Oncogenic Properties of Deoxyribonucleic Acid Isolated from Parotid Gland Tumors*

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SUMMARY

Deoxyribonucleic acid (DNA) was extracted from parotid gland tumors with phenol. The injection of this DNA into 24-hour to 1-week-old AKR mice resulted in a 65 per cent incidence of parotid tumors and/or epithelioid thymomas. Oncogenic properties of the DNA preparation were abolished by incubation with deoxyribonuclease.

Cell-free filtrates of the parotid tumors inoculated into suckling AKR mice did not result in the development of parotid gland neoplasms or thymomas.

It cannot be stated definitely from these experiments whether the oncogenic properties demonstrated are the result of transformation of normal cells by tumor cell nucleic acid or the result of the activity of infectious polyoma virus DNA.

The role of nucleic acids in the development of neoplastic disease has been the subject of several studies in recent years. It has been demonstrated by Latarjet and co-workers (9) that the injection of deoxyribonucleic acid (DNA) isolated from leukemic mouse tissues produced a low, but significant, incidence of multiple neoplasms in mice. Bielka and Graffi (2) have produced a 10 per cent incidence of leukemia in mice that had been given injections of ribonucleic acid (RNA) preparations from a mouse myeloid leukemia of presumed viral etiology. The oncogenic properties of this material were inactivated by ribonuclease. Cell-free filtrates of the same leukemic tissues produced a 72 per cent incidence of the disease. De Carvalho and his associates (3) have described the development of leukemia and other tumors in mice receiving tissue culture supernatant fluids which had been inoculated with fluorocarbon-extracted RNA from human leukemic cells. They have not, however, been able to confirm these observations. Lacour et al. (8) have reported the development of malignant tumors in mice which had received injections of RNA from a human leukemic lymph node. Di Mayorca et al. (4) have isolated DNA from polyoma virus which has produced cytopathic effects and liberation of virus in tissue culture. Ito (6) has demonstrated that the Shope papilloma virus contains infectious DNA. In our laboratory (5) we have shown that nucleic acid preparations isolated from AKR mouse leukemia cells are associated with the development of an increased incidence of leukemia when inoculated into (AKR × C3H)F1 hybrid mice and also that a small number of parotid gland tumors develop in hybrid and C3Hr/Bi mice given inoculations of nucleic acids isolated from AKR leukemic cells. The experiments reported here demonstrate the oncogenic properties of DNA prepared from these mouse parotid gland tumors.

MATERIALS AND METHODS

Mice.—The mice used in these experiments have been maintained in this laboratory as inbred strains for 5 years. The AKR/JAX mice have a 70 per cent incidence of spontaneous lymphocytic leukemia developing after 6 months of age. Mammary carcinomas are occasionally seen in female mice over 14 months of age. Neither parotid gland tumors nor epithelioid thymomas have been observed to occur spontaneously in this strain.

The hybrid mice are the F1 generation of a cross between C3Hr/Bi females and AKR males and have a 20 per cent incidence of leukemia morphologically identical to the disease in AKR mice.

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Spontaneous neoplasms are not seen in any animals of these two strains under 6 months of age.

Nucleic acid.—The nucleic acid was prepared from the parotid gland neoplasms by an adaptation of the method described by Kirby (7). Twenty ml. of a 6 per cent solution of sodium-p-amino salicylate (PAS), made with distilled water containing $5 \times 10^{-4}$ m versene, was added to 1 gm. of parotid tumor tissue, which had been removed aseptically from the animal and quickly frozen in a dry-ice isopropyl alcohol mixture. The remainder of this procedure was carried out at $4^\circ$ C. The frozen tissue was homogenized with the PAS until a smooth viscous preparation was obtained. An equal volume of aqueous phenol (water-saturated, containing no preservative) was then added, and the mixture was stirred for 1 hour.

After centrifugation at $1,500 \times g$ for 30 minutes the upper aqueous phase was removed, and 5 ml. of 6 per cent PAS was added to the remaining phenol and protein, which was shaken vigorously and recentrifuged. The aqueous phase of the second centrifugation was combined with that of the first, and the DNA was precipitated from this solution by adding an equal volume of isopropyl alcohol. The fibrous strands of DNA were removed from the stirrer and dissolved in 10 ml. of distilled water or 0.01 m sodium chloride. The nucleic acids were then reprecipitated after the addition of 600 mg. PAS and again after the addition of 400 mg. of sodium acetate. This final precipitate was dissolved in 5–15 ml. of either isotonic or hypertonic (2 per cent) sodium chloride for injection into mice. Whenever possible the animals were given injections on the same day the material was prepared, or the material was stored for 1–2 days at $4^\circ$ C. and used for injections as litters became available.

The preparations that were injected were clear and viscous and contained 200–500 $\mu$g/ml of DNA as measured by the diphenylamine reaction. Small amounts of RNA were also found to be present in these solutions. They demonstrated a single peak at 260 m$\mu$ when measured on the Cary Recording Spectrophotometer. These solutions were Biuret-negative and were considered to be qualitatively protein-free.

Nucleases.—DNase, once crystallized, and RNase, crystallized salt-free, from Worthington Biochemical Co., were prepared in concentrations of 40 $\mu$g/ml in physiological saline.

The DNA solution prepared from parotid tumors by the PAS-phenol method was divided into 3 parts, and equal volumes of DNase, RNase, and saline were added to each. Two drops of 0.01 m MgSO$_4$ were added to the DNase incubation mixture. After incubation for 20 minutes at room temperature the material was stored at $-10^\circ$ C. until used for injection into mice.

Cell-free filtrates.—Parotid tumors were homogenized in the cold. A 20 per cent solution by weight was made with addition of physiological saline. The homogenates were then centrifuged at $1,500 \times g$ for 15 minutes and $7,000 \times g$ for 10 minutes. The supernatant fractions were passed through 02 Selas filters shown to be impermeable to E. coli. These Selas filtrates were injected immediately into mice.

RESULTS

In the course of experiments (5) studying the oncogenic properties of nucleic acids isolated from leukemic mouse tissues, several animals developed bilateral parotid gland neoplasms. It was felt that these observations were similar to those of Latarjet and his associates (9) and could be related to the presence, in our nucleic acid preparations, of whole polyoma virus or polyoma DNA. In the experiments reported here we used two of these parotid tumors arising in mature (C3H $\times$ AKR)$_1$F$_1$ hybrid mice after they had received inoculations of leukemic nucleic acids.

In the first experiment a group of AKR mice received nucleic acids (DNA/RNA) from a parotid tumor developing in a 5-month-old (C3H $\times$ AKR)$_1$F$_1$ hybrid mouse. The donor animal had received, at 24 hours of age, a preparation of DNA/RNA from leukemic mouse tissues. The parotid nucleic acids were extracted by the phenol-PAS method as described above. The DNA/RNA was dissolved in 0.9 per cent sodium chloride; 0.1-ml. amounts were inoculated subcutaneously into twelve 24-hour-old mice from 1 to 3 days after preparation. The animals were observed until the time of natural death.

Four of these twelve mice developed parotid gland neoplasms, which were pleomorphic tumors composed of both glandular and mesenchymal elements. Three of the four animals had thymic epithelioid tumors. The thymic epithelioid tumors were well encapsulated, not adherent to surrounding structures, but they frequently filled three-fourths of the thoracic cavity and caused death by suffocation. They were different, both grossly and microscopically, from the thymic lymphosarcoma that occurs spontaneously in these mice. Seven mice developed leukemia at the usual age of onset.

A second group of seventeen AKR mice was given inoculations of nucleic acids prepared from a parotid tumor occurring in a 7-month-old hybrid mouse given injections when newborn of AKR leukemic DNA/RNA. All the mice received the parot-
id tumor DNA/RNA dissolved in 2 per cent sodium chloride immediately after its preparation. Three mice were given injections of 0.1 ml subcutaneously at 12 hours of age, six intrathoracically at 24 hours of age, and eight intrathoracically at 1 week of age; 0.05-ml. amounts were used for the intrathoracic injections. They were placed in the anterior mediastinum in the region of the thymus. Fifteen of these seventeen mice developed pleomorphic parotid gland neoplasms and/or epithelioid tumors of the thymus. Both tumors appeared in thirteen mice of this group. Tumors occurred in animals from 3.5 to 10.5 months of age, the majority occurring between 4 and 6 months. Of the remaining two mice, one died of a typical leukemia at 10.5 months of age, and no tumor was demonstrated in the second mouse.

Therefore, in these two experiments a total of

stance from the kidney of a parotid tumor-bearing animal, of the preceding experiment. This DNA/RNA was subjected to nuclease incubation as outlined in the Methods section of this paper. The results obtained with three different parotid nucleic acid preparations are summarized in Table 2. Fifty-five animals received, intrathoracically, 0.02–0.05 ml DNA/RNA incubated with saline at from 12 hours to 3 days of age. Nine of these animals developed parotid gland tumors and/or epithelioid thymomas. Fifty animals received the DNA/RNA, incubated with RNase, and eight developed tumors. Forty-eight animals received the DNA/RNA incubated with DNase, and no tumors developed in this group. The incidence of leukemia was similar in all three groups and was somewhat lower than that of the breeding colony, because this experiment was terminated when the mice

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tbody>
<tr>
<td>TUMORS DEVELOPING IN AKR/JAX MICE RECEIVING INJECTIONS OF DNA FROM PAROTID GLAND NEOPLASMS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group no.</th>
<th>No. mice</th>
<th>Route of injection</th>
<th>Age at injection</th>
<th>Age of tumor development (months)</th>
<th>Thymomas</th>
<th>Parotid tumors</th>
<th>Leukemia</th>
<th>No. tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>Subcutaneous</td>
<td>24 hr.</td>
<td>5–13</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>Subcutaneous</td>
<td>12 hr.</td>
<td>4–6</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>Intrathoracic</td>
<td>24 hr.</td>
<td>3–5</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total groups 1 and 2</td>
<td>29</td>
<td></td>
<td>1 week</td>
<td>4–10</td>
<td>6</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>No injection</td>
<td>8–14</td>
<td></td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>10</td>
</tr>
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* A total of nineteen of the 29 animals given injections, with one or both tumors.

29 mice received the parotid tumor DNA/RNA, and nineteen (65 per cent) of these animals developed neoplasms. Two had parotid tumors, one had a thymoma, and the remaining sixteen animals demonstrated both neoplasms occurring simultaneously.

A third group consisted of twenty noninjected AKR mice, observed simultaneously with the experimental animals. Eighteen developed leukemia. One of these had a mammary carcinoma concomitantly, and ten had no tumors. This control sampling is representative of the findings of 5 years of observation of this AKR/JAX colony. The observations of these three groups are summarized in Table 1. Control animals given inoculations of phenol extracts of normal parotid glands were not utilized.

Subsequently, DNA/RNA was isolated from three of the parotid gland tumors, and in one in-

<table>
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<th>TABLE 2</th>
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<tr>
<td>THE RESULTS OFNUCLEASE INCUBATION ON THE ONCOCENIC PROPERTIES OFNUCLEIC ACID PREPARED FROM PAROTID TUMORS AND KIDNEYS OF ANIMALS WITH PAROTID TUMORS</td>
</tr>
</tbody>
</table>

| TYPE OF TUMORS | NUCLEIC ACID INCUBATED 20 MIN. AT 37° C. IN: |
|---|---|---|
| | Saline | RNase | DNase |
| Parotid tumors | 1 | 3 | 0 |
| Epithelioid thymomas | 4 | 2 | 0 |
| Parotid tumors and thymomas | 4 | 3 | 0 |
| Leukemia | 16 | 16 | 20 |
| No tumors | 30 | 20 | 28 |
| No. mice with parotid tumors and/or thymomas/No. mice given inoculations | 9/55 | 8/50 | 0/48 |
were 8–12 months of age. The morphologic appearance of the parotid and thymic tumors was identical to that seen previously. Their lower incidence than that of the initial experiments could have been due to the twofold dilution of the nucleic acids resulting from addition of either saline or nucleases in saline solution, as well as to incubation and subsequent storage of the material. All this material was stored at −10°C for from 2 days to 2 weeks, as litters became available for injection. When undiluted, unincubated DNA/RNA from the same source was inoculated (0.05 ml.) intrathoracically immediately after preparation into five 48-hour-old AKR mice, all the animals developed massive epithelioid thymomas at 3 months of age.

Cell-free filtrates were prepared from several parotid gland tumors and immediately inoculated (0.05–0.05 ml.) intrathoracically into 35 AKR mice at 12 hours to 1 week of age. No parotid tumors or thymomas were noted in these mice.

**DISCUSSION**

In the experiments reported here it has been demonstrated that a nucleic acid preparation isolated from parotid gland tumor and containing DNA in concentrations of 200–500 µg/ml, when inoculated into AKR mice from 12 hours to 1 week of age, has rather remarkable oncogenic properties. Sixty-five per cent of the DNA/RNA-injected mice developed parotid tumors and thymomas. Incubation of the nucleic acid with DNase destroyed its tumor-producing activity, indicating that the active nucleic acid was DNA.

The question whether this nucleic acid is a cellular DNA with information necessary to transform normal cells into malignant ones, or DNA of polyoma, or some other mouse tumor virus is raised by these studies. Polyoma virus as demonstrated by Stewart et al. (11) produces multiple tumors when inoculated into mice. The parotid tumors and thymomas of polyoma-infected mice demonstrated by others (1, 11) have the same gross and microscopic appearance as those seen in this experiment. Polyoma isolated from tissue culture has been shown to contain infectious DNA, which produces cytopathic changes and formation of virus in culture, as well as tumors in animals (4, 10). It is possible, therefore, that polyoma DNA could have been the oncogenic factor in this study. Hemagglutination inhibition antibodies to this virus were demonstrated in high titer in the sera of both experimental and control mice, and so the test was not helpful in clarifying this point.

Therefore, these experiments demonstrate merely that one can isolate DNA from a tumor which induces that tumor and one other when inoculated into young mice, and that these tumors are morphologically like those described as occurring after polyoma virus infection. These studies, however, do not show whether or not we are dealing with viral DNA, tumor-cell DNA, or tumor-cell DNA altered by virus. It is hoped that experiments utilizing DNA prepared from concentrated preparations of polyoma virus, and comparing its oncogenic properties with those of DNA from parotid tumors demonstrated to be free of virus, will give an answer to this question.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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