Oncornavirus-like particles in human skin cancers
(oncogenic RNA viruses/melanoma/molecular hybridization/simultaneous detection test)

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ABSTRACT  A high-molecular-weight RNA encapsulated with an RNA-instructed DNA polymerase in particles possessing the density characteristic of the RNA tumor viruses has been detected in 13 out of 14 human malignant melanomas. The [3H]DNA synthesized by these particles in an endogenous reaction hybridizes to RNA extracted from the human melanoma particulate structures, but not to RNA from normal skin. Similar particles containing RNA and enzyme have been found in basal cell and squamous cell carcinomas of the skin. The RNA of the melanoma particles is easily distinguishable by hybridization from the RNAs found in the particles of the basal and squamous cell carcinomas.

We have shown in human leukemias, lymphomas, sarcomas, and breast cancer the presence of viral-related RNA (1-5) and of particles containing an RNA-instructed DNA polymerase (reverse transcriptase) complexed to a high-molecular-weight RNA (6-10). These particles are not found in the corresponding normal tissues. The availability of RNA tumor viruses known to cause corresponding cancers in animals made possible the identification of certain of the human malignant nucleic acid sequences as viral related. We subsequently detected similar particles in human brain tumors, in human malignancies of the gastrointestinal tract (11), and in lung (12). The lack of corresponding animal viruses for these neoplasms made it impossible to explore possible homologies with known animal tumor viruses.

The association of C-type viruses with several animal melanomas (13-15) and the detection of similar particles in a mouse melanoma (Hehlmann, Balda, and Spiegelman, in manuscript) suggested that a viral animal model may exist for this disease. Its fast progression and widespread metastases makes melanoma one of the most malignant of the human cancers.

Because of its nature, melanoma is easily detected at an early stage, and it is rare that the tumors removed are large enough to permit the kind of biochemical examinations that were required to detect the virus-like particles in our earlier studies of human malignancies. Despite such difficulties, it was decided to make the attempt, and the present paper describes the successful demonstration of particles in human melanomas possessing the density characteristic of RNA tumor viruses and encapsulating a large RNA molecule complexed to a reverse transcriptase.

MATERIALS AND METHODS

Clinical Material. After surgical removal, all tissue specimens were frozen as quickly as possible and stored at −70°.

Occasionally they were kept at −20°, if they were to be used within a few days. Repeated freezing and thawing and storage at temperatures higher than −70° for more than a week were avoided to prevent the known loss of enzyme activity that attends such procedures.

Preparation of Particle-Enriched Fraction. Tissue specimens (0.1–3 g) were minced by scissors, forming a paste, and then homogenized with a Silverson homogenizer at 4° in a buffer containing 10 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and 10 mM EDTA (TNE). Prior to sedimentation of the virus particles, nuclei and mitochondria were removed by centrifugation of the homogenate at 4000 × g for 10 min at 0–2° each. The second supernatant was spun through a 20% glycerol (in some experiments sucrose) cushion at 100,000 × g and 4° for 1 hr. The resulting supernatant was then layered on a 20% sucrose gradient in a Spinco SW-27 rotor. Usually, the resulting pellet was suspended in 50 μl of 10 mM Tris-HCl (pH 8.3) for each 0.1 g of tissue and examined for reverse transcriptase and 70S RNA. In a number of instances, the pellet was density fractionated by a further equilibrium centrifugation through a 20–50% linear sucrose gradient at 27,000 rpm and 4° for 180 min in a Spinco SW-27 rotor. The density region (1.16–1.19 g/ml) with the highest polymerase activity was collected and pooled. All assays for reverse transcriptase were carried out in the presence of actinomycin D to suppress DNA-directed DNA polymerization.

Simultaneous Detection Test. The viral-enriched fractions were used to detect reverse transcriptase associated with a high molecular weight by the procedures described previously (7-9).

Preparation of [3H]DNA Probes. The [3H]DNAs used in molecular hybridization were obtained from the 70S regions of the simultaneous detection tests performed on the particle-enriched fractions derived from tumors. If a particular reaction generated enough radioactive DNA, the 70S region of the velocity gradient was pooled and the nucleic acid precipitated with ethanol. The 70S RNA-[3H]DNA complex was then digested with 0.4 M NaOH for 18 hr at 37° to remove the DNA, and the [3H]DNA was recovered by ethanol precipitation.

Preparation of Cellular RNA. Tissues were minced and homogenized as described above for particle enrichment except that it was carried out in TNM buffer [10 mM Tris-HCl (pH 7.4)–150 mM NaCl–5 mM MgCl2]. The suspension was centrifuged at 15,000 × g for 40 min at 0°. The supernatant fluid was then layered on 20 ml of 25% sucrose in TNM buffer and spun for 180 min at 180,000 × g in a Spinco Ti rotor. The pellet (P-180) was resuspended in TNM buffer plus 1% sodium dodecyl sulfate and the RNA extracted three times with ethanol.

The nucleic acid was precipitated from the aqueous phase
by the addition of two volumes of ethanol and 0.1 volume of 4 M LiCl.

RNA-DNA Hybridizations. Purified [3H]dTTP-labeled product (2000 cpm per reaction) was first incubated at 68°C for 10 min in 50% formamide to denature the DNA. After quick chilling of the solution to 0°C, the appropriate RNA was added and the hybridization mixture was brought to 0.4 M NaCl–50% formamide, in a total volume of 100 µl, and incubated for 18 hr at 37°C.

After incubation, the reaction mixture was added to 5.5 ml of 5 mM EDTA mixed with an equal volume of saturated Cs2SO4 to yield a starting density of 1.52, and centrifuged at 44,000 rpm in a 50 Ti rotor (Spinco) for 60 hr at 20°C. Fractions of 0.4 ml were collected and assayed (8) for Cl3CCOOH-precipitable radioactivity.

RESULTS

Particle-enriched fractions were prepared from tumors by the procedures described in Materials and Methods and examined by the simultaneous detection test (8) for the presence of high-molecular-weight RNA associated with a reverse transcriptase. Typical positive outcomes are shown in Fig. 1, where the telltale peaks of newly synthesized tritiated DNA in the 70S region of the velocity gradient indicate that the newly synthesized DNA is complexed to a large RNA molecule as demonstrated by the disappearance of these peaks after ribonuclease digestion (open circles, Fig. 1B). Table 1 summarizes the results of 14 human melanomas examined in this way; of these, 13 showed clear evidence of particles containing a reverse transcriptase with a high-molecular-weight RNA. The two normal skins gave negative responses. It will be noted that “normal” skin within 5 cm of a melanoma yielded a positive response. This finding may be of interest in ultimately illuminating fast progression of this disease. Finally, two nonmelanotic skin malignancies, one a basal cell carcinoma and the other a squamous cell carcinoma, each gave strong positive reactions.

The density of the particles yielding a positive simultaneous detection test was determined by subjecting them to an equilibrium density centrifugation in sucrose as described in Materials and Methods. It was found (Fig. 2) that most of the positive particles band near the 1.16 g/ml density region of the sucrose gradient, a density characteristic of RNA tumor viruses. Some activity is detected at high densities (1.2 g/ml), and these probably represent viral cores that have lost some of their outer lipoprotein components during the course of the preparation.
It was of obvious interest to examine sequence relation of the DNA synthesized by the melanoma particles to the RNA found in such tumors as well as to the RNA found in other tumors of the skin that are unrelated to the melanomas. Such experiments could not be carried out with all of the tumors obtained since most of them were too small to provide the required amounts of tritiated DNA product. In two instances, however, sufficient [3H]DNA could be isolated to allow for hybridizations of this sort; the results are described in Fig. 3. A significant amount of hybridization occurs (Fig. 3A) between the tritiated DNA made with a melanoma particle and the RNA extracted from a melanoma. Interestingly, no hybridization could be detected with an equivalent amount of RNA prepared from a similar particulate fraction from a human basal cell carcinoma (Fig. 3B). Note, however, that the [3H]DNA probe made with particles from the basal cell carcinoma does hybridize to its own RNA. It should be noted that a similar examination (data not shown) was done with squamous cell carcinoma material (Table 1), and no homology could be found between the [3H]DNA probe from the melanoma and the RNA of the squamous cell carcinoma. Here again, the [3H]DNA synthesized endogenously by the squamous cell particles did hybridize back to the RNA from its own tumor.

**DISCUSSION**

The results described here add malignant melanoma to the list of human malignancies in which evidence for an association with an RNA tumor virus-like particle can be provided (1–12). These results with fresh tumors are consistent with earlier electron microscopic evidence (16) and the more recent simultaneous detection results with human melanoma cell lines and tumors (17, 18). In view of their strong positive reactions (Table 1), it seems likely that the squamous cell and basal cell carcinomas of the skin will also fall into this group after enough of these tumors have been examined.

Because of the limited supply, the results must be regarded as preliminary, but it would appear that the particles found in melanomas are readily distinguished from those associated with either squamous or basal cell carcinomas, indicating a high degree of histogenic specificity for the particle found in each type of tumor. This possibility is already suggested by the unique particle in human breast cancer, which shows no homology to any of the particles found in mesenchymal tumors (6). If this situation extends to other human cancers, it would encourage the exploration of using these

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**Table 1. Simultaneous detection assay of 70S RNA and RNA-instructed DNA polymerase in human malignant melanomas**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Primary tumor</th>
<th>Metastasis</th>
<th>cpm in 70S region</th>
<th>Result</th>
</tr>
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<tbody>
<tr>
<td>1. Melanomas</td>
<td>+</td>
<td>+</td>
<td>1320</td>
<td>+</td>
</tr>
<tr>
<td>2. Melanomas</td>
<td>+</td>
<td>+</td>
<td>340</td>
<td>+</td>
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<td>3. Melanomas</td>
<td>+</td>
<td>+</td>
<td>468</td>
<td>+</td>
</tr>
<tr>
<td>4. Melanomas</td>
<td>+</td>
<td>+</td>
<td>172</td>
<td>+</td>
</tr>
<tr>
<td>5. Melanomas</td>
<td>+</td>
<td>+</td>
<td>46</td>
<td>+</td>
</tr>
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<td>6. Melanomas</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>8. Melanomas</td>
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<td>+</td>
<td>300</td>
<td>+</td>
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<td>9. Melanomas</td>
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<td>+</td>
<td>1000</td>
<td>+</td>
</tr>
<tr>
<td>10. Melanomas</td>
<td>+</td>
<td>+</td>
<td>1640</td>
<td>+</td>
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<tr>
<td>11. Melanomas</td>
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<td>+</td>
<td>870</td>
<td>+</td>
</tr>
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<td>12. Melanomas</td>
<td>+</td>
<td>+</td>
<td>2640</td>
<td>+</td>
</tr>
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<td>13. Basal cell carcinoma</td>
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<td>+</td>
<td>1000</td>
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<td>14. Squamous cell carcinoma</td>
<td>+</td>
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<td>4000</td>
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</table>

Average of 14 melanomas = 730 cpm. Average of positive melanomas = 790 cpm. Average of negative melanomas = 8 cpm. Particle preparation and assay for synthesis of [3H]DNA-70S RNA complex are as described in the legend to Fig. 1. The sum of the cpm in the 70S region is recorded and corrected for a background of 12 cpm. A reaction is designated as positive if the cpm exceed 35.
particles, or their components, as organ-site and cell-specific diagnostic signals.

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