Tumors in the Rat after Injection of Neoplastic and Preneoplastic Nucleic Acids

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Summary

Hepatomas occurred in the liver of 5 of 386 ACI rats at the site of injection, 10–16 weeks previously, of cells of regenerating liver pre-incubated with DNA derived from the Morris hepatoma 3924C, transplantable in this strain. A tumor of undetermined nature developed in 1 of 62 rats 6 months after intrahepatic injection of a mixture of preneoplastic liver DNA and RNA.

No tumors occurred in 1480 other ACI rats (No. in parentheses) treated as follows: (a) intrahepatic injection of regenerating liver cells pre-incubated with (a) hepatoma RNA (340), (b) preneoplastic DNA (124), (c) normal liver DNA (40), and (d) normal liver RNA (40); (b) intrahepatic injection of (a) hepatoma DNA (280), (b) hepatoma RNA (246), (c) preneoplastic liver DNA (166), (d) preneoplastic liver RNA (184), (e) normal liver DNA (30), and (f) normal liver RNA (30).

The circumstances under which the hepatomas occurred seem to warrant the conclusion that a process of cell transformation by the hepatoma DNA was involved in their pathogenesis.

Introduction

Some years ago, it was reported from our laboratories that lymphosarcomas developed at the site of s.c. injection of a chromatin fraction obtained from Murphy lymphosarcoma cells and that hepatomas developed at the site of intrahepatic injection of a similar fraction obtained from cells of a transplantable hepatoma (30, 36, 37). The significance of these observations was questioned on the basis that the possibility of the presence of intact cells in the injected material could not be excluded with certainty.

Since then, there have been several reports of induction of tumors by nucleic acids of oncogenic viruses (12, 13, 16, 17). There is evidence, too, that biologically active nucleic acids of other types can enter somatic cells and alter synthetic processes, and that exogenous DNA had entered the recipient genome (22, 24, 31, 39). Accordingly, the concept of transfer of subcellular genie entities is no longer regarded as fanciful, as it was at the time of our initial reports.

The present study was undertaken to investigate the following possibilities: (a) that the malignant potentialities of "preneo-

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1 Supported in part by USPHS Grant Ca-07212 from the National Cancer Institute.

Received for publication June 7, 1965; revised September 9, 1965.

Materials and Methods

Nucleic acids were prepared from 2 sources: the Morris hepatoma 3924C, which was induced originally in ACI rats by diacetylaminofluorene and has been carried by s.c. transplantation in this strain in our laboratories since 1950; (b) "preneoplastic" liver, i.e., liver of male rats that had received 2-acetylaminofluorene (AAF) (0.03%) in the diet for 90 days, followed by a carcinogen-free diet for 30 days, at which time (120 days) there is no detectable morphologic evidence of malignancy in the liver. With this AAF dosage and the diet employed, hepatomas develop ultimately in 85% of male rats (156–320 days).

All animals were killed by cervical fracture. The tumors and livers were excised quickly and, after removal of excess blood by blotting, were frozen immediately either in a Dry Ice-alcohol mixture or in 2-methoxyethanol at −60°C. The frozen tissues were weighed and were processed in amounts of 20–50 gm. DNA was prepared by the method of Colter et al. (8) and RNA by the method of Kirby (20). The DNA preparations contained a small amount of RNA, no attempt having been made to remove traces of the latter. All preparations were dried in vacuo and kept at −27°C prior to use.

Two types of experiments were performed: (a) intrahepatic injection of regenerating liver cells pre-incubated with hepatoma nucleic acids; (b) intrahepatic injection of nucleic acids of hepatoma and of preneoplastic liver.

(a) Male ACI rats (150–200 gm) were partially hepatectomized (15) and were sacrificed 48 or 72 hr later by cervical fracture or by exsanguination under light ether anesthesia. Two gm of regenerating liver were disrupted in 20 ml of Hanks' solution in a glass homogenizer with a loosely fitting (to minimize disintegration of cells) Teflon pestle and were mixed thoroughly with approximately 50 mg of hepatoma DNA or RNA or preneoplastic DNA. Hepatoma DNA was employed in 386 cases, hepatoma RNA in 340, and preneoplastic DNA in 124. The cell suspension was incubated at 37°C in a shaking incubator for 2 hr and then was centrifuged; 0.1 ml of packed cells was injected into the liver in male ACI rats (150 gm) under ether anesthesia. The injection site was sealed with Surgicel. They were sacrificed...
after 2–8 months, the majority after 6 months. As controls, normal liver nucleic acids were employed in the same manner, DNA in 30 cases and RNA in 30. 

(b) Approximately 50 mg of DNA or RNA, or of a mixture of equal amounts of each, were dissolved in 5 ml of Hanks' solution. Approximately 1 mg of nucleic acid (0.1 ml) was injected into either the intact liver or the regenerating liver (72 hr after partial hepatectomy) of 150-gm male ACI rats under ether anesthesia. The injection site was sealed with Surgicel. Hepatoma DNA was employed in 280 animals and hepatoma RNA in 246. They were sacrificed 2—8 months later. 

Surgicel. Hepatoma DNA was employed in 280 animals and 
Preneoplastic liver DNA was employed in 166 animals, preneo 
under ether anesthesia. The injection site was sealed with 
DNA in 30 cases and RNA in 30. These animals were sacrificed after 6 months. 

A cell-free saline extract of the hepatoma was injected i.p. in 86 rats, hepatoma DNA i.p. in 38 and i.m. in 26, and hepatoma RNA i.p. in 34. These animals were sacrificed after 6 months.

Results

The results are presented in Table 1. Tumors occurred only in animals in 2 groups: (a) in 1 of 6 in which a mixture of preneoplastic liver DNA and RNA was injected into a regenerating liver; (b) in 5 of 386 injected with regenerating liver cells pre-incubated with hepatoma DNA.

Preneoplastic DNA and RNA. This tumor was found 6 months after injection. It measured 2.5 cm in longest diameter, was well encapsulated, presented beneath the skin of the epigastrium, and was adherent to the anterior surface of the liver.

It consisted mainly of irregularly rounded, but also some polyhedral and spindle-shaped, cells of varying size, arranged in cords, islets, or sheets, with an abundant stroma (Figs. 1, 2). The nuclei, usually spheroidal, were relatively large, and mitotic figures were scarce. In some areas, the longitudinal arrangement of polyhedral cells was suggestive of liver cords. Nothing was seen that suggested bile ducts or bile pigment. There were vascular channels, resembling sinusoids, in the lining of which there were numbers of phagocytizing cells.

Because of the unusual morphologic characteristics of this tumor and the circumstances under which it developed, slides were submitted to Dr. Harold L. Stewart, who stated as follows: "Ten or twelve of us have examined the slides of this tumor. We are all agreed that this is a malignant tumor, but none of us can recall ever having seen a tumor like this in a rat. The majority feel that it is a sarcoma, but two of us are not sure that it is not a carcinoma. A number of possible types of tumor were considered, as follows: reticulum cell sarcoma, chondrosarcoma, alveolar soft parts sarcoma, glomus tumor, hemangiopericytoma, ganglioneuroma, hypernephroma, and undifferentiated carcinoma. Our group could not agree on any one of these diagnoses. None of us suggested an origin from the liver."

Regenerating Liver Cells Pre-incubated with Hepatoma DNA. The 5 tumors in this group were found at the intrahepatic injection site, 1 at 10 weeks, the others at 16 weeks after injection. All were well encapsulated and sharply demarcated from the adjacent normal liver, measuring 1.5–3 cm in longest diameter (Figs. 7–11). 

On microscopic examination these nodules did not differ significantly from the Morris hepatoma 3924C from which the DNA had been obtained (Figs. 3–6). There was complete absence of normal liver architecture. The tumor in each instance was composed of atypical epithelial cells, varying in size, shape, and nuclear chromatin content. The majority were polyhedral, with basophilic cytoplasm and large nuclei, many of which were hyperchromatic. There were many mitotic figures. The cells generally were arranged in irregular fashion, but occasionally showed a cordlike arrangement. Bile ducts and bile pigment could be identified.

Two of these tumors, transplanted in the groin in adult ACI rats, gave rise to hepatomas indistinguishable from the original Morris hepatoma 3924C. As is the case with the latter tumor, no growth occurred when transplantation was attempted in Wistar rats. Another of these nodules, disrupted and injected i.p in ACI rats, was established as an ascites tumor, which has been carried successfully in both solid and ascites forms through 12 transplant generations.

Discussion

There is substantial evidence that the nucleic acids of certain oncogenic viruses can cause malignant transformation of animal cells in the same manner as do the intact viruses (12, 13, 16, 17). It has been demonstrated also that neither the virus nor its DNA in infectious form is present in the transformed cells (10, 38). Nor was it found possible to produce active virus or DNA by measures that are effective in activating prophage, i.e., exposure to ultraviolet or X-rays, or temporary thymidine starvation (38). There has been general agreement that infectious virus is not produced in cells transformed by carcinogenic adenoviruses nor in cells transformed by SV40 (10). However, Ito and Evans (17), working with the Shope papilloma virus, found that the DNA extracted from the papillomas induced in domestic rabbits also was tumorigenic, although at a lower level than that obtained from wild cottontail rabbits, despite the fact that the tumors in the domestic rabbits fail to yield evidence of virus when tested by usual procedures.

The possibility that similar transformations of mammalian

Effect of Neoplastic and Preneoplastic Nucleic Acids

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<tr>
<th>SOURCE OF NUCLEIC ACID</th>
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<td>Hepatoma RNA</td>
<td>0/340</td>
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<td>Preneoplastic liver RNA</td>
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* Intrahepatic injection of regenerating liver cells pre-incubated 2 hr with nucleic acid.

* Intrahepatic injection of nucleic acid.

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cells may be effected by exposure to nucleic acids of cells with different potentialities has received increasing attention in recent years. This possibility has received theoretical support from observations that exogenous DNA can be taken into the nuclei of other cells, including (a) cells of transplantable mouse leukemias (34), (b) HeLa cells (4, 7), (c) L cells (3), (d) various ascites tumor cells (6, 19, 21, 32), and (e) Henle tissue culture cells (11). However, studies of this type do not establish conclusively that the exogenous DNA enters the nucleus of the recipient cell as the intact molecule and influences its biologic potential.

More significant support of this possibility is provided by demonstrations of the transfer of biologic characteristics to the recipient cells. Resistance to 8-azaguanine (5) and to sarcolysin (31) has been reported to have been induced in tumor cells sensitive to these tumoricidal agents by the DNA of cells resistant to them. Cells of normal bone marrow, producing only normal adult hemoglobin (HbA), have been induced to produce sickle-cell hemoglobin (Hbs) by incubation with DNA (22) and RNA (39) extracted from the bone marrow of subjects with sickle cell anemia. RNA of rabbits immunized to skin homografts has transferred this immunity to normal lymphocytes upon incubation (24). RNA of normal mouse liver cells has been reported to induce enzyme formation in tumor cells (27–29).

It has also been reported that morphologic changes have apparently been induced in cells by exposure to nucleic acids of other types of cells in vitro, i.e., inhibition of growth of fibroblasts in tissue culture by exposure to DNA of cells of Ehrlich ascites tumor and of Sarcoma 180 (35), and development of an appearance resembling that of malignant cells by normal hamster kidney cells exposed to kidney tumor RNA in tissue culture (18).

Direct approaches to this matter from the standpoint of carcinogenesis have taken two courses: (a) attempts to transform normal cells to neoplastic cells by exposing them to nucleic acids of neoplastic cells; (b) attempts to modify the neoplastic potential of tumor cells by exposing them to nucleic acids of normal cells. Lacour et al. (23) reported the occasional development of morphologically unusual tumors in newborn Swiss mice following i.p. injection of RNA extracted from lymph nodes of patients with lymphomatosis. Peculiar tumors occurred occasionally also in newborn and adult mice after i.p. injection of RNA extracted from ascites tumor cells. Hewer and Meeks (14) observed intestinal tumors following injection of herring sperm DNA in 3-week-old C3H mice. DeCarvalho and Rand (9) reported the occurrence of tumors (a) in 30% of adult rats following i.p. injection of Novikoff hepatoma RNA, (b) in 10% of the offspring of rats following i.v. injection of this material during pregnancy, and (c) in 5% of rats given s.c. injections cutaneously shortly after birth. There was no adequate description of these tumors.

Although bizarre effects might conceivably be produced by such procedures, it is difficult to understand how nucleic acids of a specific type of neoplastic cell can reproduce the original tumor with such frequency when administered parenterally. We have not observed any tumors in ACI rats after i.p. or intrahepatic injection of RNA or DNA extracted from both solid and ascites forms of the Morris hepatoma 3924C, transplantable in this strain. Other negative results of experiments of this type have been reported (2), and undoubtedly many more negative results have not. More successful results might be anticipated if the tumor cell nucleic acid were to be placed in contact with normal homologous cells under conditions that would favor its entrance into these cells. There are few reports of experiments of this type, although this is the procedure that has been employed regularly for the purpose of demonstrating that exogenous nucleic acids can enter cells and can modify their biologic characteristics.

The Novikoff hepatoma RNA that was reported to induce tumors in 30% of rats when injected i.p. was also stated to induce malignant transformation of normal liver cells (20% of cases) upon incubation at 2°C for 15 hr (9). There have been reports also of neoplastic transformation of heterologous tissues, i.e., normal mouse kidney by incubation with ascites tumor RNA (25) and chick chorioallantoic membrane by incubation with Novikoff hepatoma RNA (26).

The converse has been reported, i.e., modification of the neoplastic potential of tumor cells by incubation with RNA of normal cells. The capacity for growth was stated to be markedly reduced in the case of Nelson ascites tumor cells (Swiss mouse) after incubation with normal mouse liver RNA (28) and cells of transplantable rat hepatomas after incubation with normal rat liver RNA (1, 9).

The most important implication of the development of the tumors in the present experiment is that they represent a process of cell transformation, involving genic recombination mechanisms similar to those established for certain bacteria. Other possibilities must be considered: (a) that they are spontaneous tumors, unrelated to the experimental procedure; or (b) that they represent tumor production by a virus or by the nucleic acid of a virus, rather than by that of the hepatoma cell.

In the case of the tumor that occurred after injection of preneoplastic nucleic acids, there is an additional remote possibility that it arose as a result of transfer, into the incubated cells or the injected liver, of carcinogen (AAF) bound to the nucleic acids. There is little that can be stated concerning the pathogenesis of this tumor. It may have occurred spontaneously, although a number of pathologists with extensive experience in rodent tumors had not encountered one like it previously. Moreover, there is no precise information as to how much, if any, AAF is bound to liver nucleic acids 30 days after discontinuing administration of the carcinogen. There is therefore no certainty that minute amounts may not have been introduced into the liver with the preneoplastic nucleic acids and may possibly have contributed to the development of this peculiar tumor of undetermined nature several months later.

In considering the pathogenesis of the 5 hepatomas, the following observations are pertinent:

(a) All occurred at the intrahepatic injection site in animals in which the liver had been observed to be grossly normal at the time of injection 10–16 weeks previously. This would appear to eliminate the possibility of spontaneous origin of these tumors.

(b) All were morphologically essentially identical with the original Morris hepatoma 3924C from which the DNA had been obtained. Two were successfully transplanted s.c. and another gave rise to an ascites tumor upon i.p. injection and has been carried in this form through 12 transplant generations. Their neoplastic nature has therefore been established.
(c). All occurred following injection of regenerating liver cells pre-incubated with hepatoma DNA (5 of 386 rats). Although the DNA was contaminated with traces of RNA, it seems justifiable to attribute the tumor induction to the DNA, inasmuch as none occurred in 340 rats given injections of regenerating liver cells incubated with hepatoma RNA.

(d). No tumors occurred in rats given injections of regenerating liver cells pre-incubated with preneoplastic liver DNA (124 rats), normal liver DNA (30 rats), or normal liver RNA (30 rats). This seems to warrant the conclusion that the effect produced was related to the neoplastic origin of the DNA and not to some possible nonspecific action of nucleic acids.

The report of production of mammary tumors in rats by injection of nucleic acids and cell-free preparations of mammary cancers induced by 7,12-dimethylbenzanthracene implies viral involvement in the production of these tumors (33). Although this possibility cannot be excluded in the present instance, it seems unlikely. One would have to presuppose the persistence of active virus after 15 yr of passage through adult hosts, i.e., about 200 transplant generations. No tumors have occurred following i.p. or i.m. injection of hepatoma DNA or RNA or of cell-free saline extracts of the tumors. Moreover, none has occurred within 2—8 months following intrahepatic injection of hepatoma DNA in 280 rats and RNA in 246, the majority of which were examined after 6 months. If the nucleic acid regarded as hepatoma DNA were in reality an oncogenic virus DNA, one would expect tumors to occur at least as frequently following direct intrahepatic injection of the nucleic acid as with the incubation procedure.

On the basis of these observations it seems reasonable to conclude that these hepatomas were induced by the experimental procedure and that the probability is strong that a process of cell transformation by hepatoma DNA was involved in their pathogenesis.

Acknowledgment

The authors are indebted to Dr. Harold L. Stewart, National Cancer Institute, for his opinion on 3 of the tumors reported here.

References


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FIG. 1. Tumor of undetermined nature that followed injection of mixture of preneoplastic liver DNA and RNA. Compact arrangement of rounded and polyhedral cells in tumor of undetermined nature. × 160.

FIG. 2. Same as Fig. 1. Suggestive trabecular arrangement of cells in tumor of undetermined nature. × 350.

FIG. 3. Characteristic appearance of the 5 hepatomas that followed intrahepatic injection of cells of regenerating liver pre-incubated with hepatoma DNA. Hepatoma sharply demarcated from adjacent normal liver. × 160.

FIG. 4. Same as Fig. 3. Hepatoma. Polyhedral cells in cordlike arrangement. × 350.

FIG. 5. Same as Fig. 3. Hepatoma. Newly formed bile ducts. × 350.

FIG. 6. Characteristic appearance of the Morris hepatoma 3924C from which the DNA was obtained. × 350.
FIGS. 7–11. Gross photographs of the livers containing hepatomas that occurred following intrahepatic injection of regenerating liver cells pre-incubated with hepatoma DNA. The tumor in Fig. 11 has been cut longitudinally.
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