the hybridization of these sequences, indicating that the fraction of this RNA that is virus specific is 3.0 x 10\(^{-8}\). When the [3H]cDNA was hybridized with RNA extracted from the thymus of lymphomatous mice, a different pattern of hybridization kinetics was observed. At low C{l/s}'s, the curve resembles that observed when the probe is hybridized with RNA from normal tissue; however, a much higher number of viral genome equivalents are expressed per cell. At higher C{l/s} values, new base sequences homologous to the Moloney probe are detected. Tentatively, the curve can be considered the sum of 2 plots with C{l/s} of 6.3 x 10\(^{-1}\) and 7.9 x 10\(^{-4}\) mol × sec/liter, respectively, corresponding to hybridization of 2 classes of RNA (A and B). The fractions of the RNA preparation corresponding to Class A and Class B sequences are 4.8 x 10\(^{-4}\) and 3.8 x 10\(^{-4}\).

The number of copies of viral-related RNA per cell was estimated from the number of cells per unit weight of thymic tissue and the amount of RNA extracted from a given amount of thymic tissue. An average value of 2.6 x 10\(^{6}\) cells/mg of thymic tissue was obtained from several determinations with 2-month-old mice. The amount of RNA extracted from the thymic tissue of 2-month-old and lymphomatous mice was 2.0 and 2.9 x 10\(^{-6}\) µg/cell, respectively. As a value of 3.4 x 10\(^{6}\) for the molecular weight of the subunit form of M-MuLV RNA (25) and assuming that Class A sequences are identical with the sequences detected in normal thymus, it was calculated that there are approximately 100 copies/cell in 2-month-old mice and about 2400 copies per cell in lymphomatous tissue. A possible contribution to the calculated number of copies by extracellular particles cannot now be excluded. Viral RNA sharing additional homology (Class B sequences) with the Moloney probe is present in lymphomatous tissue at 10 to 20 copies/cell.

Thermal denaturation profiles of the hybrids obtained with the Moloney cDNA probe and RNA extracted from the normal and lymphomatous thymic tissues were identical, with a melting temperature (T\(_{m}\)) of 83° (Chart 5). A higher degree of base pairing was observed, however, for the DNA-

![Chart 3. Phosphocellulose chromatography of DNA polymerase activities from thymic particles. Approximately 5 g of thymic tissue were obtained from 1-month-old mice and from retired breeders. Postmitochondrial pellets (27K) were resuspended in 3 ml of TNE buffer at protein concentrations of 3 and 5 mg/ml, respectively, and centrifuged on parallel gradients. Fractions with densities between 1.10 and 1.19 g/ml were pooled, reband, and used for enzyme purification. A, 1-month-old mice. [3H]TTP, 5000 cpm, and [3H]dGTP, 1200 cpm, were incorporated by the enzymatic activity in Chart 3A in the reaction of polymerization directed by poly(A) and poly(C), respectively. B, lymphomatous mice. [3H]TTP and [3H]dGTP, 190,000 and 30,000 cpm, were incorporated in an analogous assay with the use of Fraction 31. Thirty-µl aliquots were assayed.

![Chart 4. Hybridization of M-MuLV cDNA with M-MuLV 70S RNA and with RNA extracted from the thymus of 2-month-old and lymphomatous AKR mice. Ten (A) and 100 (A) ng of M-MuLV RNA were hybridized with 0.1 ng of M-MuLV cDNA for 0, 15, 30, 60, and 120 min (A); 0, 15, 30, and 60 min; and 2, 4, 8, and 40 hr (A). The last point on the curve was obtained with 1 µg of RNA and 40 hr of incubation. One-tenth to 240 µg of RNA from the thymus of 2-month-old mice (B) or 0.1 to 180 µg of RNA from lymphomatous thymus (C) were hybridized with 0.1 ng of cDNA; the reaction time was 40 hr. The maximal value of hybridization of cDNA with its homologous 70S RNA was 80%; this value was normalized to 100%, and all experimental values were adjusted accordingly. C{l/s} values were multiplied by 0.55 to obtain equivalent C{l/s} values relative to 0.18 µm Na\(^{+}\) (5). Each kinetic analysis was conducted 2 to 3 times with similar results. Representative analyses are shown.

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leukemia could result from the synergistic interaction of a highly replicating helper MuLV present in preleukemic stage and a replication-defective transforming agent and could be similar in nature to tumor induction by murine sarcoma complexes. Since, however, the structural nature of the thymic particles observed in this study remains to be elucidated, the possibility that these particles can be implicated in the process of leukemogenesis is as yet a matter of speculation.

Direct evidence of biochemical changes in the expression of viral-specific information, which might be implicated in the age-dependent process of leukemogenesis, has been obtained in our hybridization studies. The rationale for the use of the cDNA of M-MuLV is that M-MuLV shares sequence homology with the ecotropic AKR virus and induces the same type of thymic lymphoma as the Gross passage A virus, the leukemogenic strain of AKR MuLV. The viral RNA sequences detected in the thymus of young mice are partially related to the Moloney genome, as hybridization and thermal denaturation data indicate. Very probably, they are representative of the endogenous AKR virus sharing a partial homology with M-MuLV. As an alternate hypothesis the partial yield of hybridization might be indicative of an incomplete expression of endogenous viral genome. Further hybridization studies with cDNA synthesized from AKR virus should shed light on this question. The low density of the cytoplasmic particles would then be a consequence of the partial expression of the relevant viral genome, reflected in structural differences and/or different packing of viral components.

The increased number of equivalent viral genomes observed in the lymphomatous tissue is in agreement with previous reports that the titer in the thymus of infectious endogenous MuLV increases dramatically with the onset of leukemia (26). The expression of new base sequences related to the Moloney genome supports a correlation with the onset of the disease and could be interpreted in the light of recent studies (8, 14) that have proposed as a candidate for the leukemogenic agent a genetic recombinant between the ecotropic and a xenotropic MuLV. Thus, the late sequences detected with the Moloney probe would be related to the new classes of virus activated in the preleukemic stage. The inference would be therefore that the Moloney genome arose as a recombinant of an endogenous murine virus and genetic information of viral or cellular origin that provided additional information for transformation of lymphocytes. Alternatively, uncloned M-MuLV stock may contain several component viruses, and therefore the 2 sets of sequences detected in lymphomatous thymus could correspond to different viral agents.

ACKNOWLEDGMENTS

We are grateful to Dr. Hsu-San Lin and Dr. Frederick Valerio, Department of Radiology, Section of Cancer Biology, Washington University School of Medicine, for their interest in the development of this work and their hospitality to Corrado Gurgo during its initial phase. We thank Dr. Maurice Green for his critical reading of the paper and Dr. Gary Gerard for useful discussion. We also acknowledge the skilful technical assistance of Mary Hunter.

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