The Mammary Tumor Agent in Extracts of Frozen and Unfrozen Mammary Cancers

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Gye and his associates (15–17, 20, 21, 24) have reported on the development of mouse tumors, either sarcomas or carcinomas, at the subcutaneous site of injection of either frozen and/or frozen-dried tumor suspensions. They interpreted their data to imply that the tumors were induced because of the action of a virus upon the normal cells, and the virus they called “the continuing cause of cancer.” Recently, this theory has been applied to certain clinical problems (20, 21, 24).

In the case of mammary cancer, Mann (20, 21, 24) stated that the tumors develop because of the “selective infectivity” of the mammary tumor agent, made “active” because of freezing, when the active virus encounters a tubule of the normal mammary gland (20). The tumors appeared within a matter of days, or at most a few weeks, following the subcutaneous injection of the frozen tumor

This report is concerned with the tumor-producing activity of the mammary tumor agent obtained from centrifugates of frozen or unfrozen transplanted mammary carcinoma and with the tumors which arose following the injection of the thawed tumor mince. Preliminary data, tabulated 76–185 days after the administration of these extracts, have been reported (5).

<table>
<thead>
<tr>
<th>TABLE 1</th>
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ASSAY FOR THE MAMMARY TUMOR AGENT IN FRESH AND FROZEN TISSUE OF THE AZFi MAMMARY CARCINOMA, NO. 8415A

A tumor which developed following the injection of the thawed tumor mince was called 8415B (1st passage)

<table>
<thead>
<tr>
<th>TUMOR PASSAGE</th>
<th>TISSUE</th>
<th>GM. EQUIV.</th>
</tr>
</thead>
<tbody>
<tr>
<td>8415A 1st</td>
<td>Unfrozen</td>
<td>$2 \times 10^{-4}$</td>
</tr>
<tr>
<td>8415A 1st</td>
<td>Unfrozen</td>
<td>$10^{-3}$</td>
</tr>
<tr>
<td>8415A 9th</td>
<td>Unfrozen</td>
<td>$5 \times 10^{-3}$</td>
</tr>
<tr>
<td>8415B 1st</td>
<td>Unfrozen</td>
<td>$10^{-3}$</td>
</tr>
<tr>
<td>8415B 1st</td>
<td>Frozen</td>
<td>$2 \times 10^{-3}$</td>
</tr>
<tr>
<td>8415B 1st</td>
<td>Frozen</td>
<td>$10^{-3}$</td>
</tr>
<tr>
<td>8415B 3d</td>
<td>Unfrozen</td>
<td>$2 \times 10^{-3}$</td>
</tr>
<tr>
<td>8415B 3d</td>
<td>Unfrozen</td>
<td>$10^{-3}$</td>
</tr>
</tbody>
</table>

MATERIALS AND METHODS

Tumor No. 8415, a spontaneous mammary carcinoma, developed in a breeding female of the AZFi (A $\varphi \times Zc$) generation. This tumor was transplanted into AxZbFi, or ZbAxZf hybrids produced by mating animals of the fostered (without the mammary tumor agent) Ax and Zb lines of the parental strains. The tumor was continued for 31 passages, and the grafts showed progressive growth in all of the 226 inoculated animals.

Fresh tissues from tumors of the first and ninth passages were used for the mammary tumor agent, and these are referred to as unfrozen tissue of tumor No. 8415A, 1st and 9th passages (Table 1). Transplants of the first passage of tumor No. 8415A were frozen to $-79^\circ$ C. in cellophane and dry ice, as previously described (5), following the technic outlined by Gye et al. After storage for 7 days, the ampules were placed in a water bath maintained at $37^\circ$ C. until the tissue had thawed.

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Saline was added to the tumor mince, and the suspension was injected subcutaneously into F1 mice without the agent. Some tumors developed at the site of injection (5); the largest (3.5 gm.) was called 8415B (1st passage) and was assayed for the agent. Tumor 8415B was transplanted for two additional passages when tissue was again tested for the agent.

The test animals were ZBC mice without the mammary tumor agent, 20–24 days of age, of which less than 2 per cent develop spontaneous mammary cancer (5) when maintained as controls. The experimental animals were continued as breeders and given Purina Fox Chow and tap water.

The tissue to be assayed was ground with sand, and distilled water was added to give a 10 per cent suspension (wet weight). This was centrifuged for 10 minutes at approximately 2,500 r.p.m., and the supernatant was recentrifuged for either 10 or 20 minutes at the same speed. The final supernatant was further diluted so that the injection of 1 cc. contained the agent extracted from the amount of tissue given in the table.

To compensate for any differential mortality between the various groups of injected mice, only noncancerous animals which lived to the average cancer age have been tabulated. The average cancer and noncancerous ages for all mice were 355 and 603 days, respectively.

RESULTS AND DISCUSSION

The results observed following the administration of various groups of injected mice, only noncancerous animals which lived to the average cancer age have been tabulated. The average cancer and noncancerous ages for all mice were 355 and 603 days, respectively.

To demonstrate the presence of the mammary tumor agent in either normal or cancerous tissues, cell-free extracts of these tissues are injected into young animals of strains which are susceptible to spontaneous mammary cancer, but which do not possess the agent. If the extract contains the agent, the incidence and time of appearance of mammary cancer in the test animals will be found to be comparable, in general, to those observed in some high cancer stocks.

The mammary tumor agent of mice (1) has been found to have the properties of an infectious agent or virus and will remain active following filtration (3), lyophilization (2), and desiccation (8, 10–13).

The conclusion of Gye et al. (16–17) and Mann (20, 21, 24) that tumor cells, including mammary carcinoma, would not survive freezing to low temperatures is contrary to the results obtained by many workers previous to the date of their first publication and to the many reports that have appeared since that time (5, 7–9, 18, 19, 25–29).

Passey et al. (26) demonstrated the presence of viable cells in lyophilized tumor material, where active growth was obtained in tissue culture, provided the frozen tumor was not desiccated for longer than 75 minutes (approximately the length of time employed by Gye).

It also has been noted that the injection of the suspended thawed tumor mince would produce tumors at the site of injection only in mice of stocks or their hybrids which were susceptible to grafts of the fresh tumor being used. These tumors would behave, upon further transplantation, like the original tumor (5, 9), showing that they did not develop from the cells of the injected host. The mammary tumor agent, on the other hand, will induce tumors in mice of other strains (see 3, 4) or, as stated by Gye (15), in mice with a susceptibility different from that of the strain from which the agent was obtained.

By using a transplanted mammary tumor, No. 8415A, which developed in an ACF hybrid female, observations have been obtained on the activity of the mammary tumor agent in fresh and frozen tumors of the same passage.

When unfrozen tumors of the first passage were assayed for the agent, higher incidences and earlier cancer ages were observed in the mice which received the extracts subcutaneously than in those which were given the same fractions intraperitoneally. After the tumor had been transplanted for nine passages, transplants were tested, but the mice served as controls for another study, and only intraperitoneal injections were made. The results were comparable to those obtained for the first-passage tumors, regardless of the route of injection, showing that the tumor-producing activity of the agent had not decreased during the interval (Table 1). Transplants of the twelfth and 31st passages have been assayed, and preliminary data demonstrate the survival of the agent in both series.

Transplants of the first passage of tumor 8415A were frozen in celllosolve and dry ice at —79° C., stored for 7 days, thawed at 37° C., extracted in the usual way, and the final supernatant injected into agent-free young mice. The extracts were as active as those of the unfrozen tissue of the same passage, and the tumors appeared as early in mice injected intraperitoneally as subcutaneously. No variation was seen in the age of the mice at the time of appearance of the first tumors in the four groups, the range being from 103 to 206 days after birth.

Following the subcutaneous injection of the thawed tumor suspension of tumor 8415A, frozen for 7 days, the largest tumor to develop was called 8415B, to distinguish it from transplants descended from grafts of the frozen tissue of 8415A. The cell-free centrifugates of tumor 8415B
showed a lower tumor-producing activity than did extracts of tumor 8415A, except when the mice received a dose of $2 \times 10^{-2}$ gm. equivalents of the material intraperitoneally. The same fraction, administered subcutaneously, gave a low incidence. When the extract of 8415B was diluted 1000-fold, fewer mice developed tumors, regardless of the route of injection. In this series, the first groups to be injected were the mice which received the fractions subcutaneously, and litter-mate controls were employed for all groups. The unfrozen tissue of the third passage of tumor 8415B showed further loss of activity following subcutaneous administration but remained relatively the same for intraperitoneal injection of the extracts.

In all groups tested with extracts of tumor 8415B, which appeared after the injection of the thawed suspension of tumor 8415A, the incidence in the experimental animals was dependent not only upon the amount of the agent to be injected but also the site of inoculation. This was not the case with tumor 8415A, when unfrozen tissue of the first and ninth passages and frozen tissue of transplants of the first passage were used.

Considering all groups, regardless of the tissue used or the concentration of the extracts, the average cancer age for mice which received subcutaneous injections was 347 days, as compared to 360 days for those which obtained the extracts intraperitoneally; the average noncancer ages for the respective groups were 664 and 543 days. The average noncancer age for the mice which received extracts of tumor 8415B subcutaneously was 654 days. These mice were continued as breeders, and the average number of litters born to each female was 5.9.

It is obvious that, at this time, no definite explanation may be suggested to account for the results obtained following the injection of the extracts of the tumor, 8415B, which were descended from the viable cells present in the thawed tumor mince. Further studies are contemplated on the problem.

From these studies, it is evident that the mammary tumor agent, an infectious agent or virus, does not become "active" following freezing and induce mammary cancer within a few days or weeks, as claimed by Mann (20, 21, 24). After the injection of cell-free supernatants, the first tumors appeared as early in mice which received extracts of the unfrozen tumors as when frozen tissue was used. Also, the results showed that the agent did not have to come in contact with tubules of the normal gland to produce mammary cancer, since, in most series, intraperitoneal injection was more effective than subcutaneous administration of the same fraction.

**SUMMARY**

No difference was observed in the tumor-producing activity of the mammary tumor agent in cell-free supernatants of frozen and unfrozen transplanted mammary carcinoma, when tumors of the same passage were used as the source material.

There was no decrease in the activity of the agent derived from fresh tissue after the tumor had been transplanted for nine passages.

When extracts of frozen tumors of the first passage were used, no variation was seen either in the incidence of mammary cancer or the average cancer age in mice injected intraperitoneally or subcutaneously. The first tumor appeared at 193 days.

Tumors appeared at the injection site of the thawed tumor mince. Extracts of these tumors, and their transplants, gave fewer tumors in the test animals when injected subcutaneously than intraperitoneally.

From these studies, no evidence was obtained to suggest that the mammary tumor agent is activated by freezing.

**REFERENCES**

2. ———. The Preservation by Freezing and Drying in Focus of the Milk-Influence for the Development of Breast Cancer in Mice. Ibid., 181:587-89, 1941.
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