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Particles with RNA of High Molecular Weight and RNA-Directed DNA Polymerase in Human Brain Tumors

(simultaneous detection test/oncornaviruses)

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ABSTRACT **We have previously shown that neoplastic cells of human breast cancers, leukemias, lymphomas, and sarcomas contain particles similar to the viruses that have been established as etiologic agents of these diseases in mice. The present paper concerns tumors of the central nervous system for which no suitable animal model or corresponding virus exists. Nevertheless, using the simultaneous detection test, we showed that human brain tumors contain 70S RNA and RNA-directed DNA polymerase encapsulated in a particulate component possessing a density of 1.17 g/ml. These particles satisfy the three diagnostic criteria that characterize RNA tumor viruses of animals. 24 Out of 26 (92%) of the most malignant (glioblastoma and medulloblastoma) brain tumors examined contained these virus-like entities.**

Molecular hybridization (1) with radioactive DNA probes synthesized with the aid of appropriate murine RNA tumor viruses has demonstrated the presence of viral-related RNA in human neoplasias (2–5). Development of the simultaneous detection test (6) permitted us to establish that the RNA we were detecting in human tumors was 70 S in size and encapsulated with an RNA-dependent DNA polymerase (7–9) in a particle possessing the density of animal oncornaviruses (8). Thus, human neoplastic cells contain entities that satisfy three of the criteria diagnostic of viral etiological agents identified with homologous diseases in animals.

We used [^{3}H]DNA made with animal oncogenic particles to probe for homologous information in the RNA of human tumors. The particles identified in human neoplastic tissues can be a source of the [^{3}H]DNA required to determine sequence relationship between the RNA of the human particles and that of the corresponding animal oncornaviruses. When [^{3}H]DNA synthesized with particles obtained from human breast cancer and from human leukemias were challenged with various RNAs from murine and avian oncornaviruses, the [^{3}H]DNA synthesized by the particles derived from various human cancers hybridized uniquely to the RNAs of the viruses that caused the corresponding neoplastic diseases in mice (8, 9).

In our present approach, we focused on adenocarcinoma of breast (2), leukemias (3), sarcomas (4), and lymphomas (5). These were chosen because excellent murine models were known and the corresponding viral agents were well characterized and readily available. Thus, we could produce the required [^{3}H]DNA probes. The degree of homology between the murine agents and the human tumor sequences was small but adequate for detectability. While confirming the homology between RNA in human leukemic cells and that of murine Rauscher leukemia virus, Gallo and his associates (10) showed that, had it been available at the time in adequate quantity, a primate sarcoma virus would have made such experiments much easier.

Neoplasms of the central nervous system are unique in being prevalent in *Homo sapiens*. Spontaneously occurring brain tumors in animals are rare (11, 12). Although some viruses produce tumors in animals when directly inoculated into the brain in fairly massive doses, no acceptable evidence for putative etiologic viral agents has been provided (13).

We tried [^{3}H]DNA probes made with Rauscher leukemia virus, murine mammary tumor virus, and visna. 26 Malignant brain tumors were examined, including glioblastoma multiforme, medulloblastoma, and oligodendroglioma. None exhibited evidence of RNA homologous to that of these viruses. We therefore had to bypass hybridization with a known [^{3}H]DNA probe to detect viral-related RNA. Instead, the simultaneous detection test was used to answer the following questions: (*1*) Do human neoplasms of the central nervous system contain particulate elements encapsulating a high-molecular-weight RNA with an associated RNA-directed DNA polymerase in a particle possessing the density characteristic of the RNA oncornaviruses? (*2*) Does the resulting DNA synthesized exhibit specific sequence relatedness to RNA from the homologous tumor or to known animal RNA tumor viruses?

Our results demonstrate that human tumors of the central nervous system do contain particulate entities possessing many of the diagnostic characteristics of RNA tumor viruses. The particles found in human brain tumors showed little, if any, sequence homology to RNAs of the animal agents implicated with mammary tumors, leukemias, lymphomas, or sarcomas. They do, however, show homology uniquely to the RNA extracted from homologous human tumors. The implication is that these particulate entities are unique to neoplastic cells and are organ-specific.

MATERIALS AND METHODS

Clinical Material. 51 Individual human brain tumors were used. These were fresh surgical specimens frozen immediately at −70° after excision. Thirteen samples of histologically

Abbreviations: TNE buffer, 10 mM Tris·HCl (pH 7.4)–0.15 M NaCl–10 mM EDTA; TNM buffer, 10 mM Tris·HCl (pH 7.4)–0.15 M NaCl–5 mM $MgCl_2$.

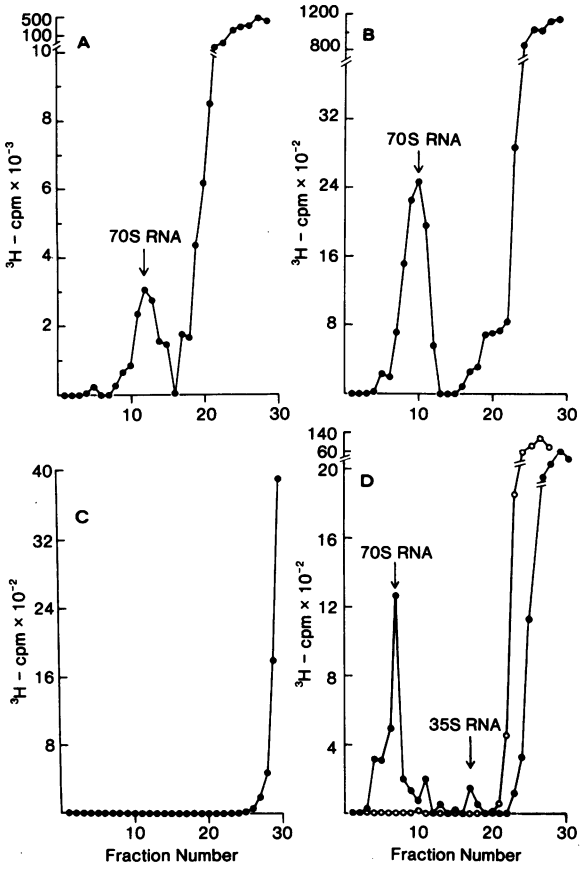

FIG. 1. Simultaneous detection of the 70S RNA–[^{3}H]DNA complex in brain tissue. (*A*), (*B*), and (*D*) are malignant tumors and (*C*) is normal tissue. Effect of ribonuclease on the high-molecular-weight RNA–[^{3}H]DNA complex was tested (*D*). The p-180 pellet was resuspended in 10 mM Tris·HCl (pH 8.3) and divided into two equal parts. A standard RNA-directed DNA polymerase reaction was performed on one part of the p-180 fraction. After incubation for 15 min at 37°, the nucleic acid complex was extracted with phenol–cresol. Its size was determined on a 10–30% linear glycerol gradient(●). After detergent disruption, the other half of the p-180 pellet was preincubated in the presence of RNase A (50 μg/ml) and RNase T_1 (50 μg/ml) for 15 min at 37°. A similar RNA-directed DNA polymerase reaction was then performed (○).

normal human brain tissues from patients with nonneoplastic or unrelated diseases served as controls.

Simultaneous Detection of 70S RNA and RNA-Directed DNA Polymerase (*7*). 1–4 g of tissue was finely minced and disrupted with a Silverson homogenizer at 4° in TNE buffer [10 mM Tris·HCl (pH 7.4)–0.15 M NaCl–10 mM EDTA]. This tissue homogenate was then centrifuged at 4000 × *g* for 10 min at 0°, and the supernatant was recentrifuged at 10,000 × *g* for 10 min at 0°. The resulting supernatant was then layered on a 15-ml column of 25% glycerol in TNE buffer and spun at 100,000 × *g* for 1 hr at 4° in a Spinco SW-27 rotor. The resulting pellet (p-180) was suspended in 10 mM Tris·HCl (pH 8.3) (50 μl of 10 mM Tris·HCl/g of tumor tissue). The suspension was preincubated at 0° in 0.3% NP-40 detergent (Shell), 25 mM dithiothreitol and then added to a standard endogenous RNA-directed DNA polymerase reaction mixture containing 6.25 μmol of Tris·HCl (pH 8.3), 1 μmol of $MgCl_2$, 1.25 μmol of NaCl, 0.2 μmol each of dGTP and dCTP, and

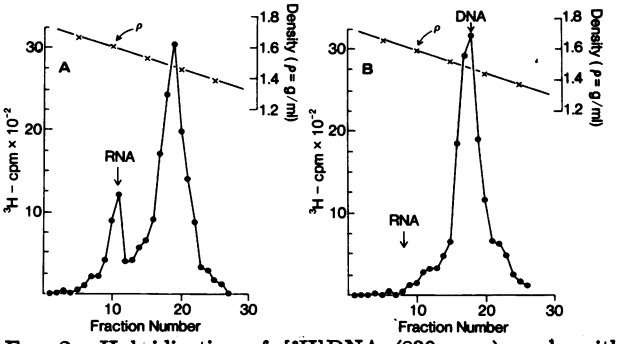

FIG. 2. Hybridization of [^{3}H]DNA (830 cpm) made with particles from a glioblastoma to polysome RNAs from a glioblastoma (160 μg of RNA) (*A*) and normal brain tissue (135 μg of RNA) (*B*).

0.2 ml of [^{3}H] dTTP (50 Ci/mmol). After 15 min of preincubation at 37°, the reaction was terminated by addition of 0.4 M NaCl and 1% sodium dodecyl sulfate. RNA was extracted by addition of an equal volume of a phenol–cresol 7:1 mixture containing 8-hydroxyquinoline (0.2 g/100-ml mixture). This was shaken at 25° for 5 min and centrifuged at 5000 × *g* for 5 min at the same temperature. The aqueous phase was then layered over a linear glycerol gradient (10–30% in TNE buffer) and centrifuged at 40,000 rpm for 210 min at 4° with a Spinco SW-41 rotor. External size markers were 70S [^{3}H]RNA from avian myeloblastosis virus. Fractions were collected from below; 50-μl aliquots were assayed for trichloroacetic acid-precipitable radioactivity. If the 50-μl aliquots were positive and contained enough radioactive DNA to warrant further characterization, then the corresponding portions were pooled and ethanol precipitation (2 × volume of absolute alcohol) was done. The resulting [^{3}H]DNA was then used for hybridization studies.

Preparation of Cytoplasmic p-180 RNA. Malignant and normal tissues were minced and then disrupted with a Silverson homogenizer at 4° in 2 volumes of 5% sucrose in TNM buffer [10 mM Tris·HCl (pH 7.4)–0.15 M NaCl–5 mM $MgCl_2$]. Suspension was then spun at 4000 × *g* for 10 min at 0°; the resulting supernatant was centrifuged at 10,000 × *g* for 10 min at 0°. This latter supernatant was layered on 20 ml of 25% sucrose in TNM buffer and centrifuged at 180,000 × *g* for 180 min at 4° in a Spinco 60 Ti rotor. This pellet (p-180) was suspended in TNM and 1% sodium dodecyl sulfate; the RNA was extracted three times with an equal volume of phenol–cresol (pH 8.4). Nucleic acids were precipitated by addition of 2 volumes of ethanol and 0.1 volume of 4 M LiCl.

Hybridization. [^{3}H]DNA (from 550–1800 cpm depending on quantity available) was annealed to cytoplasmic RNAs of the tumors of origin where possible and, in some instances, to RNAs of the same type of neoplasia. [^{3}H]DNA was first melted in 50% formamide at 80° for 10 min. After quick chilling to 0°, the RNAs were added. The mixture was brought to 0.4 M NaCl–50% formamide in a total volume of 100 μl and was then incubated for 24 hr at 37°. The reaction mixture was added to 5.4 ml of 5 mM EDTA with an equal volume of saturated Cs_2SO_4 to yield a starting density of 1.52. This was centrifuged at 44,000 rpm in a 50 Ti rotor (Spinco) for 60 hr at 15°. Fractions of 0.4 ml were collected and assayed for acid-precipitable radioactivity.

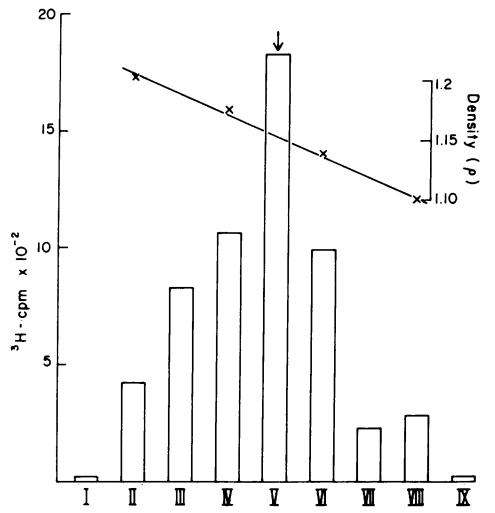

FIG. 3. Density of particles containing 70S RNA and RNA-directed DNA polymerase. The p-180 fraction from an oligodendroglioma was banded to equilibrium, and the fractions were assayed by the simultaneous detection test.

Hybridizations were also done between tumor [^{3}H]DNA and viral RNA. Preparation of viral RNAs has been described (14).

Density of Particles Containing 70S RNA and RNA-Directed DNA Polymerase. A polysome RNA (p-180), prepared from tumor tissues as described above, was suspended in TNE buffer, layered on a linear gradient of 20–50% sucrose in TNE buffer, and centrifuged at 25,000 rpm in a Spinco SW-27 rotor at 4° for 210 min. 10 Equal fractions were gathered from below and diluted with TNE buffer to a sucrose concentration of less than 15%. Simultaneous detection for 70S RNA and RNA-directed DNA polymerase was done on each fraction, and each was spun at 100,000 × *g*. The amount of 70S [^{3}H]DNA synthesized by an endogenous RNA-directed DNA polymerase was determined by glycerol velocity centrifugation.

RESULTS

Representative outcomes of simultaneous detection assays on malignant brain tumors and on normal brain tissue are described in Fig. 1. All three malignant tissues (Figs. 1*A*, *B*, and *D*) show clear evidence of well-defined ^{3}H-labeled DNA in the 70S position of the glycerol gradient. Furthermore, pretreatment with ribonuclease leads to the disappearance of [^{3}H]DNA from the 70S region. Finally, no such peak appears with a similar fraction prepared from normal brain tissue (Fig. 1*C*).

Specificity of [^{3}H]DNA Synthesized. The appearance of [^{3}H]DNA in the 70S region of the glycerol gradients and the response to the ribonuclease demonstrate the existence of a ribonuclease-sensitive synthesis of an RNA–[^{3}H]DNA complex with a sedimentation coefficient of 70 S. This does not, however, establish that a RNA-directed DNA polymerase has been identified. More rigorous proof requires showing that the [^{3}H]DNA synthesized can be hybridized to its presumed template, which in the present instance exists in the particulate elements isolated from malignant tumor (Fig. 2). The [^{3}H]-DNA synthesized by the particles from the glioblastoma hybridizes back to the RNA derived from the same tumor

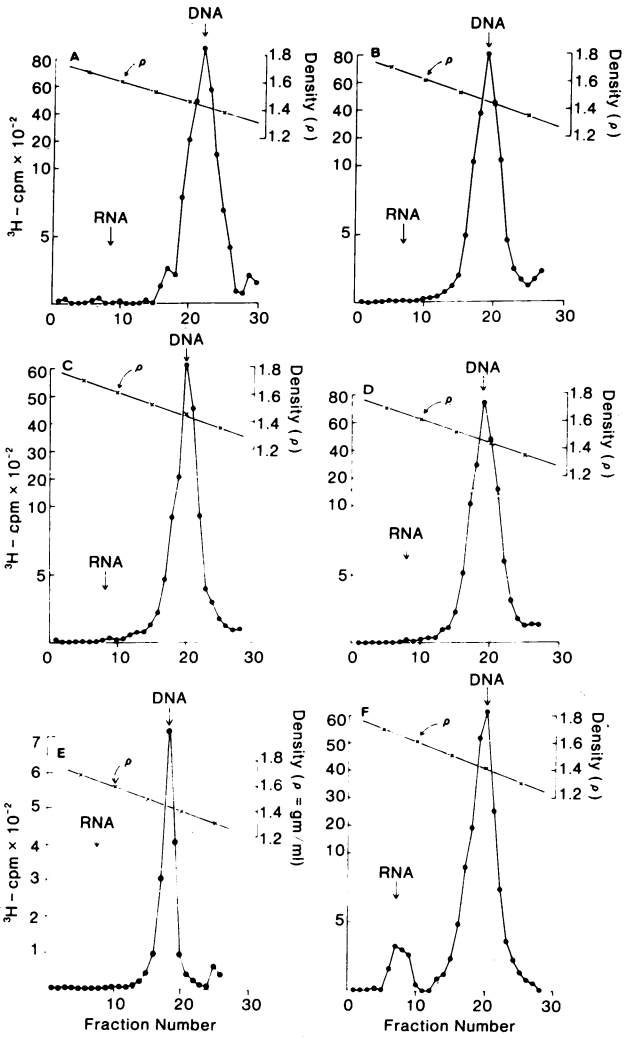

FIG. 4. Annealing reaction between glioblastoma [^{3}H]DNA and various viral RNAs. The amounts of the RNAs used are as follows: (*A*) Rous sarcoma virus, 2 μg; (*B*) Rauscher leukemia virus, 1 μg; (*C*) mouse mammary tumor virus, 0.4 μg; (*D*) avian myeloblastosis virus, 1 μg; and (*E*) visna virus, 1.1 μg. The amount of glioblastoma [^{3}H]DNA used in each corresponded to 2140 cpm. The positive control (*F*) consisted of 147 μg of polysome RNA from the same glioblastoma.

(Fig. 2*A*). No such complex formation, however, occurs with similar preparations of cytoplasmic RNA from normal brain tissue (Fig. 2*B*).

Density of Particles Encapsulating 70S RNA and RNA-Directed DNA Polymerase. The very fact that the p-180 fraction of the malignant tissue yielded a 70S RNA–[^{3}H]DNA complex in an endogenous reaction implies that the enyzme involved must be physically associated with its 70S RNA template. We wanted to determine whether RNA–enzyme complex was found in particles possessing the density characteristic of an RNA tumor virus. The p-180 fraction was thus subjected to an equilibrium centrifugation in a linear sucrose density gradient. The contents were collected in 10 fractions, and the particles in each were recovered by centrifugation. Simultaneous detection assays were then run (Fig. 3). The peak of the activity (*arrow*) centers at 1.17 g/ml, the density characteristic of RNA tumor viruses.

TABLE 1. *Simultaneous detection assay of 70S RNA and RNA-directed DNA polymerase in human brain tumors*

Brain tissue	70S Peak cpm	Results	Hybridization with homologous polysome RNA
Glioblastoma multiforme			
(KR)	1847	+	+
(HR)	1500	+	+
(RY)	2300	+	+
(FS)	500	+	+
(HM)	500	+	
(AS)	2500	+	+ (2)
(GA)	3500	+	+ (2)
(RD)	2000	+	+
(RS)	1040	+	
(JK)	2500	+	+
(CS)	100	+	
(ST)	800	+	
(FN)	2700	+	+ (3)
(CC)	1799	+	+
(HM 2)	6	−	
(MZ)	20	−	
(RM)	1661	+	+
(GB)	2800	+	+
(SF)	8500	+	+ (3)
(SN)	3150	+	+
(NV)	1860	+	
Meningioma			
(AC)	163	+	
(AR)	3462	+	−
(NO)	680	+	
(JD)	5	−	
(VK)	17	−	
(GA)	15	−	
(OO)	15	−	
(GN)	8	−	
(CB)	3		
(VM)	4	−	
(CB)	26	−	
Astrocytoma GR. I			
(JB)	20	−	
(JS)	202	+	
Astrocytoma GR. II			
(PC)	15	−	
Medulloblastoma			
(JB)	3000	+	
(RM)	805	+	+ (2)
(R)	2800	+	+ (3)
Hemangioblastoma			
(AB)	2000	+	+
(CH)	15	−	
Schwanoma [Acoustic]			
(AT)	20	−	
(JS)	2	−	
Schwanoma			
(SS)	30	−	
Ependymoma			
(CF)	8	−	
(TX)	18	−	
Mixed oligodendroglioma & astrocytoma			
(RE)	20	−	
Oligodendroglioma			
(DG)	3000	+	+ (2)
Chromophobe adenoma			
(WH)	30	−	
Craniopharyngioma			
(MP)	7	−	
(TT)	800	+	
Optic glioma			
(PC)	2000	+	
Noninvolved brain tissue			
WR (Tuberous sclerosis)	0	−	
NP (Meningitis)	2	−	
FM (Metastatic cancer)	8	−	
Fetus (Aborted normal)	6	−	
JW (Pneumonia)	20	−	
WF (Pneumonia)	11	−	
FF (Pneumonia)	8	−	
HV (Myocardial infarction)	20	−	
CG (Myocardial infarction)	9	−	
JO (Cirrhosis of liver)	10	−	
RH (Cancer of breast)	10	−	
CG (Prostatic tumor)	10	−	

Examination of Sequence Homology to Known Oncornaviruses. We pointed out in the introductory paragraphs that the attempt to use [^{3}H]DNA probes synthesized with known murine or avian RNA oncornaviruses failed to detect complementary RNA in various human brain tumors. It was nevertheless of interest to perform the adverse experiment and ask whether the [^{3}H]DNA synthesized by the particles from a brain tumor exhibits any homology to the RNAs isolated from known oncornaviruses. This was done with [^{3}H]DNA synthesized by particles from several kinds of brain tumors (Fig. 4). RNAs were prepared from avian myeloblastosis virus, Rous sarcoma virus, Rauscher leukemia virus, mouse mammary tumor virus and visna virus, which causes a "slow" neurological disease in sheep. The RNAs of none of these animal viruses possess any detectable homology to the DNA synthesized by the particles from the glioblastoma (Fig. 4). This same [^{3}H]DNA makes an excellent complex with glioblastoma cytoplasmic RNA (Fig. 4*F*).

A Survey of Brain Tumors for the Presence of Particles Containing 70S RNA and RNA-Directed DNA Polymerase. By the procedures described in *Methods*, and exemplified by the results described in Figs. 1 and 2, 51 brain tumors and 12 nonmalignant brain tissues were assayed for the presence of particulate elements containing 70S RNA and RNA-directed DNA polymerase (Table 1). The average counts per min in the 70S region of the nonneoplastic brain tissue were 9.5 cpm, whereas the responses observed with malignant tissue yielded an average of 2219 cpm. The highest count observed in normal tissues was 20 cpm, and the lowest amongst the positives was 160 cpm. There was, therefore, no difficulty in cataloguing a reaction as either positive or negative. In 17

of the positive tumors, enough [^{3}H]DNA was synthesized by the tumor particles to permit hybridization tests. Wherever this could be tested, homology was observed with the polysome RNA fraction from the same or similar tumor. In a few cases the amounts of [^{3}H]DNA available permitted several repetitive tests, indicated by the number in parentheses. The results were always concordant. 30 (or 59%) Of the tumors examined showed clear evidence of particulate elements possessing 70S RNA in association with RNA-directed DNA polymerase. In contrast, these particles could not be detected in any of the nonneoplastic brain tissues examined.

Although the numbers available do not permit a definitive conclusion, there is an interesting trend discernible amongst these tumors, which is more clearly seen in Table 2, in which the tumors are arranged in descending order of malignancy. The first four are the most malignant and show the highest incidence of particulate elements. On the other hand, the probability of finding these particles appears to be lower in the last three tumor types, which are considerably less malignant. This situation recalls the analogous finding (2) with breast cancer in which particles are found in malignant adenocarcinomas of breast but not in benign fibroadenomas.

TABLE 2. *Distribution of positive simultaneous detection assays in human brain tumors*

Brain tumors	Total	Positive	%
Glioblastoma multiforme	21	19	90
Medulloblastoma	3	3	100
Optic glioma	1	1	100
Oligodengroglioma	1	1	100
Craniopharyngioma	2	1	50
Hemangioblastoma	2	1	50
Astrocytoma (GR 1 & 2)	3	1	33
Ependymoma	2	0	0
Meningioma	11	3*	27
Schwanoma	3	0	0

* Of the three positives, one was found to be a meningeal sarcoma and another, an extremely cellular angioblastic type.

DISCUSSION

The present investigation into the etiology of human brain tumors is the first in which we have attempted to apply the methodologies of molecular biology in the absence of an available animal model with corresponding viral agent. We therefore could not use hybridization as the first step in the search for evidence for viral-related information in human brain tumors. The experiments described, however, demonstrate that it is possible to get around this deficiency by the use of the simultaneous detection test (6). With this device no prior availability of a suitable animal viral probe is required. The simultaneous detection test can directly provide evidence for the existence of a particle having the critical diagnostic characteristic of RNA tumor viruses. It is relatively easy to detect in human malignant brain tumors particulate elements possessing the tell-tale 70S RNA and RNA-directed DNA polymerase. Further, these particles possess a density characteristic of an RNA tumor virus.

The negative reactions with some of the tumors may raise questions of universality. However, a negative outcome cannot be accepted as evidence for the *absence* of these particles, whether the tissue being tested is neoplastic or normal. The presence of interfering reactions such as those of nucleases or the limit of sensitivity may prevent a positive result.

The existence of these virus-like particles in human brain tumors does not establish a viral etiology for this disease. It will be necessary ultimately to isolate these particles and demonstrate their infectivity and transforming capacity.

Since [^{3}H]DNA probes can be made from human brain tumors, it is now possible to examine homologies among the different brain-tumor particles, as well as similar particles associated with malignant tumors in other organ sites. This examination should provide insight into the number and relatedness of the kinds of virus-like particles found in various human neoplasias. If they are distinct, a possibility suggested by the unique particle in breast cancer, a pathway towards an organ site-specific diagnostic procedure might be provided.

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