The Effect of a Murine Leukemia Virus on RNA Metabolism

ROBERT SILBER, BERNARD GOLDSTEIN, ELLEN BERMAN, JULIAN DECTER, AND CHARLOTTE FRIEND

Department of Medicine, New York University School of Medicine, and Division of Microbiology, Sloan-Kettering Institute for Cancer Research, New York, New York 10016

SUMMARY

RNA metabolism in the spleens of DBA/2 mice infected with a murine leukemia virus (Friend) was investigated. The spleen cells were tested in an in vitro system. Incorporation of uridine-14C into the RNA of spleen cells 4 days after the mice were inoculated with the virus was 4 times higher than that observed in normal spleen cell preparations. Subsequently, the incorporation of uridine-14C declined to control levels. RNA synthesis in both control and leukemic spleen cells was sensitive to the action of actinomycin D. RNA methylase increased in activity early after infection, reaching 3.5 times the control levels on the 5th post-inoculation day, and declining gradually to normal thereafter. Ribonuclease, on the other hand, declined to 25% of the normal activity by the 4th postinoculation day and remained at this level (or lower) throughout the course of the disease.

INTRODUCTION

In the past decade the viral etiology of several murine leukemias has been firmly established (13). In contrast to the widely documented enzymatic alterations in neoplasms such as hepatocellular carcinomas (15), relatively little is known about the biochemical changes that occur during the development of the viral leukemias.

A recent study from this laboratory has shown increases in the activity of tetrahydrofolate-dependent enzymes in spleen cells during the first 2 weeks of infection with a murine leukemia virus (19). In the light of the role of RNA in the transduction and translation of the genetic information controlling protein synthesis, the possibility that increases in the activity of specific enzymes are preceded by virus-induced alterations in RNA synthesis was explored. This report presents the results of studies on various aspects of RNA metabolism in the course of the development of a virus-induced mouse leukemia.

MATERIALS AND METHODS

Reagents. Uridine-2-14C was obtained from the New England Nuclear Co., and methyl-14C-S-adenosyl methionine was purchased from Tracerlab. Crude bentonite was obtained from Fisher Scientific Co. and prepared according to Fraenkel-Conrat et al. (8). Methyl-deficient soluble RNA (sRNA) was prepared from Escherichia coli S-61 by the method of Hurwitz et al. (10). Actinomycin D was obtained from Merck Sharp and Dohme.

Inoculation of Animals. DBA/2 mice, approximately 2 months of age, were inoculated with a murine leukemia virus originally isolated by one of us (9). The material was prepared as follows: A 20% homogenate of leukemic spleen in buffered Locke-Ringer solution was prepared with a Teflon homogenizer. The homogenate was centrifuged for 10 minutes at 775 × g (4°C) and the supernatant fluid filtered through a Selas 03 filter. In general, animals were inoculated intraperitoneally with 0.2 ml of the filtrate while controls received 0.2 ml of the buffer. Occasionally a twice-centrifuged unfiltered supernatant was used as the inoculum. Animals were sacrificed at 2- or 3-day intervals, as specified in the text, and the spleens immediately removed for assay.

Uridine-14C Incorporation Studies. Cell suspensions from spleen were prepared by gently teasing the tissue in Eagle's minimal essential medium (MEM) and filtering the suspension through a thin layer of glass wool. The spleens from 5 to 15 control animals and 2 to 6 spleens from leukemic animals were pooled for each experiment. The cells were centrifuged at 900 × g for 10 minutes and resuspended in MEM, supplemented with 10−4 M CaCl2 and essential amino acids in a concentration of 10−4 M. After the cell concentration was adjusted to 50,000 cells/ml, uridine-14C (specific activity, 25 µc/µmole) was added to a final concentration of 1 µc/ml. The method used for the extraction of RNA from cells has been previously described (20). The sedimentation velocities for mouse spleen RNA were determined by comparison with HeLa cell RNA and E. coli B RNA as standards. Specific activities were calculated by dividing the acid-precipitable radioactivity incorporated into the 28 S, 16 S, and 4 S RNA fractions by the absorbance of the extracted RNA at 260 millimicrons. Agreement between duplicate determinations was within 30%.

Preparation of Homogenates. For the preparation of cell-free homogenates 3–10 normal or leukemic spleens were suspended in 20 volumes (w/v) of 0.05 M potassium phosphate buffer, pH 6.5, and homogenized for 1 minute at 45,000 rpm in a VirTis 45 homogenizer. The homogenate was centrifuged at 10,000 × g. The supernatant fluid after 15 minutes of centrifugation was used for ribonuclease assays, and the supernatant fluid after 90 minutes of centrifugation was used for RNA methylase assays.

RNA Methylase Assay. The methylation of sRNA was measured by the method of Hurwitz et al. (10) modified with respect to pH and S-adenosyl methionine concentration. The assay mixture
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RESULTS

Incorporation of Uridine-\(^{14}\)C into RNA. When cell suspensions prepared from leukemic or control spleens were incubated \textit{in vitro}, the incorporation of uridine-\(^{14}\)C into RNA was linear up to a 3-hour period. The qualititative distribution of the radioactive label was the same in control and leukemic animals (Chart 1). Within 30 minutes considerable radioactivity had appeared in the 4–14 S regions of the gradient. Synthesis of ribosomal RNA is evident from the incorporation of label into 16 S and 28 S RNA. After 60 minutes of incubation the radioactivity peaks coincided with the ultraviolet absorption pattern.

The ribonuclease sensitivity of the leukemic RNA was established as follows: 0.5 mg of RNA isolated 8 days after inoculation from the spleenic cells of 3 mice was incubated with 10 \(\mu\)g/ml of crystalline pancreatic ribonuclease in 0.05 M tris(hydroxymethyl)aminomethane-HCl, pH 7.5, for 60 minutes. The control and ribonuclease-treated preparations were then sedimented for 12 hours at 23,000 rpm on 5–20% sucrose gradients, using the SW-39 rotor. Chart 2 shows the effect of ribonuclease. The alteration in the optical density pattern and the loss of acid-precipitable counts shown in the lower graph are evidence against the presence of ribonuclease-resistant material in spleen RNA in this type of murine leukemia. Similar results were obtained when the ultracentrifugation was carried out at 15,000 rpm for 12 hours using an SW-25.3 rotor, thereby ruling out the presence of ribonuclease-resistant material with a sedimentation velocity between 50 S and 200 S.

Cells obtained from spleens 4 days after inoculation were incubated for 30 minutes with 2.5 \(\mu\)g/ml of actinomycin D; uridine-\(^{14}\)C was then added to the incubation mixture for 60 minutes, after which RNA was isolated and ultracentrifuged in the customary manner. As shown in the sucrose gradient ultracentrifugation pattern (Chart 3), in the presence of actinomycin D, a striking inhibition of uridine incorporation occurred. Similar inhibition by actinomycin D was noted when spleen cells from animals sacrificed 8 and 15 days after inoculation with virus were tested. As could be expected, this dose of actinomycin D also inhibited uridine incorporation into RNA extracted from normal mouse spleen cells.

The \textit{in vitro} rates of uridine-\(^{14}\)C incorporation into spleen RNA in the course of the leukemia are shown in Chart 4. On the 2nd day after inoculation the specific activity observed in leukemic animals was still within the normal range in the experiment shown, although in 1 group of infected animals, specific activity contained the following: 3 \(\mu\)moles of methyl-\(^{14}\)C-S-adenosyl methionine (specific activity, 30–50 \(\mu\)c/\(\mu\)mole), 1 \(\mu\)mole of MgCl\(_2\), 2 \(\mu\)moles of 2-mercaptoethanol, 10 \(\mu\)moles of triethanolamine buffer (pH 9.0), 500 \(\mu\)moles of nucleotide residues as methyl-deficient sRNA, and 0.2–1.0 mg of homogenate protein in a volume of 0.3 ml. sRNA was omitted from the blank. After incubation for 45 minutes at 37°C, 5 \(\mu\)moles of sodium pyrophosphate, 0.05 ml of 0.5% human serum albumin, and 0.2 ml of 7% HClO\(_4\) were added. The precipitate was washed twice in 2 mM sodium pyrophosphate and dissolved in 2 ml of 2 M NH\(_4\)OH. The radioactivity was determined in a low-background gas-flow counter. The methylation of sRNA was determined by the amount of radioactive activity converted into an acid-insoluble form, 1 unit of enzyme being that amount which transfers 1 \(\mu\)mole of methyl group to sRNA in 1 hour.

Ribonuclease Assay. Ribonuclease was assayed by the method of Anfinsen et al. (1) with the following minor modifications: The incubation mixture contained 0.6 ml of 0.05 M potassium phosphate buffer, pH 6.5, 1 mg of yeast RNA which had been dialyzed against this buffer for 48 hours, and 1–5 mg of protein homogenate. The final volume was brought to 1.8 ml with 0.05 M phosphate buffer, pH 6.5. The reaction was carried out at room temperature. Three-tents-ml aliquots were removed at 0, 2, 4, 6, and 8 minutes and deproteinized with 0.1 ml of uranyl acetate in 25% perchloric acid. After a 10-minute centrifugation at 2000 \(\times\) g, 0.1 ml of supernatant was diluted to 1 ml with water and its absorbance was determined at 260 m\(\mu\). All assays were run at 2 or more concentrations of homogenate to insure that the reaction rate was linear. Duplicate determinations agreed within 20%. One enzyme unit is that activity resulting in an increase of 1 optical density unit at 260 m\(\mu\) per ml per min of incubation. Specific activity is expressed in units per mg of homogenate protein. Every result shown in this report was found in at least 3 groups of animals inoculated with the virus.
twice that of the controls was observed. Over the next two days there was a sharp increase in the rate of incorporation of uridine-$^{14}$C into 4 S, 16 S, and 28 S RNA, which on Day 4 showed a peak specific activity approximately 4 times higher than the average for normal spleen cells. In 3 other groups of experimental animals, the specific activity at Day 4 ranged from 2 to 5 times the normal level. Thereafter, an abrupt decline occurred, and by Day 6 after inoculation the specific activities had reached normal or, in some cases, even slightly subnormal levels in spite of the fact that the leukemic spleens were rapidly growing at this time.

RNA Methylase Assay. A somewhat different pattern was observed in the activity of the RNA methylase. Chart 5 shows that while identical levels were seen in leukemic and control spleens on Day 2 after inoculation, a 3.5-fold increase in activity is noted in the leukemic animals on Day 5. A gradual decline in activity is observed with a return to normal on the 12th day. In some experiments the methylase activity was still 50% above normal at the end of 2 weeks. It must be noted that these assays were performed with methionine-starved E. coli sRNA as acceptor and therefore represent the capacity of the mouse enzymes to methylate this acceptor rather than that of mouse RNA.

Ribonuclease. In contrast to the increases observed in uridine-$^{14}$C incorporation, the activity of pancreatic ribonuclease was sharply reduced in the leukemic spleens. These reductions were apparent on Day 2 after inoculation and were maintained throughout the remainder of the experiment. The results indicate that leukemic spleens have a reduced capacity to degrade RNA, thus accounting for the increased synthesis of RNA observed in these animals.
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**Chart 5.** Levels of RNA methylase in spleen of normal and virus-infected mice. ●●, leukemic. ▲▲, control.

**Chart 6.** Levels of ribonuclease in spleen of normal and virus-infected mice. ●●, leukemic. ▲▲, control. The range of activities in 2 other groups of experimental animals is indicated in the shaded area.

d-U-14C incorporation and RNA methylase level in the spleen of leukemic animals, ribonuclease activity by the 4th day after inoculation had decreased to less than one-third the level of control animals (Chart 6). The activity in the spleen of infected animals ranged from one-fifth to one-tenth that of the controls for the remainder of the experiment.

**DISCUSSION**

Infection with the strain of murine leukemia virus used in these studies has a characteristic pattern of progression. Very early in the disease a viremia occurs (6). Between the 2nd and 4th postinoculation day scattered neoplastic cells appear in the spleen. An increase in splenic size occurs after Day 3 with a quadrupling in weight by Day 6. Death generally occurs after 3 weeks.

In vivo studies (11) have shown an increased uptake of 32P by the spleen of animals infected with several types of murine leukemia, which was not observed in other diseases with spleen involvement. The increase in specific activity observed before significant splenic enlargement occurred was limited to microsomal and supernatant lipids and nucleic acids (12). Recently Budillon et al. (3) reported that infection with the same murine leukemia virus used in the present study resulted in increased incorporation of labeled amino acids into spleen protein in vivo and by cell-free preparations in vitro.

From the present studies a highly reproducible sequence of biochemical alterations emerges, which can best be interpreted in relation to the histologic changes. Between the 2nd and 4th day after inoculation with virus, there is a striking increase in the in vitro incorporation of uridine-14C into spleen RNA. It must be...
emphasized that the incorporation of uridine-14C into RNA cannot be used as an absolute measurement of RNA synthesis, since changes in permeability, alterations in the pool size of endogenous nucleotides and a variety of other metabolic events can influence the incorporation of labeled precursors into RNA. Despite these limitations it is likely that increased synthesis in vitro of soluble and ribosomal RNA occurs very early in the course of the leukemic cell proliferation. Since infiltration of the spleen by the leukemic cells is minimal at this stage, there must be either an extremely high rate of RNA synthesis in the relatively few neoplastic cells present at this time or an increase in RNA synthesis in the normal cells following viral infection. The latter interpretation is compatible with the observation that while numerous virions are present in the spleen by four days, nonneoplastic cells are also able to synthesize virus (5).

Since an increase in RNA synthesis does occur, the question arises whether the material made is virus RNA or cell RNA. Recently, Rich et al. (18) have shown that spleens of mice infected with this murine leukemia virus contain increased concentrations of RNA whose base ratios differ from that of controls. Unfortunately, the virus has not been sufficiently purified to determine its base composition. Homology tests in which denatured DNA from specific hybrids with homologous RNA have not been done to date between the newly made RNA and mouse spleen DNA or RNA. The exact nature of this newly made RNA must, therefore, remain uncertain.

Following the infection of cells by single-stranded RNA viruses, such as those causing encephalomyocarditis or poliomyelitis, the formation of virus-specific RNA with the properties of a single-stranded RNA is resistant to the action of pancreatic ribonuclease. A single-stranded RNA in the leukemic cells was the sensitivity of the synthesized RNA to pancreatic ribonuclease, since certain pertinent exceptions to the resistance of RNA viruses to this antibiotic have been reported. Among these is the sensitivity of RNA synthesis to actinomycin D, which in low concentration inhibits DNA-dependent RNA synthesis. Since this inhibition depends on the binding of actinomycin D to guanine in DNA primer, this antibiotic is generally ineffective in the suppression of autoreplication of RNA derived from RNA viruses. However, the sensitivity of RNA synthesis to actinomycin D in the leukemic cell cannot be taken as definite evidence against viral RNA as the template for further viral RNA synthesis, since certain pertinent exceptions to the resistance of RNA viruses to this antibiotic have been reported. Among these is the inhibition of poliovirus synthesis by actinomycin D, which is possibly secondary to effects of the drug on host DNA. Another important exception is the case of some avian tumor RNA viruses where both actinomycin D and inhibitors of DNA synthesis will block viral multiplication (21). Additional evidence against the presence of detectable double-stranded viral RNA in the leukemic cells was the sensitivity of the synthesized RNA to pancreatic ribonuclease, since this type of RNA would be resistant to the hydrolytic action of the enzyme. A single-stranded, ribonuclease-sensitive RNA of high molecular weight has recently been reported in another murine leukemia (Rauscher) (7).

The return to normal or subnormal rates of RNA synthesis after Day 6 is of interest since the spleen will again triple in weight over the next 10 days. Although extrapolations from an in vitro to an in vivo situation must remain conjectural, a hypothesis to account for continued growth in mass in vivo in the presence of a normal RNA synthesis in vivo can be derived from Pitot's suggestion (16) that in the neoplastic cell there may be increased stability of the RNA template. This raises the familiar question in oncogenesis whether the increase in mass may not necessarily present increased production of cells but rather a decreased rate of maturation and longer survival of the neoplastic cell. The increased RNA synthesis is accompanied by a sharp drop in ribonuclease and followed by an increase in tetrahydrofolic-dependent enzymes (19). The enhanced activity of the enzymes of one carbon metabolism and the decrease in ribonuclease in the leukemic cells could be ascribed either to the activation and inhibition of preexisting enzymes or to changes in synthesis of biologically active proteins. It is conceivable that the RNA made between Days 2 and 4 after infection carries this control information.

The uncertain biologic function of ribonuclease makes interpretation of the decreased activity difficult. It must be noted, however, that low levels of ribonuclease have been described in a variety of neoplastic tissues. It is tempting to speculate whether the decrease in ribonuclease is related to the altered template stability described in neoplasms (17).

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REFERENCES

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