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RNA in Human Leukemic Cells Related to the RNA of a Mouse Leukemia Virus

(leukocytes/RNA–DNA hybridization/Rauscher virus/polysomal RNA)

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ABSTRACT **Molecular hybridization with radioactively labeled DNA complementary to the RNA of the Rauscher leukemia virus was used to probe for homologous RNA in the polysome fraction of human leukemic cells. The leukocytes of 24 out of 27 patients examined contained RNA possessing homology to that of the mouse leukemia agent, but not to that of the unrelated viruses causing mammary tumors in mice or myeloblastosis in chickens. Further, no control human leukocytes or other adult and fetal tissues showed significant levels of the leukemia-specific RNA. It would appear that human leukemic cells contain RNA sequences homologous to those found in a viral agent known to cause leukemia in an experimental animal. The fact that human sarcomas have also been shown to contain this type of RNA points to a remarkable parallelism in the leukemias and sarcomas of mice and men.**

The search for evidence implicating oncogenic RNA viruses in human neoplasia constitutes a major endeavor in present-day cancer research. One approach recently explored stemmed from an attempt to determine via RNA–DNA hybridization (1) whether human breast carcinomas contain RNA molecules homologous to an analogous virus of proven oncogenic potential. Human breast cancer was chosen for the initial effort because of the recent observations (2) in human milk of particles similar to the mouse mammary tumor virus and the demonstration (ref. 3 and manuscript in preparation) that the human particles also contain the enzyme and high molecular weight (70S) RNA characteristic of the animal RNA tumor viruses.

As a necessary prelude to attempts with human material, we examined the technical feasibility of using the molecular hybridization technique with mouse mammary tumors and the corresponding viral agent as a suitable experimental model. Hybridizations with radioactively labeled DNA complementary to the RNA of the mouse mammary tumor virus revealed (4) that it was in fact possible to identify in tumor extracts viral-specific RNA in the fraction of ribosomes actively engaged in protein synthesis.

The experiments with the mouse model produced the detailed technology required to handle human tissue specimens. The mouse mammary tumor virus was chosen to produce the radioactively labeled DNA needed to search for homologous RNA in human tumors. The results obtained (5) were satisfyingly clear-cut. Of 29 malignant breast tumors examined, 67% showed unmistakable evidence of RNA that can hybridize with mouse mammary tumor virus DNA. None of the 30 preparations from normal or nonmalignant breast tissues contained detectable quantities of this type of RNA. Further, the malignant RNA samples that hybridized with mouse mammary tumor virus DNA showed no ability to complex with DNA complementary to the RNA of two leukemogenic agents, the avian myeloblastosis virus and the murine Rauscher leukemia virus.

These last observations in particular gave added credence to the belief that the positive responses observed between RNA from malignant human breast tumors and the mouse–viral DNA were specific and meaningful. In any event, as soon as it became apparent that the experiments with breast tumor material were yielding interesting results, the present investigation into human leukemia, and a parallel one in human sarcomas (manuscript in preparation), were instituted.

A particle of established leukemogenic potential, the murine Rauscher leukemia virus (RLV), was chosen to produce the radioactively labeled DNA required. We report here that 89% of leukemic patients in the active phase of the disease contained, in their leukocytes, RNA possessing homology to that of the Rauscher leukemia virus but not to the RNAs of the unrelated mouse mammary tumor virus or the avian myeloblastosis virus. Further, no control leukocytes or other tissues examined showed significant levels of this specific RNA.

METHODS

Leukocytes were obtained by fractionation of citrated blood samples in inverted syringes. Leukemic cells prepared in other laboratories were sent to us frozen in dry ice. The cells were collected by centrifugation, washed in saline, and then suspended in 0.01 M Tris·HCl (pH 7.4)–0.15 M NaCl–2 mM $MgCl_2$ containing 5% sucrose. They were ruptured with 5–12 strokes of a Dounce homogenizer. Cell membranes and nuclei were removed and the cytoplasmic pellets were collected and used for the isolation of polysomal RNA (4). All annealings were at 37°C in 50% formamide, to minimize fragmentation of the RNA during the hybridization. Hybrids were detected by isopycnic separation in Cs_2SO_4 gradients (6).

RESULTS

For comparison, and to see what might be optimally expected with human material, it was of interest to examine the outcomes of analogous hybridizations in instances of two animal leukemias known to be associated with a viral agent. Fig. 1*A* shows the result of hybridization of RLV-[^{3}H]DNA to polysomal plus monosomal RNA, hereafter designated polysomal RNA, prepared from the spleen of an RLV-infected mouse. A peak of [^{3}H]DNA, corresponding to about 3% of the input DNA, is found in the RNA region of the density gradient. The density position of the hybrid indicates that the RNA

Abbreviation: RLV, Rauscher leukemia virus.

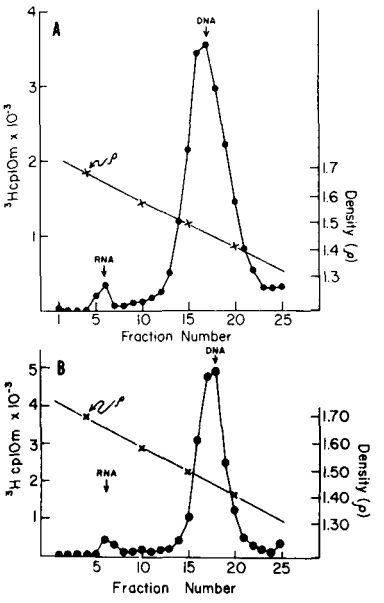

FIG. 1. (*A*) Cs_2SO_4 density profile of Rauscher leukemia virus (RLV) [^{3}H]DNA hybridized to polysomal RNA obtained from the spleen of an RLV-infected mouse. RLV-[^{3}H]DNA was synthesized as follows: A 1-ml reaction mixture, containing 100 μg of viral protein purified (6) from plasma, 50 μmol of Tris·HCl (pH 8.3)–40 μmol KCl–6 μmol $MgCl_2$–2.5 μmol dithiothreitol–0.00125% NP-40 detergent (Shell Chemical Co.)–100 μmol (each) of dGTP, dATP, and dCTP–5 $\times$ 10^4 pmol of [^{3}H]TTP (8000 cpm/pmol), was incubated at 37°C for 180 min. After addition of 0.5% sodium dodecyl sulfate and extraction with an equal volume of phenol–cresol, the [^{3}H]DNA product was purified by Sephadex G-50 chromatography and treated with 0.5 M NaOH at 43°C for 24 hr to hydrolyze any viral RNA present.

In studies such as these, it must be shown before use that the radioactive DNA product bands solely in the DNA density region of a Cs_2SO_4 gradient and that it hybridizes with homologous RNA and not to normal cellular RNA.

The RNA (designated as pRNA) used in the hybridizations is derived from a cytoplasmic pellet consisting of a mixture of monosomes and polysomes (4). Suitable care must be exercised to insure the complete removal of protein contaminants. For the preparation of polysomal RNA, the spleen was disrupted by a Silverson homogenizer at 4°C in two volumes of 5% sucrose in TNM buffer [0.01 M Tris·HCl (pH 7.4)–0.15 M NaCl–2 mM $MgCl_2$]. The suspension was centrifuged at 20,000 *g* for 15 min at 0°C. The supernatant fluid was then layered on 20 ml of 25% sucrose in TNM buffer containing 200 μg of polyvinyl sulfate per ml and spun for 180 min at 180,000 $\times$ *g* in a 60 Ti rotor (Spinco). The pellet was resuspended in TNM buffer with 0.5% sodium dodecyl sulfate and the RNA was extracted twice with an equal volume of cresol–phenol (pH 8.0). Nucleic acid was precipitated from the aqueous phase by addition of two volumes of ethanol and 0.1 volume of 4 M NaCl. The polysomal RNA was redissolved in 50% formamide–50% 5 mM EDTA. The [^{3}H]DNA was denatured by incubation at 80°C for 15 min in 70% formamide and subsequent quick chilling. 480 μg of polysomal RNA were hybridized to 2000 cpm of RLV-[^{3}H]DNA in 60 μl of 0.4 M NaCl containing 50% formamide. The reaction was incubated for 18 hr at 37°C, mixed with 10 ml of 50%-saturated Cs_2SO_4 (starting density 1.52) and centrifuged at 44,000 cpm for 60 hr at 20°C in a 50 Ti rotor (Spinco). 0.4-ml (8-drop) Fractions were collected through an 18-gauge needle from the bottom of the tube and assayed for Cl_3CCOOH-precipitable radioactivity.

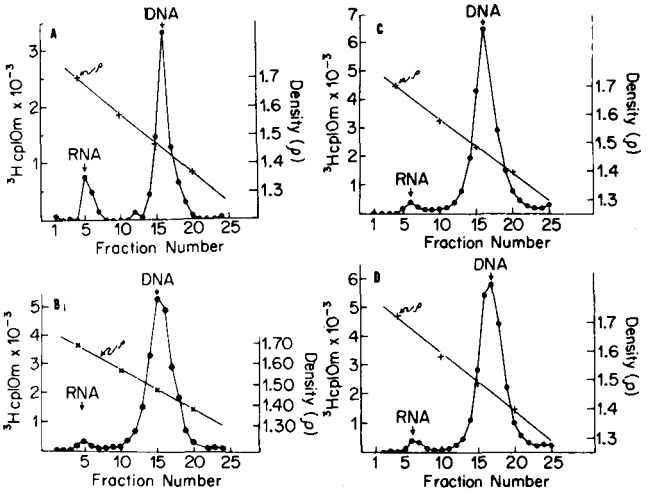

FIG. 2. (*A–D*) Cs_2SO_4 density profiles of RLV-[^{3}H]DNA hybridized to polysomal RNAs of four leukemic samples. Polysomal RNA was isolated from buffy coats of patients showing clinical manifestation of acute lymphocytic leukemia (*A*, *C*, and *D*) and of acute myelogenous leukemia (*B*). The cells were disrupted with a Dounce homogenizer, and cytoplasmic pellets were prepared as described under Fig. 1*B*. 300 μg of polysomal RNA were hybridized to RLV-[^{3}H]DNA in 60-μl volumes, and the reactions were analyzed by Cs_2SO_4 density centrifugation.

molecule in the complex is much larger than the 4 S–6 S average size of the product DNA. Fig. 1*B* shows the similar outcome with a rat ascitic monocytic leukemia, originally induced (7) with 20-methylcholanthrene, that produces low levels of a C-type particle (8) of unknown specificity. Again, about 3–4% of the input [^{3}H]DNA is found in the RNA region at the concentration of polysomal RNA used in the annealing reaction. Similar results have been obtained in hybridizations of RLV-[^{3}H]DNA with polysomal RNA from cat lymphocytes infected with feline leukemia virus and hamster cells (HT2) transformed with the Moloney sarcoma virus.

Fig. 2*A–D* shows representative profiles in Cs_2SO_4 gradients of annealing reactions between RLV-[^{3}H]DNA and polysomal RNA preparations derived from human-leukemic leukocytes (buffy coats). As may be seen, the results are comparable to those obtained with the animal systems (Fig. 1*A* and *B*). In Fig. 2*A*, 15% of the input RLV-DNA is hybridized; the other three samples gave 3–5% formation of the hybrid complex. Again, as in the hybridizations with polysomal RNA from the animal leukemic material (Fig. 1*A* and *B*), the locations of the complexes in the gradients of Fig. 2 indicate that the RNA is much larger than the DNA, and determines the density of the DNA–RNA hybrid structure.

The positive responses observed (Fig. 2*A–D*) with the leukemic polysomal RNAs are in contrast to the negative

Data are expressed in this and subsequent figures as *cp10m*, counts per 10 min.

(*B*) Cs_2SO_4 equilibrium gradient centrifugation of RLV-[^{3}H]DNA annealed to 600 μg of polysomal RNA obtained from ascites cells of a rat leukemia. The cells were collected from the rat ascitic fluid, washed in phosphate-buffered saline (pH 7.4) by low-speed centrifugation (2500 rpm, Sorvall), and resuspended in two volumes of TNM buffer containing 5% sucrose. The cells were disrupted by 5–12 strokes of a Dounce homogenizer, and treated as in part *A*.

reactions with polysomal RNAs from normal leukocytes (Fig. 3*A*), from phytohemagglutinin-stimulated lymphocytes (Fig. 3*B*), from leukocytes of a leukemic patient in clinical remission (Fig. 3*C*), and from human fetal lung (Fig. 3*D*). In none of these samples does one find significant amounts of [^{3}H]DNA in the RNA region of the density gradient.

An informative way to compare positive and negative reactions is via a concentration curve, as shown in Fig. 4. Here, the same amount of RLV-[^{3}H]DNA is annealed to increasing concentrations of polysomal RNA and the percent of [^{3}H]-DNA complexed to the RNA is determined by isopycnic separation in Cs_2SO_4 gradients. It is clear that no detectable response with polysomal RNA from normal leukocytes is observed, even at 14 mg of RNA per ml. On the other hand, the percent of RLV–DNA complexed to polysomal RNA from the buffy coat of a patient with acute myelogenous leukemia shows no evidence of saturation within the concentration range tested. Several leukemic buffy coats were obtained in amounts sufficient to permit the determination of such concentration curves, and comparable results were obtained. It should be further noted that similar concentration curves were also run with polysomal RNA preparations from phytohemagglutinin-stimulated lymphocytes with results not significantly different from those seen with normal leukocytes (Fig. 4).

Since it is impractical to present the Cs_2SO_4 gradient profile of every sample examined, a more convenient recording of our findings was used. After correction for background counts, the sum of the tritium counts in the RNA density region (between densities 1.62 and 1.68 g/ml) was used to measure the amount of DNA complexed to RNA. To achieve the accuracy desired, 10-min counts were taken on each sample. An operational mean background and its standard deviation (*S*) were empirically determined for individual machines by the total counts/10 min of three tubes in the negative regions (e.g., tubes 2, 3, and 4) of each of 60 gradients. The convention was adopted that all specimens with less than three standard deviations of the background mean counts/10 min would be considered negative.

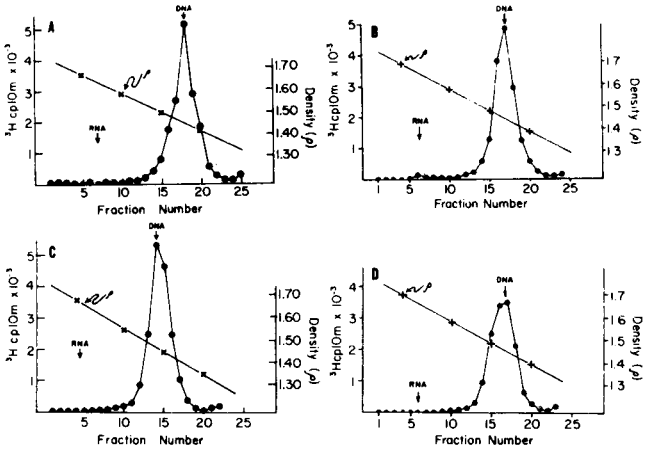

FIG. 3. (*A–D*) Cs_2SO_4 density centrifugation of RLV-[^{3}H]-DNA after annealing to polysomal RNA isolated from (*A*) normal white buffy coat, (*B*) phytohemagglutinin-stimulated lymphocytes (*C*), buffy coat of a leukemic patient in clinical remission, and (*D*) fetal lung. The polysomal RNA input was 300 μg, except in (*C*) where 1000 μg were used per 60-μl hybridization reaction. Subsequent analysis on Cs_2SO_4 was described in Fig. 1.

Table 1 lists the leukemic buffy coats tested for RNA complementary to RLV–DNA, with results recorded as the total counts/10 min (corrected for background) in the RNA density region, and as multiples of the mean background standard deviation. The samples came from patients in the active phase of the disease, as determined by differential counts of their peripheral leukocytes, and include acute myelogenous leukemia, acute lymphocytic leukemia, chronic lymphocytic

TABLE 1. *Test for viral-specific RNA in human leukemic cells*

Tissue	RNA-region Counts/10 min	Counts per 10 min/*S*	Reaction
Acute lymphatic leukemias			
(Y)	402	4.7	+
(Hl)	515	6.0	+
(E)	387	4.5	+
(V)	233	2.7	−
(Gr)	1413	16.4	+
(S)	308	3.6	+
(W)	832	9.7	+
(O)	393	4.6	+
(Bx)	1271	14.8	+
Chronic lymphatic leukemias			
(LS_{68})	478	5.6	+
(LS_{71})	328	3.8	+
Acute myelogenous leukemias			
(B)	631	7.3	+
(D)	813	9.5	+
(Hr)	564	6.6	+
(GM)	912	10.6	+
(WS)	118	1.4	−
(H_{69})	682	7.9	+
(H_{70})	450	5.2	+
(L)	684	8.0	+
(R)	361	4.2	+
(C)	322	3.7	+
(M)	665	7.7	+
(G)	220	2.6	−
(F)	1315	15.3	+
Monocytic leukemias			
(WR)	528	6.1	+
(RG)	590	6.9	+
(Sy)	550	6.4	+

Results of hybridization reactions between RLV-[^{3}H]DNA and polysomal RNA isolated from human leukemic cells. In two cases (*H* and *LS*) cells were taken in 1- and 3-year intervals. 200–1000 μg of polysomal RNA of each sample were hybridized to 2000 cpm of RLV-[^{3}H]DNA and the reactions were analyzed by Cs_2SO_4 equilibrium centrifugation. The amount of DNA banding in the RNA region of the gradient (between densities 1.62 and 1.68) was then determined. The results are expressed as counts/10 min (corrected for background) banding in the RNA region for each RNA sample tested, and as multiples of *S*, the operational standard deviation (see text and legend to Fig. 5). The annealing reaction is considered positive only if the counts/10 min per RNA region is greater than 3*S*, thus providing 99.9% confidence statistically. Of the 27 samples tested, 89% were positive.

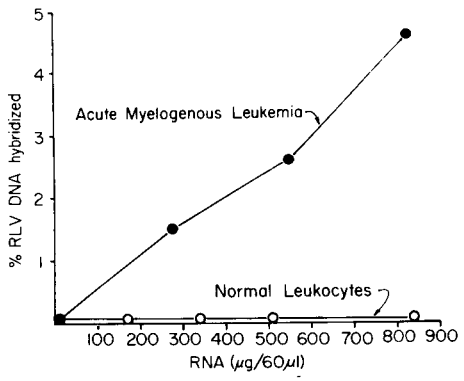

FIG. 4. Comparison of hybridization reactions between RLV-[^{3}H]DNA, leukemic polysomal RNA, and normal buffy-coat polysomal RNA at various RNA concentrations. The individual annealing reactions were analyzed by Cs_2SO_4 density centrifugation, and the percent DNA hybridized was determined by the counts/10 min of [^{3}H]DNA (corrected for background) banding in the RNA region (between densities 1.62 and 1.68) of the gradients.

leukemia, and monocytic leukemia. Fig. 5 presents a convenient pictorial summary of the reactions observed with leukemic (Table 1) and various control tissues.

Of the 27 leukemic samples examined, only three fell in the negative range. The remaining 24 showed positive reactions, with some exceeding those observed with the animal leukemic material (Fig. 1*A* and *B*). In contrast, none of the polysomal RNA preparations from 33 normal tissues gave a reaction that could be unambiguously designated as positive. These preparations include RNAs from normal leukocytes, phytohemagglutinin-stimulated lymphocytes, and various normal human adult and fetal tissues.

The fact that 89% of the leukemic polysomal RNAs yielded unmistakable positive hybridizations with RLV-DNA, whereas none of the 33 control tissues exhibited this type of response, argues for the specificity of the annealing reactions.

Further support for this conclusion can be obtained by the use of a [^{3}H]DNA complementary to the RNA of the mouse mammary tumor virus or to that of the avian myeloblastosis virus. We have shown (manuscript in preparation) that RLV-DNA and the homologous viral RNA do not crosshybridize significantly with the corresponding nucleic acids of either the mammary tumor virus or the avian agent. If the annealing reaction is specific, one would not expect a leukemic polysomal RNA, positive for a reaction with RLV-DNA to show the ability to hybridize either with mouse mammary tumor viral-DNA or avian myeloblastosis viral-DNA. Fig. 6 shows that these expectations are realized. None of three other leukemic polysomal RNAs giving a positive (550–910 counts/10 min above background) reaction with RLV-DNA responded to either of the other two viral DNAs.

Stimulated by the known viral etiology of animal leukemias (9, 10), others have sought for similar evidence in the human disease. Particles resembling the C-type viruses of the avian and murine leukemias have been occasionally detected by electron microscopy (11). Of perhaps more immediate relevance is the report (12) of components in human leukemic cells and sera that can interact with fluorescent-labeled antibodies against the Rauscher leukemia virus.

The present investigation is not directly aimed at resolving the issue of a viral etiology of human cancer. It is concerned rather with determining whether viral-related information, in terms of sequence similarities, can be detected in human neoplastic cells.

It is worth explicitly noting the questions that remain unanswered by the data described here. They provide no measure of the degree of homology between the RLV-DNA and the RNA detected in human leukemic cells. To settle this issue will require the synthesis of RLV-DNA in amounts adequate to permit comparative saturation curves of labeled RLV-RNA and the relevent RNA strands from human leukemic cells. The fact that hybrids are found in the RNA region of the density gradient implies that the RNA is much larger than the DNA product. However, experiments must now be designed and performed that will determine how large this RNA is, how much viral sequence it contains, and whether the viral information is covalently linked to normal cellular message. The latter point is particularly relevant to whether the viral information is integrated into the genome of the cancer cell. Finally, more highly purified mRNA fractions will be required to make the quantitative aspects more certain. In particular, it is apparent that a negative outcome of a hybridiza-

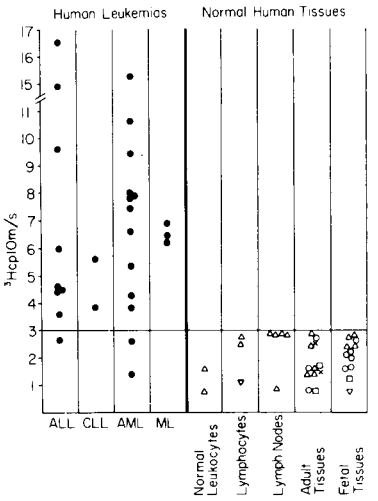

FIG. 5. Results of hybridization reactions with RLV-[^{3}H]-DNA and polysomal RNA from leukemic and normal human cells. The RNA was derived from leukemic and normal leukocytes, phytohemagglutinin-stimulated lymphocytes (△), a human lymphocyte cell line, NC37 (▽), lymph nodes, other adult tissues: liver (△), spleen (×), intestine (○), striated muscle (□), and fetal tissues: liver (△), lung (▽), limbs (○), placenta (□). The reactions were then subjected to Cs_2SO_4 equilibrium density centrifugation as described under Fig. 1. The amount of [^{3}H]DNA, expressed as cp10m corrected for background banding in the density region of RNA (between densities 1.62 and 1.68), was determined for each reaction. An operational mean and standard deviation (*S*) were determined for each counter by the total cp10m of three tubes [e.g., 2, 3, 4] of each of 60 gradients. The cp10m [^{3}H]DNA corrected for background banding in the RNA region of the gradient was then divided by the appropriate operational standard deviation. Any reaction with less than 3*S* cp10m in the RNA region is considered negative, thus providing 99.9% confidence that those reactions retained as positive (greater than 3*S*) are statistically significant. *ALL*, acute lymphatic leukemia; *CLL*, chronic lymphatic leukemia; *AML*, acute myelogenous leukemia; *ML*, monocytic leukemia.

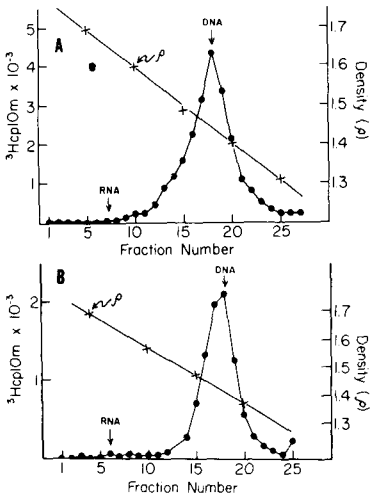

FIG. 6. Cs_2SO_4 equilibrium gradient centrifugation of (*A*) mouse mammary tumor virus [^{3}H]DNA and (*B*) avian myeloblastosis virus [^{3}H]DNA hydridized (each) to 400 μg of human leukemic polysomal RNA. Hybridization conditions and Cs_2SO_4 gradient analysis as in Fig. 1.

tion test cannot be accepted as evidence for absence of the relevant RNA.

From the data presented, one can conclude at best that the probability of finding RNA homologous to Rauscher leukemia viral RNA is much greater in human leukemic cells than in normal tissues. Indeed, if the provirus (13) or oncogene hypotheses (14) are valid, some part of this information might be found with more sensitive tests in presumably normal cells derived from fetal tissues or phytohemagglutinin-stimulated lymphocytes, both of which have been reported (14, 15) to possess the group-specific antigen of the mammalian leukemogenic viruses.

While the experiments described here do not constitute definitive proof of a viral etiology of human leukemia, they provide rather compelling evidence for the presence in human leukemic cells of RNA posessing sequences homologous to those found in a viral agent known to cause leukemia in an experimental animal. From what we know of animal systems, one would predict that a similar situation would be found in human sarcomas, a prediction that has been confirmed and will be detailed in another publication.

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