Embryogeneic Cell Type, Organ Site Sequence Specificity in Human Cancers

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SUMMARY

An extensive series of cross-hybridization studies were carried out with the DNA-RNA molecular hybridization technique. Molecular 70 S [3H]DNA probes synthesized from human central nervous system, gastrointestinal, pulmonary, and prostatic carcinomas were hybridized to cytoplasmic RNA's isolated from cancers of virtually all organ sites of the human body. Results indicated sequence homology between cancers of the same organ or cell type but not with cancers of different cell types. Thus cell types based on embryological origins determine the organ site specificity of the involved sequences. The designation of 70 S [3H]DNA denotes those [3H]DNA's that were copied off the template 70 S RNA, as distinguished from total [3H]DNA product, which includes all DNA's synthesized. It does not necessarily follow nor is it to be inferred that the 70 S [3H]DNA thus designated contains the full complement of the sequences found in the 70 S RNA template.

INTRODUCTION

Some investigators have reported that many human tumors contain a HMW RNA with a sedimentation coefficient in the vicinity of the 70 S region, a RNA-directed DNA polymerase capable of synthesizing DNA with the use of the HMW RNA as a template, and particulate elements with densities of 1.16 to 1.18 g/ml sucrose gradient. Because these same biochemical characteristics are reported to be invariably found in RNA oncornaviruses, the question of etiologic relationship between the 2 inevitably arises. Although some degree of sequence homology has been shown to exist between human breast cancer, human leukemias, sarcomas, lymphomas, and corresponding murine RNA oncornaviruses, in many others, no such homology can be demonstrated. The likelihood of the implication of a viral agent as the cause of human cancer is further dimmed by the inability of any researcher to isolate any proven human virus from human tumors. Accordingly, it may ultimately prove to be more fruitful to seek such answers at a genetic level rather than at a viral level. This project was started to seek out at a molecular level any genetic sequence relationship between different tumors from different organs of the human body. The technique chosen was the DNA-RNA molecular hybridization originally developed by Hall and Spiegelman. Although this technique has its limitations, mainly because hybridizations cannot be carried to a high R0t value (concentration of RNA multiplied by time), nevertheless it is sufficiently accurate as a screening technique when dealing with a great number of hybridizations between a vast array of tumors. Temperature melt analysis would be the more accurate and ideal technique but would be virtually impossible to execute when dealing with such vast quantities of tumors. Again because of the vast number of hybridizations involved, it is difficult to synthesize sufficient 70 S [3H]DNA molecular probes to carry out hybridizations between all the tumors in 1 or even a few series of experiments. Accordingly, the hybridizations were carried out under identical laboratory settings in many sets or series involving from 2 to 4 tumors/experiment. Furthermore, because of the great number of figures involved, representative figures from many sets of hybridization studies were selected. For the sake of lucidity and clarity, they were occasionally graphed together, hence the discrepancy in the amounts of 70 S [3H]DNA probes used. We now describe the results of these extensive cross-hybridization studies.

MATERIALS AND METHODS

Surgical specimens of human cancer were obtained and frozen at −70°C within 1 hr after removal. On infrequent occasions, autopsied specimens were used, primarily for cytoplasmic RNA extractions. Where possible, histological normal tissues from identical organs from the same patients were also obtained to serve as controls.

Synthesis of [3H]DNA from Tumor 70 S RNA. One to 4 g tumor tissue were finely minced and disrupted with a Silverson homogenizer at 4°C in TNE buffer. This tissue homogenate was then centrifuged at 4000 × g for 10 min at 0°C, and the supernatant was recentrifuged at 10,000 × g for 10 min at 0°C. The resulting supernatant was layered on a 15-ml column of 20% glycerol in TNE buffer and spun at 100,000 × g for 1 hr at 1°C in a Spinco SW-27 rotor. The resulting pellet was resuspended in 0.01 M Tris-HCl, pH 8.3 (100 μl 0.01 M Tris-HCl per g tumor tissue). The suspension was preincubated at 0°C in concentrations of 1.0% NP40 detergent (Shell Oil Co., New York, N. Y.): 0.1 M dithiothreitol for 10 min. Actinomycin D (5 mg/ml), 0.1 volume, and 4 M NaCl, 0.05 volume, were added, and then these were...
added to a standard endogenous RNA-instructed DNA polymerase reaction mixture containing 10 mM Tris-HCl, pH 8.3, 8 mM MgCl₂, 1.7 mM concentrations each of dGTP, dCTP, dATP; and 1 mCi [³H]TTP per 200 µl of suspension. After 7 min of incubation at 37°, the reaction was terminated by chilling at 0° and adding 0.1 volume 4 M NaCl and 10% sodium dodecyl sulfate. Extraction was achieved by the addition of an equal volume of phenol:creosol (7:1) containing 8-hydroxyquinoline (0.2 g/100-ml mixture). This was shaken in a vortex at 25° for 3 min and centrifuged in a Sorval centrifuge at 16,300 × g for 10 min at the same temperature. The aqueous phase was then layered over a linear glycerol gradient (10 to 30% in TNE buffer) and centrifuged at 41,000 rpm for 210 min at 4° with a Spinco SW-41 rotor. External size markers were 70 S [³H]RNA from avian myeloblastosis virus. Fractions were collected from below and 50-µl aliquots assayed for trichloroacetic acid-precipitable radioactivity. If the 50-µl aliquots proved to contain enough 70 S RNA-[³H]DNA to warrant further characterization, then the corresponding portions were pooled and ethanol precipitation (2 volumes of absolute alcohol and 0.4 M LiCl) was carried out. After alkaline destruction of the complexed RNA's, the resulting 70 S [³H]DNA was used for hybridization studies.

Preparation of Cytoplasmic 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. The suspension was spun at 4000 × g for 10 min at 0° and the resulting supernatant was centrifuged at 10,000 × g for 10 min at the same temperature. This latter supernatant was layered on 15 ml of 25% sucrose in TNE buffer and centrifuged at 180,000 × g for 180 min at 4° in a Spinco Ti 60 rotor. The resulting pellet was suspended in TNM buffer, 0.4 M NaCl, and 1% sodium dodecyl sulfate, and the RNA was extracted 3 times with an equal volume of phenol:creosol (pH 8.4). The nucleic acids were precipitated by the addition of 2 volumes of ethanol and 0.1 volume of 4 M LiCl.

Hybridization. The 70 S [³H]DNA (from 550 to 1800 cpm) was annealed to cytoplasmic RNA's of the tumors of origin where possible and, in some instances, to RNA's of the same type of neoplasia. The 70 S [³H]DNA was first melted in a 50% formamide concentration at 80° for 10 min. After quick chilling at 0°, the HMW RNA's were added and the mixture was brought to 0.4 M NaCl:50% formamide in a total volume of 50 µl. This was then incubated for 24 to 36 hr at 37°. Thereafter the reaction mixture was added to 5.4 ml of 5 mM EDTA with an equal volume of saturated Cs₂SO₄ to yield a starting density of 1.52. This was centrifuged at 44,000 rpm in a Ti 50 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 60 hr at 15°. Fractions of 0.4 ml were collected and assayed for acid-precipitable radioactivity. The Rₐf value (concentration of RNA multiplied by time) was from 2 to 3 × 10⁸.

RESULTS

It has been shown that the molecular 70 S [³H]DNA probes synthesized by the method described above were indeed copied off 70 S RNA (4, 5, 12). In this study, external size markers in the form of 70 S [³H]RNA from avian myeloblastosis virus were used to ensure the accuracy of the analysis. The cytoplasmic RNA's were shown to contain polyosomal HMW RNA's (4, 5). All the tumors involved in the synthesis of 70 S [³H]DNA have displayed the 3 biochemical attributes mentioned above. The presence of positive hybridization between tumors would signify that these tumors contained homologous sequences in their HMW RNA's. The degree or percentage of positive hybrid formation would depend on the number of homologous sequences, assuming optimal laboratory conditions.

We began by synthesizing 70 S [³H]DNA from 3 types of brain tumors. These tumors were selected because of their general availability and because they produced more of the 70 S [³H]DNA necessary for the hybridization studies per experiment. The lower grades of astrocytomas were also available but were deficient in terms of 70 S [³H]DNA synthesis. Hybridization was then instituted in various combinations in series of 2 or more depending on the amount of 70 S [³H]DNA synthesized. Controls with their own HMW RNA and/or equivalent normal tissues were always included in each series of hybridizations. The hybridizations were carried out in either direction to ensure accuracy (e.g., medulloblastoma 70 S [³H]DNA + glioblastoma HMW RNA, glioblastoma 70 S [³H]DNA + medulloblastoma HMW RNA). Hybridizations were done at least twice in both directions. The same procedures were carried out with lung, GI, and prostatic neoplasms. In some instances, enough 70 S [³H]DNA was synthesized from 1 single tumor to enable us to carry out hybridizations between 10 separate tumors in 1 sitting (Figs. 1 and 5). More often they were done in a series of 2 to 4 tumors, the limiting factor being the amount of 70 S [³H]DNA available. At no time was there any mixing of 70 S [³H]DNA's from 2 separate tumors, even though they were of the same histopathological type. The same applies to the HMW RNA's. Hybridization was carried to a Rₐf of between 2000 and 3000. In our experience it is not advisable to increase the Rₐf values beyond 3000 with the existing technique because in order to do so one must either lengthen the duration of hybridization at 37° or increase the concentration of HMW RNA. The hybridization beyond 36 hr carries with it the danger of degradation of the RNA and/or the hybrid. Increasing the concentration of HMW RNA would increase the viscosity to a point where it would become technically difficult to achieve good mixing. Hence we have not been able to carry out the hybridization beyond a Rₐf of 3 × 10⁸ with any degree of success. This is actually the limitation of the DNA-RNA hybridization.

Hybridization was carried out between tumors from the same organ (e.g., brain, lung, stomach, colon, rectum, and prostate) and then between different organs of the above listing. Finally, 70 S [³H]DNA's were synthesized from the tumors of these organs and hybridized to HMW RNA's from tumors from virtually all other organs of the human body (Table 1). They represented all 3 embryological strata and all cell lines: mesodermal, endodermal, and ectodermal. The results indicated that the sole determinant of this aspect of sequence homology or sequence specificity appears to be organ-site or cell-type differentiation based on the difference in embryological origins (Table 1). A hybridization of at least 15% spread over at least 3 points
Extensive cross-hybridization studies with 70 S \(^{3}H\) DNA probes derived from CNS, GI, pulmonary, and prostatic cancers with HMW RNA's from various neoplasias

Some tumors were not tested because of inadequate materials. They were matched, nevertheless, with at least 1 tumor in each group. A definable pattern was thereby established.

<table>
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<tr>
<th>CNS</th>
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<td><strong>Uterine cancer</strong></td>
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<sup>a</sup> +, positive results; -, negative results; NT, not tested.

Chart 1. Hybridization of 70 S \(^{3}H\)DNA made from particles of medulloblastoma to, respectively, normal brain RNA A, medulloblastoma HMW RNA B, and glioblastoma HMW RNA C and from glioblastoma to oligodendroglioma D. Details are described in "Materials and Methods."
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is considered positive. Probably because of the relatively low $R_0$ value, it is rare to achieve hybridization of 50% or more with the use of this technique and with human materials. [In only 2 instances were we able to obtain hybrids of 50% or more, one was a prostatic cancer (70%) and the other was a glioblastoma (52%).] Since there are too many figures in this study, we will show only a representative few.

Chart 1 shows cross-hybridization studies between the 3 types of brain tumors. Because these were from 2 separate experiments, there was some discrepancy between the 70 S $[^3H]$DNA input probe cpm and also the total cpm. There was clearly positive hybrid formation between the 3 types of brain tumors but not with normal brain tissue which served as controls. Chart 2 represents those studies conducted with various GI cancers. These represent 2 separate experiments. Nevertheless, there was no obvious homology between the various GI tumors. Chart 3 represents studies with the use of stomach cancer against cancers from other organs outside the GI tract. This is 1 of the 2 instances in which we were able to synthesize sufficient 70 S $[^3H]$DNA from 1 single huge tumor to execute hybridization studies with 9 separate tumors. Again, there is no detectable homology except with its own RNA which serves as a control. Chart 4 is a mixture of hybridization studies with lung and colon cancers. No obvious homology can be detected between the 2. Corresponding normal and neoplastic tissues serve as controls. The 2nd instance, when sufficient 70 S $[^3H]$DNA was synthesized from 1 single tumor for hybridization studies against 10 separate tumors, is represented by Chart 5. In these studies, there is no obvious sequence homology between these tumors, despite the fact that some of the tumors arise from what is classified anatomically as the genitourinary system.

The amounts of 70 S $[^3H]$DNA used per hybrid ranged from 550 to 1800 cpm and were identical in each experiment. However, the amounts of HMW RNA used differed because the tumors from which they were extracted differed for obvious reasons, even in the same experiments. They ranged from 160 to 400 $\mu$g. There is no difficulty in cataloging these studies as either positive or negative based not only on the percentage of hybridization (from a low of 16% to a high of 41%) but also the pattern and configuration of hybrid peaks.

**DISCUSSION**

Although negative results are fraught with some degree of uncertainty because of the limitations of the methods used and the difficulty of relating degrees of change in any biological system, with the available data, by these methods, and within the limits of their sensitivity, the absence of sequence homology between cancers of the stomach, colon, and rectum and tumors of the genitourinary system, despite their anatomical proximity, prompted us to con-

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**Chart 2. Molecular DNA-RNA hybridizations with the use of 70 S $[^3H]$DNA probes from stomach carcinomas (CA) with, respectively, HMW RNA templates from cancer of the stomach (A), from a normal stomach (B), from cancer of the colon (C), and from cancer of the rectum (D).**
Chart 3. 70 S [3H]DNA probes were synthesized from a single gastric carcinoma (CA). Then they were annealed to the HMW RNA's of simian sarcoma virus, liposarcoma, hepatoma, hypernephroma, cancer of the breast, cancer of the prostate, cancer of the ovary, cancer of the cervix, and cancer of the pancreas. As controls, they were annealed to their own RNA's which showed a 16% homology. No homology was clearly discernible with the other tumors.

Chart 4. 70 S [3H]DNA was synthesized from lung and colon carcinomas (CA) and annealed to its own HMW RNA and corresponding normal tissues and to each other. There is no sequence homology except to their own RNA's (28 and 17%).

dlude that cell types based on embryological origins determine the organ site specificity of the involved sequences. An examination of the GI tumors revealed that the embryological origin for colon cancer is different from that of rectal carcinoma. Their cell types are probably subtly different although not grossly obvious. Similarly, prostatic and bladder carcinomas do not display sequence homology, since they have different cell types and embryological origins. When we relate these data to the brain tumors, it becomes obvious that all those examined have a common embryological origin, namely the glial cells. A perusal of this array of tumors in Table 1 reveals the same common denominator as a basis for sequence homology. At a molecular level it is possible that the sequences may reside in the final DNA of the DNA±RNA±DNA pathway (13). This in turn may be related to the membrane-associated cytoplasmic DNA's that allegedly control cellular differentiation and provide for the specific cellular immunological response through their influence on immunogen recognition (11). The diagnostic and therapeutic implications become quite apparent. An immunological diagnostic device can conceivably be programmed to detect cancers in specific organs. Therapy may be directed accordingly along similar lines. It would seem that tumors from the same cell type share a
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Chart 5. Molecular 70 S rRNA probes were synthesized from single prostate cancer (CA) and then annealed to HMM RNA from cancer of the bladder, cancer of the ovary, and cancer of the breast. No sequence homology was seen. There is a 25% homology with its own HMM RNA.
common or similar antigen, at least on a molecular level. The data obtained from these experiments appear to complement those obtained from temperature melt experiments in which tumor-specific sequences are found in human central nervous system tumors. Since these sequences have been shown to be similar irrespective of degree of malignancy, their specificity must be modified or determined by their embryological origin. Thus, these so-called tumor-specific sequences are specific only with respect to cell type and organ site based on embryological origin of that particular tumor or group of tumors. They are not necessarily specific with respect to the type of cells that make up the tumor.

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With the use of hydroxyapatite chromatography columns, temperature melt analysis was made on a series of human central nervous system tumors. 70 S ([3H]DNA probes were 1st exhaustively annealed to normal brain DNA. When the repeat sequences were removed, the resulting recycled probe was reannealed to brain tumor DNA to seek out the nonrepeat sequences or tumor-specific sequences. These same probes were then used against the various grades of astrocytomas. The results indicated that these sequences are similar irrespective of grade of malignancy.

REFERENCES

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