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Viral-Related RNA in Hodgkins' Disease and Other Human Lymphomas

(DNA–RNA hybridization/Rauscher murine leukemia virus/cancer/neoplasia/tumor)

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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 human lymphomas. 22 of 32 specimens contained RNA possessing homology to the RNA of the mouse leukemia virus, but not to that of the unrelated viruses causing mammary tumors in mice or myeloblastosis in chickens. Normal adult and fetal tissues failed to show significant levels of the leukemia-specific RNA. It appears that human lymphomas contain RNA sequences homologous to those found in a viral agent known to cause leukemia and lymphomas in an experimental animal. The fact that human leukemias and sarcomas also contain this type of RNA further emphasizes a remarkable similarity between the corresponding neoplasias of murine and human origin.**

We have used molecular hybridization to detect virus-specific RNA in tumors (1), and have found that corresponding neoplasias of murine and human origin exhibit remarkable similarities. Thus, human breast carcinomas contained (2) RNA possessing sequence homology to that of mouse mammary tumor virus (MMTV). This type of RNA was unique to the malignant adeno- and medullary-carcinomas, being undetectable in normal breast tissue and in such benign pathologies as fibrocystic disease and fibroadenoma. In keeping with the known unrelatedness of the leukemogenic and mammary tumor viruses, we found that breast cancer RNA did not hydridize to DNA complementary to the RNA of Rauscher leukemia virus (RLV). Finally, and most compelling, was the demonstration that human leukemic cells (3) and human sarcomas (4) both contain RNA showing homology to that of Rauscher leukemia virus, and not to that of mouse mammary tumor virus. It was of obvious interest to pursue this intriguing concordance between the neoplasias of mice and men.

From the viewpoint of etiology and cellular pathology, lymphomas in mice are linked to the leukemias and sarcomas. In addition, it may be noted that some human lymphomas are accompanied by the clinical appearance of a peripheral leukemia. In any event, if human neoplasias parallel those observed in mice it might be expected that human lymphomas would, like the leukemias and sarcomas, contain RNA uniquely homologous to that of a mouse leukemia agent.

We report here a confirmation of this expectation. Human lymphomas, including Hodgkins' disease, lymphosarcomas, and reticulum cell sarcomas contain RNA exhibiting homology to the RNA of Rauscher leukemia virus, but not to the unrelated RNAs of either mouse mammary tumor virus or of avian myeloblastosis virus (AMV).

Abbreviations: RLV, Rauscher (murine) leukemia virus; pRNA, cytoplasmic (polysomal) RNA; AMV, avian myeloblastosis virus; MMTV, mouse mammary tumor virus.

METHODS AND MATERIALS

Preparation of Nucleic Acids. The preparation of labeled DNA is exemplified in the case of RLV-[^{3}H]DNA. A 1-ml reaction mixture, containing 100 μg of viral protein prepared from virus purified from plasma (5), 50 mM Tris·HCl (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.00125% Nonidet (NP-40), 100 mM (each) of dGTP, dATP, and dCTP, and 5×10^4 pmol of [^{3}H]TTP (8000 cpm/pmol), was incubated at 37° for 180 min. After the addition of sodium dodecyl sulphate to 0.5%, the nucleic acid was extracted with an equal volume of phenol–cresol, and the [^{3}H]DNA product was purified by passage through a Sephadex G-50 column, followed by incubation in 0.5 M NaOH at 43° for 24 hr to remove viral RNA. MMTV-[^{3}H]DNA and AMV-[^{3}H]DNA were prepared in a similar manner.

For the preparation of cytoplasmic RNA (designated as pRNA), the tumors were disrupted with a Silverson homogenizer at 4° in 2 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 $\times$ g for 15 min at 0° to remove nuclei and cell debris. The supernatant fluid was layered on 20 ml of 25% sucrose containing 200 μg of polyvinyl sulfate per ml and centrifuged at 4° for 180 min at 180,000 $\times$ g in a 60-Ti rotor (Spinco). The pellet was resuspended in TNM buffer containing 0.5% sodium dodecyl sulfate and the RNA was extracted twice with an equal volume of cresol–phenyl (pH 8.0). The aqueous phase was adjusted to 0.4 M NaCl, and the nucleic acid was precipitated with two volumes of ethanol. The resulting pRNA precipitate was dissolved in minimum volume of 50% formamide–3 mM EDTA.

Annealing Reactions Between pRNA and [^{3}H]DNA. Immediately before use, [^{3}H]DNA was denatured by incubation for 10 min at 80° in 75% formamide, followed by quick chilling at 0°. The annealing mixture usually contained 350 μg of pRNA and 2000 cpm of [^{3}H]DNA in 60 μl of 0.4 M NaCl, adjusted to 50% formamide and pH 7.4. Annealing was for 18 hr at 37°, after which 10 ml of Cs_2SO_4 at 50% saturation (density: 1.52 g/ml) was added; the resulting solution was centrifuged at 44,000 rpm for 60 hr at 15° in a 50-Ti rotor (Spinco). Fractions (0.4 ml) were collected through a needle inserted in the bottom of the tube and assayed (3) for acid-precipitable (10% trichloroacetic acid) radioactivity.

Pitfalls to be Guarded Against. The experiments described here and in our previous studies on breast cancer (2), the leukemias (3) and the sarcomas (4) were designed to detect in the neighborhood of 0.1 ng or less of complementary RNA in the pRNA preparations being tested. The sensitivity of the method is therefore being pushed towards its limit. Under the

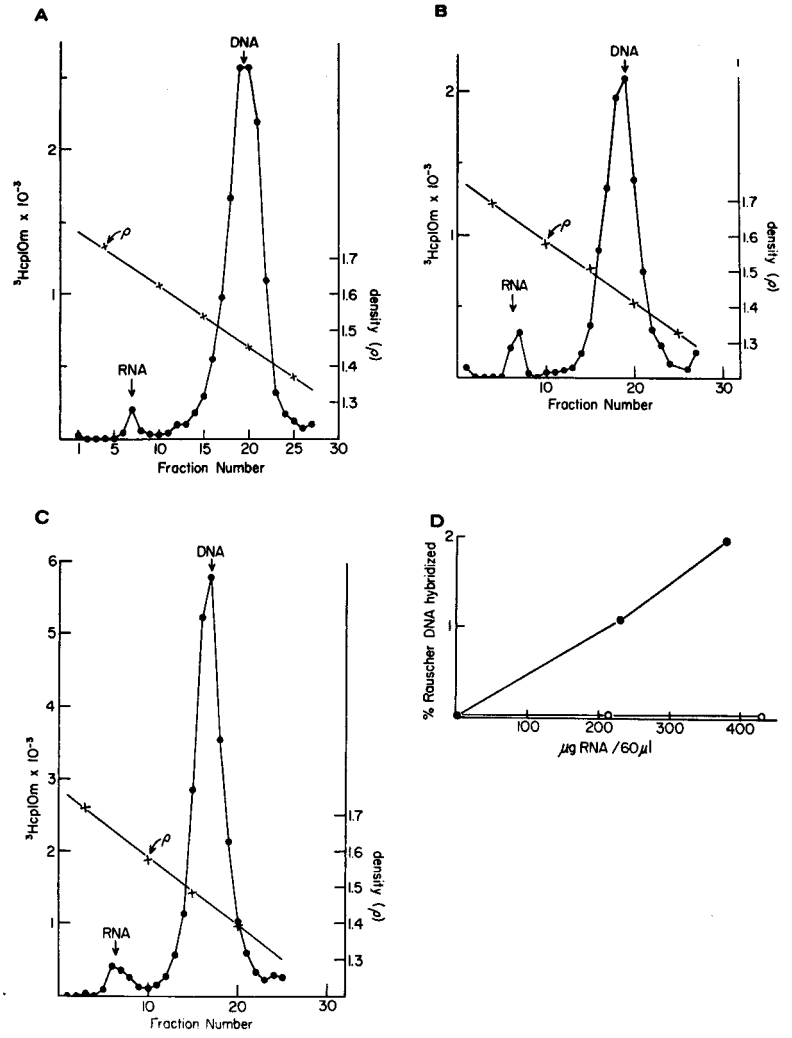

FIG. 1. (*A–C*). Cs_2SO_4 density profiles of RLV-[^{3}H]DNA hybridized to pRNA obtained from human lymphomas. Polysomal RNA was isolated from tissues of patients with Hodgkins' lymphoma (*A*), reticulosarcoma (*B*), and lymphosarcoma (*C*). 300 μg of polysomal RNA was hybridized to RLV-[^{3}H]DNA in 60-μl volumes, and the reactions were analyzed by Cs_2SO_4 density centrifugation. (*D*) Comparison of hybridization reactions between RLV-[^{3}H]DNA and various pRNA concentrations of a spleen from a patient with Hodgkins' lymphoma (●——●) and of a normal human spleen (○——○). The individual annealing reactions were analyzed by Cs_2SO_4 density centrifugation, and the % 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 1.69) of the gradients.

circumstances, it is necessary to exercise extreme care in the preparation of both the pRNA and the [^{3}H]DNA used as a probe. Complete removal of the contaminating protein from the pRNA is necessary to avoid nonspecific trapping of [^{3}H]-DNA in nonrelevant portions of the density gradient.

To insure the interpretability of the results, it is imperative that every purified [^{3}H]DNA preparation used be monitored for adequacy by being subjected to the following tests: (*a*) It must be shown to band cleanly in the appropriate DNA region of the density gradient to insure that it is free of contaminating viral RNA; (*b*) It must be extensively hybridizable to the homologous viral RNA used as the template in the original synthesis. This can exceed 90% in the presence of excess viral RNA if actinomycin D, at a concentration of 100 μg/ml, is included to prevent DNA-directed DNA synthesis, a possibility particularly prevalent in crude enzyme preparations; and (*c*) The [^{3}H]DNA product should not hybridize to unrelated viral RNA or to RNA from normal tissues. We customarily use the RNAs from AMV, RLV, and MMTV as test objects for cross-hybridizability checks. A suitable synthetic DNA complementary to any one of them will not hybridize to either of the other two or to pRNA from normal tissue.

It is not a trivial matter to obtain nucleic acid preparations that will satisfy all of these criteria. It is, unfortunately, not uncommon to obtain DNA preparations that fail the third requisite of specific hybridizability to its homologous RNA. This failure may stem from slight contamination with cellular DNA in the synthesis mixture, in which case the product will contain labeled DNA that will hybridize to both normal and tumor RNA. Another source of confusion is the possible complementary copying of long stretches of adenylate residues that are present in oncogenic RNAs (6, 7) and in normal cellular messages (8, 9). [^{3}H]DNA with the resulting blocks of T residues will hybridize to any RNA containing corresponding blocks of A. This difficulty is greatly magnified by the use of oligo(dT) as a primer to enhance synthesis, a procedure that should be avoided when specific probes are made. One

can readily test for the occurrence of this type of pairing by examining the effect of prehybridizing with unlabeled oligo(rU).

Every [^{3}H]DNA used as a probe in the present and previous (1–4) studies successfully passed the three tests outlined above. We cannot overemphasize the need for exercise of the precautions noted in investigations along these lines. Unless the materials used satisfy the criteria stipulated, the resulting experiments are likely to generate more confusion than information.

RESULTS

We first illustrate (Fig. 1*A–C*) representative outcomes of hybridizations between RLV[^{3}H]DNA and the pRNA prepared from three types of human lymphomas. Between 2 and 6% of the [^{3}H]DNA is shifted to the RNA region of the Cs_2SO_4 gradient. The position of the complex implies that the RNA is much larger than the DNA and, thus, determines the density of the hybrid structure. The amounts of RLV[^{3}H]-DNA involved in the complexes are comparable to those we have seen in similar experiments with pRNA from human sarcomas (4) and human leukemic white blood cells (3).

Fig. 1*D* compares the responses of pRNA preparations from spleens of normal humans and those with Hodgkins' disease at various concentrations of pRNA. No detectable reaction is obtained with normal spleen pRNA, even at the highest concentration tested, which was in excess of 7 mg/ml. Within the same range, the pRNA derived from the spleen of the patient with Hodgkins' disease complexed with somewhat more than 2% of the RLV[^{3}H]DNA, with no evidence of saturation at 6.7 mg of pRNA per ml.

Since it is unwieldy to detail the Cs_2SO_4 gradient profile of every sample tested, we have adopted a more convenient recording of our data. After correction for background, the tritium counts in the density region of RNA (1.62–1.69 g/ml) were taken as a measure of the amount of [^{3}H]DNA complexed to RNA. To attain the statistical accuracy desired, 10-min counts (cp10m) were taken on each sample. The convention was adopted that hybridization was negative if the sum of the cp10m in the RNA density region was less than three standard deviations above the mean background in the same region.

Table 1 lists the lymphoma samples tested for RNA related to the DNA of RLV. The results are recorded in counts per 10 min (corrected for background) in the RNA density region, and as multiples of the mean background standard deviation. Included are 24 Hodgkins' lymphomas, three reticulum cell sarcomas, and five lymphosarcomas. Of the 32 lymphomas, 22 (or 69%) gave clear indications of containing RNA homologous to the RNA of RLV. This result is in sharp contrast to the results obtained with pRNA from normal adult and fetal tissues (Table 2). None of the 48 normal preparations tested gave a reaction that could be accepted unambiguously as positive. Fig. 2 provides a convenient pictorial summary as multiples of the mean background standard deviation.

The fact that 69% of the pRNAs derived from lymphomas yielded positive hybridizations with RLV-DNA, whereas none of the 48 control tissues (Table 2, Fig. 1*D*) was positive, already argues for the specific significance of the positive reactions. Further support for this conclusion can be provided by the use of [^{3}H]DNA complementary to irrelevant viral RNAs in hybridizations with lymphoma pRNAs positive for a reaction with RLV–DNA. We have shown (manuscript in preparation) that RLV–DNA and RNA do not crosshybridize significantly with the corresponding nucleic acids of either AMV or MMTV. If the annealing reaction is specific, one would not expect a lymphoma pRNA positive for a reaction with RLV–DNA to show any ability to hybridize either with MMTV–

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

Lymphomas	RNA-region cp10m	cp10m/S	Reaction
Hodgkins' lymphomas			
1417 (*S*)*	292	3.40	+
1/145 L (*L*)	269	3.13	+
1/145 Sp (*S*)	223	2.59	−
726 (*L*)	306	3.56	+
476 (*S*)	166	1.93	−
821 (*LN*)	52	0.60	−
731 (*S*)	609	7.08	+
1413 (*S*)	66	0.77	−
622 (*LN*)	48	0.56	−
1413 (*LN*)	83	0.97	−
1217420 (*S*)	600	6.98	+
1121260 (*S*)	649	7.55	+
1241240 (*S*)	50	0.58	−
1238972 (*S*)	285	3.31	+
93 (*S*)	32	0.37	−
030893 (*S*)	447	5.20	+
1059649 (*S*)	463	5.38	+
1245268 (*S*)	265	3.10	+
1244787 (*S*)	274	3.20	+
1241721 (*S*)	296	3.44	+
1239151 (*S*)	419	4.87	+
229 (*S*)	289	3.36	+
254 (*S*)	318	3.70	+
L (*S*)	429	5.00	+
Reticulum cell sarcomas			
Lymphoma 24317 (*LN*)	88	1.02	−
Histiocytic lymphoma (*LN*)	438	5.10	+
Reticulum cell sarcoma 24282 (*LN*)	565	6.57	+
Lymphosarcomas			
Lymphosarcoma Hz (*Ly*)	371	4.31	+
Lymphosarcoma Hv (*Ly*)	515	6.00	+
Lymphosarcoma Ev (*Ly*)	387	4.50	+
Lymphosarcoma 101 (*Ly*)	1217	14.90	+
Lymphosarcoma 241 (*LN*)	178	2.07	−

32 Tumors tested, 69% positive.

Results of hybridization reactions between RLV-[^{3}H]DNA and pRNA isolated from human lymphomas. 200–1000 μg of pRNA in 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 1.69) was then determined. The results are expressed as cp10m (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. 2). The annealing reaction is considered positive only if the cp10m per RNA region is greater than 3*S*, thus providing 99.9% confidence statistically.

* The tissue tested is given parenthetically: *S*, spleen; *L*, liver; *LN*, lymph node; *Ly*, lymphocyte.

TABLE 2. *Test for viral-specific RNA in normal human tissues*

Tissue	RNA-region cp10m	cp10m/*S*	Reaction
Normal white blood cells	68	0.80	—
Phytohemagglutinin-stimulated lymphocytes (39)	212	2.50	—
Phytohemagglutinin-stimulated lymphocytes (44)	241	2.80	—
10–14 (*LN*)*	74	0.90	—
24192 (*LN*)	249	2.90	—
24207 (*LN*)	253	2.90	—
24196 (*LN*)	253	2.90	—
24193 (*LN*)	254	2.95	—
24282 (*LN*)	105	1.22	—
24257 (*LN*)	85	0.99	—
24300 (*LN*)	—	0.0	—
24317 (*LN*)	39	0.45	—
24318 (*LN*)	176	2.05	—
64ym (*S*)	130	1.50	—
24207 (*S*)	226	2.60	—
24238 (*S*)	175	2.03	—
24239 (*S*)	203	2.36	—
24240 (*S*)	180	2.09	—
24241 (*S*)	214	2.49	—
24297 (*S*)	34	0.40	—
24300 (*S*)	127	1.48	—
24302 (*S*)	—	0.0	—
1072584 (*S*)	70	0.81	—
24318 (*S*)	128	1.49	—
24314 (*S*)	—	0.0	—
W.R. (*L*)	116	1.40	—
24206 (*L*)	133	1.60	—
24207 (*L*)	247	2.90	—
Intestine 24207	242	2.80	—
Fetal lung	61	0.70	—
Fetal liver (12 week)	209	2.40	—
Fetal liver (14 week)	232	2.70	—
Fetal liver (15 week)	207	2.40	—
Fetal liver (16 week)	238	2.80	—
Cell line NC37 (*Ly*)	62	1.17	—
Placenta 28005	64	1.21	—
24204 (*L*)	73	1.38	—
24205 (*L*)	131	2.48	—
Intestine 24205	84	1.59	—
Intestine 24204	42	0.80	—
Striated muscle 24204	88	1.66	—
Striated muscle 24205	43	0.81	—
Fetal limbs (16 week)	124	2.34	—
Fetal limbs (14 week)	88	1.66	—
Fetal limbs (14 week)	144	2.72	—
Fetal limbs (16 week)	86	1.62	—
Fetal limbs (16 week)	116	2.19	—
Fetal limbs (24 week)	115	2.17	—

48 Samples tested, 0% positive.

Results of hybridization reactions between RLV-[^{3}H]DNA and pRNA isolated from normal human tissues. 200–1000 μg of pRNA in each sample were annealed to 2000 cpm of RLV-[^{3}H]DNA, and the reactions were subjected to Cs_2SO_4 equilibrium centrifugation. The data are expressed as cp10m (corrected for background) that bands in the RNA region of the gradient, and as multiples of the standard deviation (*S*), as described in Fig. 2 and Table 1.

* See Table 1.

DNA or AMV–DNA. Fig. 3*A* and *B* show that these expectations are realized. Two Hodgkins' lymphoma pRNAs, positive for reactions with RLV–DNA, show no significant reactions with either AMV–DNA or MMTV–DNA.

DISCUSSION

The absence of positive reactions with the pRNA preparations from certain of the lymphomas may raise questions of universality in the minds of some. However, it must be recognized that a negative outcome cannot be accepted as evidence for the nonexistence of the relevant RNA, whether the tissue being tested is neoplastic or normal. The sensitivity of the method is, at present, principally limited by the amount of RNA that can be dissolved in the annealing mixture, a difficulty that will be greatly obviated by the development of a suitable enrichment procedure for the pertinent RNA fraction. As in the instances of the leukemias (3) and sarcomas (4), we can at best conclude that the probability of finding RNA homologous to RLV–RNA is much greater in human lymphoma cells than in normal tissues. Indeed, if the provirus (10) or oncogene (11) hypotheses are valid, some part of this oncogenic information could be expressed and detected with the aid of more sensitive tests in certain normal tissues that have been reported (12) to exhibit group-specific antigens of the mammalian leukemogenic viruses.

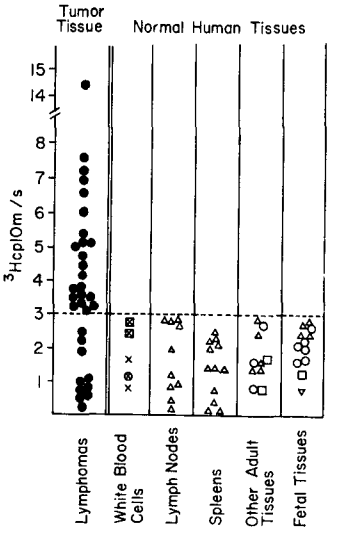

FIG. 2. Results of hybridization reactions with RLV-[^{3}H]-DNA and pRNA from human lymphomas and normal human cells. The normal tissues tested are: normal white blood cells (×), phytohemagglutinin-stimulated lymphocytes (⊠), a human lymphocyte cell line, NC37 (⊗), lymph nodes, spleens; other adult tissues: liver (△), intestine (○), and striated muscle (□); and fetal tissues: liver (△), lung (▽), limbs (○), and placenta (□). The reactions were centifuged in Cs_2SO_4 equilibrium density gradients. The amount of [^{3}H]DNA, expressed as cp10m corrected for background, banding in the density region of RNA (between densities 1.62 and 1.69), was determined for each reaction. An operational mean and standard deviation (*S*) were then determined for each counter by the total cp10m of three tubes (e.g., 2,3,4) of each of 60 gradients. The number of [^{3}H]DNA cp10m corrected for background banding in the RNA region of the gradient was then divided by the appropriate operational standard deviation. Any reaction with cp10m in the RNA region less than 3*S* is considered negative, thus providing 99.9% confidence that those reactions retained as positive (greater than 3*S*) are significant.

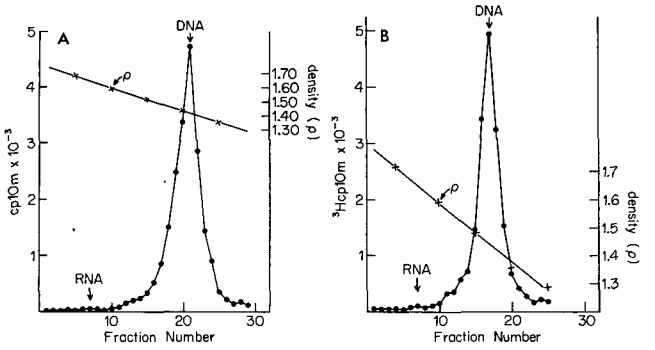

FIG. 3. Cs_2SO_4 equilibrium gradient centrifugation of (*A*) AMV-[^{3}H]DNA and (*B*) MMTV-[^{3}H]DNA hybridized, each to 400 μg of Hodgkins' lymphoma pRNA. Hybridization conditions and Cs_2SO_4 gradient analysis are detailed in *Methods*.

The existence of viral-related RNA in human lymphomas does not of course establish a viral etiology for this disease. One must now perform experiments designed to answer the following questions: (*i*) How large is the RNA being detected? (*ii*) How much homology does it in fact have to the RLV–RNA? and (*iii*) Is the viral-related RNA associated with a reverse transcriptase (RNA-instructed DNA polymerase) and is it located in structures characteristic of incomplete or complete virus particles? The requisite techniques have been developed to answer these questions with respect to the human neoplasias we have studied, and the experiments are underway.

Discovery of RNA homologous to that of the murine leukemia virus in human lymphomas immediately raises the issue of Burkitt's disease, a malignant lymphoma found in children living in a geographical belt extending across Central Africa. There exists serological (13) and nucleic acid hybridization (14) evidence linking this disease to the Epstein–Barr virus (EBV), a herpes-like virus that contains DNA detected in (15), and isolated (16), from Burkitt's lymphoma cells grown in culture. Since a causal relation between Epstein–Barr virus and Burkitt's tumor has not been established, it is pertinent to inquire whether this lymphoma, like the others examined here, also contains RLV-related RNA.

In many ways the most noteworthy features of our studies of human neoplasias emerge when they are compared with one another, and to this end a composite of the results we have thus far accumulated is presented in Table 3. Breast cancer contains RNA homologous only to that of the murine mammary tumor virus. Leukemias, sarcomas, and lymphomas all contain RNA homologous to that of the Rauscher leukemia virus and not to MMTV–RNA. Finally, none of the human tumors contains RNA detectably related to the RNA of the avian myeloblastosis virus.

TABLE 3. *Homologies among human neoplastic RNAs and animal tumor viral RNAs*

Viral RNA	Human neoplastic RNAs			
	Breast cancer	Leukemia	Sarcoma	Lymphoma
Mouse mammary tumor virus	+	−	−	−
Rauscher murine leukemia virus	−	+	+	+
Avian myeloblastosis virus	−	−	−	−

The results of molecular hybridization between [^{3}H]DNA complementary to the various viral RNAs and pRNA preparations from the indicated neoplastic tissues. A *plus* sign indicates that hybridizations were positive and a *minus* sign that no hybridization could be detected.

It is clear that with all four human neoplasias examined, the specificity pattern of the RNA they contain is in complete accord with what has been observed in the corresponding viral-induced malignancies in the mouse.

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