Comparative Oncogenic Properties of Deoxyribonucleic Acid from Primary Parotid Gland Tumors, Passage Parotid Gland Tumors, and Polyoma Virus*

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SUMMARY

These experiments have demonstrated that DNA extracted from cells infected with polyoma virus has oncogenic properties similar to DNA from primary parotid gland neoplasms when inoculated into newborn mice of the AKR strain.

DNA prepared from parotid tumor cells in the third, fourth, and sixth generation of cell transfer resulted in tumor development in only one animal.

No tumor induction was noted when primary parotid tumor DNA was inoculated into mice over 2 weeks of age.

It is concluded that the tumorigenic DNA isolated from primary parotid tumors is that of polyoma virus and not that of the cells themselves. This viral nucleic acid can be isolated in an active form in the presence of antiviral antibody and has a high degree of infectivity.

In a previous publication from this laboratory (3), it was demonstrated that deoxyribonucleic acid extracted from mouse parotid gland tumors and inoculated into newborn AKR mice resulted in a high incidence of these tumors and epithelioid thymomas in animals of 3–4 months of age. The oncogenic properties of the nucleic acid were abolished by incubation with deoxyribonuclease but not by ribonuclease. It was not clear from these studies whether the observed tumors were the result of malignant cellular transformation by the tumor cell DNA or due to infectious DNA of polyoma virus. The latter possibility was considered because these tumors were morphologically identical to those resulting from inoculation of newborn mice with polyoma (8), and this virus has been shown to contain infectious DNA in a tissue culture system (2). The experiments comprising the present report were designed to answer the question by comparing oncogenic properties in AKR mice of DNA extracted from the primary parotid tumors with those of polyoma virus DNA and DNA from a parotid tumor that had been transferred by cells for several generations.

MATERIALS AND METHODS

Mice.—The mice used in these experiments were of the inbred AKR/Jax strain from the breeding colony maintained in the laboratory. These animals have 70 per cent incidence of spontaneous lymphocytic leukemia developing after 6 months of age. No parotid tumors or epithelioid thymomas have been observed to occur spontaneously in this strain, and no neoplasms of any kind have been found in animals under 6 months of age.

In all cases we used litters from mothers who had not nursed young given injections of polyoma virus or its nucleic acid, realizing that these mothers could passively transmit polyoma antibodies to their offspring, which would make them resistant to infection with the virus (5). However, some mothers nursing animals used in this experiment had had previous litters that had been given injections of supernatants of mouse embryo tissue cultures and cell-free filtrates of AKR leukemic tissue in connection with other experiments, and these mothers could have been a source of polyoma antibody transmission.

Gross and microscopic observation.—The animals were sacrificed within 1–2 weeks of the observation of parotid tumors or when dyspnea, protruding eyes, and bulging thorax suggested the presence of thymoma. Since it was observed in earlier experiments that 90 per cent of the tumors occurred in animals under 24 weeks of age, tumor-free animals were sacrificed at 24–36 weeks after inoculation. Complete autopsies were performed in each case. The tumors were fixed in Bouin’s fluid, and hematoxylin and eosin-stained sections were prepared for microscopic examination.

Source of tumors.—The parotid tumors used for DNA extraction had arisen from inoculation of parotid tumor...
DNA into suckling AKR mice as described in a previous experiment (3).

The “passage” parotid tumors used for DNA extraction were in their third, fourth, and sixth transfer generation. The source of the original tumor was from a 5-month-old AKR mouse inoculated when newborn with parotid tumor DNA. The transfer was performed by aseptically removing a 2-mm fragment of tumor, incubating it for 5 min. at room temperature in Tyrode solution containing 800 units/ml of penicillin and streptomycin, and implanting it subcutaneously in the axilla of the recipient mice. The subcutaneous tumors were removed 2—7 months after transfer for DNA extraction.

Nucleic acid extraction.—The nucleic acid from the primary and transmitted parotid tumors was prepared according to Kirby’s method as described previously (3).

The viral DNA was prepared from polyoma virus which was kindly supplied by Dr. Clyde Goodheart of the Division of Virology of the Los Angeles Children’s Hospital. This virus resulted in the development of parotid tumors and thymomas when inoculated into newborn mice of our AKR strain. Ten secondary mouse embryo cultures in 60-mm. plastic Petri dishes were infected with polyoma virus. The growth medium (N-16 with 15 per cent fetal calf serum) was changed at 3-day intervals. Seven to ten days after infection, cytopathic effects were observed in the nuclei of the cells, and the cultures were sacrificed for DNA extraction. The method used for extraction of DNA from the infected monolayers was a modification of that described by Weil (9). Extractions were carried out from two groups of infected monolayers with slight differences in method. The supernatant fluid was removed, and the monolayers were washed three times with Hanks balanced salt solution (BSS). Versenate buffer, 2 ml., was added to each dish and allowed to stand for 5 min. at room temperature. The loosened cell sheets were removed with a pipette and pooled. The subsequent procedures in each instance were carried out at 4° C. In the first preparation, designated DNA 49, the cell sheets were homogenized in versenate buffer for 3 min. In the second preparation (DNA 51), the cell sheets were frozen (—70° C.), thawed, and then homogenized. Six milliliters of 6 per cent sodium p-aminosalicylate (PAS) were then added to the versenate mixtures, which were homogenized for an additional 3 min. This material was then stirred for 1 hr. with an equal volume of phenol. The phenol-PAS mixture was centrifuged for 30 min. at 3500 × g. In the DNA 49 preparation, the aqueous portion was removed and shaken with 2 volumes of water-saturated ether. The residual ether was removed by bubbling nitrogen through the mixture for 10 min. The aqueous phase from the phenol-PAS mixture for DNA 51 was extracted two additional times with an equal volume of phenol. The final aqueous supernatant was shaken five times with 2 volumes of ether. Thereafter, nitrogen was bubbled through for 10 min. The DNA concentration of the material extracted from both the tumors and infected monolayers was determined by the diphenylamine reaction. In the case of tumor DNA, the test was performed directly on an aliquot of the saline suspension of DNA injected into the mice. However, since the viral DNA was suspended in PAS, which interferes with this reaction, DNA concentration of this material was determined by precipitation of the nucleic acid with alcohol and resuspending it in an equal volume of water for measurement with the diphenylamine reaction. Biuret tests performed on all of the nucleic acid solutions revealed them to be qualitatively free of protein.

The DNA solutions, 0.05 ml., were inoculated into mice 0—10 days of age. Injections of older mice were adjusted to body weight, the volumes ranging from 0.07 to 0.3 ml. The injections were made in the first or second intercostal space just to the right of the sternum with the use of a 30-gauge needle.

RESULTS

DNA prepared from parotid gland tumors.—The nucleic acid preparations used in this group were prepared from four different parotid tumors arising from animals previously injected with parotid tumor DNA. The concentration of DNA in these preparations was 400—500 μg/ml. There were 39 mice in this group. Thirty of the animals from eight of the nine litters that had been given injections developed bilateral parotid tumors and/or epithelioid thymomas at the age of 14—26 weeks (mean, 16 weeks) after inoculation. All of the animals were inoculated with the material immediately after its preparation at 0—6 days of age. These parotid tumors and all subsequent ones were bilateral lobulated neoplasms which grew locally. Their histology showed the presence of both glandular and mesenchymal elements. The thymomas usually filled the thoracic cavity. They were smooth and not adherent to the chest wall, and the division between the two thymuses was distinct. They were sometimes hemorrhagic. Microscopically they were composed of thymic epithelial cells. The thymomas grew locally, causing death by suffocation. Metastases were not seen from either tumor.

To determine if DNA from this same source might induce tumors when given at an older age, 26 animals 2—8 weeks old were given intrathoracic injections of the parotid tumor DNA. The amount of DNA administered was adjusted according to body weight; three separate preparations were given to five litters. The animals were observed for 24—36 weeks after inoculation. Two littermates in this group developed parotid tumors at 32 and 36 weeks of age. Both had received 0.7 ml. of a preparation containing DNA in a concentration of 900 μg/ml at 2 weeks of age. There were no other tumors in these animals inoculated at 2—8 weeks of age. The data from these and the subsequent groups are summarized in Table 1.

DNA prepared from polyoma-infected mouse embryo cultures.—This group consisted of 42 AKR mice from ten litters given DNA extracted from mouse embryo cultures infected with polyoma virus. The animals were 0—10 days of age at the time of inoculation. The two preparations used were designated DNA 49 and DNA 51. Each 0.5-ml. intrathoracic injection contained DNA in a concentration of approximately 80 μg/ml. Twenty-one animals developed bilateral parotid tumors and/or epithe-
lioid thymomas at an average age of 14 weeks. Tumors occurred from ages 8–25 weeks. Fifteen of the tumor-bearing animals, representing two litters given DNA 49 and two litters given DNA 51, received the nucleic acid immediately after its preparation. The remaining six animals with tumors were littermates and were inoculated with DNA 49 which had been stored at −70°C for 6 months. All of the animals in the five litters bearing tumors developed neoplasms, while no neoplasms were seen in the other five DNA-inoculated litters.

Hemagglutination inhibition titers to polyoma virus were performed on the sera of representative tumor-bearing mice receiving parotid tumor and viral DNA when newborn, by means of the method described by Rowe et al. (7). All animals tested had positive titers in a dilution of 1:5000 or greater.

### TABLE 1

Tumors Developing in AKR Mice Receiving Several DNA Preparations

<table>
<thead>
<tr>
<th>Material Injected</th>
<th>Age at Injection</th>
<th>No. of mice</th>
<th>Parotid tumors and/or thymomas</th>
<th>Tumor Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parotid tumor DNA</td>
<td>0–6 days</td>
<td>39</td>
<td>30</td>
<td>76</td>
</tr>
<tr>
<td>Parotid tumor DNA</td>
<td>2–8 weeks</td>
<td>26</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Polyoma DNA</td>
<td>0–10 days</td>
<td>42</td>
<td>21</td>
<td>50</td>
</tr>
<tr>
<td>Passage parotid tumor DNA</td>
<td>0–10 days</td>
<td>57</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Controls</td>
<td>—</td>
<td>57</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

DNA prepared from passage parotid tumors.—This third group consisted of mice receiving DNA prepared from the third, fourth, and sixth subcutaneous transplantation of a parotid gland tumor. These tumors first appeared at about 2 months after transfer but continued to grow locally at a very slow rate. They did not seem to alter the animals’ general health and were not seen to metastasize. Their gross morphology resembled the primary parotid tumors in that they had a lobulated appearance with some cystic spaces. Microscopically they were composed of cells with large oval nuclei with nucleoli and scant cytoplasm. These cells resembled those of the mesenchymal elements of the primary parotid tumor. Only rare glandular elements were seen in contrast to the primary neoplasm, where glandular elements were plentiful. Mitoses were abundant in these transmitted neoplasms.

This “passage” DNA was injected immediately after its preparation into 57 mice representing fourteen litters at 0–10 days of age. Three preparations of DNA were used. The concentration of DNA in these preparations was as follows: 150 μg/ml from the third transfer; 280 μg/ml from the fourth transfer; and 950 μg/ml from the sixth transfer tumor. One mouse receiving the 150 μg/ml preparation at 4 days of age developed bilateral parotid tumors and a thymoma at 22 weeks of age. No other tumors were seen in the remaining 56 animals. Unfortunately, no polyoma antibody studies were performed on the serum of this one tumor-bearing animal. The hemagglutination inhibition titer to polyoma in the serum of the tumor donor was 1:160.
Controls.—Fifty-seven animals of alternate litters of AKR mice that were not treated with injections demonstrated the usual incidence of spontaneous lymphocytic lymphomas, but no tumors of other types were seen in these animals. They were observed until 14 months of age.

DISCUSSION

These experiments were undertaken to determine if the oncogenic properties of DNA extracted from parotid tumors were the result of an inoculation of infectious polyoma virus DNA or due to an actual cellular transformation mediated by the DNA of the tumor cells. It was assumed that DNA extracted from polyoma-infected C3H mouse embryo monolayers would represent, for the most part, viral nucleic acid. The transplanted parotid tumor cells were used as a source of cellular DNA. Oncogenic properties of polyoma DNA were demonstrated, as was a relative lack of tumor induction in animals receiving DNA prepared from the transmitted tumors. These observations suggest that viral DNA was responsible for the tumorigenic properties of the nucleic acid preparations of the primary parotid tumors, and that the DNA of the malignant cells themselves could not effectively produce a neoplastic transformation.

A similar observation with a different tumor virus system has been made by Hodes et al. (4), who have shown that DNA extracted from Shope papilloma tissue containing virus readily produces papillomas at the site of inoculation in wild and domestic rabbits, whereas two carcinomas originally arising in papilloma virus-infected rabbits, carried for many generations by serial cell transfers and known to be virus-free, yielded DNA without oncogenic properties.

The fact that a tumorigenic DNA could be extracted from the primary parotid neoplasms with oncogenic properties similar to that of the viral DNA suggests that there is virus present in these tumors. Support for this assumption is given by the finding of hemagglutination inhibition titers to polyoma in the sera of the tumor-bearing mice which were positive in dilution of 1:5000 or higher. Antibody titers of this magnitude were also found in the sera of the animals used as donors of the primary parotid tumors from which the DNA was obtained. This demonstrates that infectious viral nucleic acid can be extracted in the presence of high titer antiviral antibody. It was previously shown that cell-free filtrates of the parotid tumors from animals with high antibody titers did not produce neoplasms when inoculated into newborn mice (3). This was presumably due to neutralization of virus present in the tumor by antibody in the serum.

Another indication that viral nucleic acid is present is that the nucleic acid from the primary parotid neoplasms did not result in tumors in mice over 2 weeks of age and was most effective in newborn mice. Resistance to onogenesis in mice by polyoma is practically complete by the fifteenth day of life (1). Also the age distribution of the DNA-induced tumors was found to be similar to that of polyoma virus-induced neoplasms as illustrated in Chart 1.

The "litter effect" observed in animals inoculated with parotid tumor DNA and viral DNA, i.e., some litters with every member involved with tumor and others completely negative, is like that found in tumorigenesis occurring after infection with polyoma virus and is presumably due to the transmission of maternal antibodies resulting in virus neutralization (5). It is indeed likely that some of the mothers previously inoculated with cell-free filtrates and tissue culture supernatant fluids could have been infected with polyoma and passively transferred the antibodies to the DNA-inoculated offspring. If the negative results in certain litters inoculated with the nucleic acid preparations are interpreted as due to transmitted maternal antiviral antibodies, the degree of infectivity of the primary tumor and viral nucleic acid preparations in susceptible animals approaches 100 per cent. The nucleic acid concentration of the material isolated from the monolayers was 80 \( \mu g/ml \), and presumably the concentration of viral nucleic acid in the tumor cell preparations is only a fraction of that measured. This then would indicate a high degree of infectivity in vivo for viral DNA. This has not been observed with tumor virus RNA. Weil et al. (10) were unable to extract infective RNA from Rous sarcoma virus; Maloney leukemia virus RNA produced this disease in 11.8 per cent of BALB/c mice inoculated (6). Intact Maloney virus produces a 100 per cent incidence of leukemia in this same strain.

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REFERENCES

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