Assay of Frozen Mouse Mammary Carcinoma for the Mammary Tumor Milk Agent*

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In a series of publications during the past year Gye and his associates (28, 29, 33–35) interpreted their data to imply that when various types of cancers from mice were maintained at −79°C, the malignant cells "are readily killed by extreme cold, but the dead malignant cells are nevertheless capable of starting a new, strictly new, tumour in virtue of the intrinsic virus which they contain" (28). It was suggested that the active form of the virus, changed from a latent form because of freezing, was capable of altering the connective tissue of the injected host to sarcoma or the normal mammary gland to mammary carcinoma, depending upon the type of cancer being tested. Normal and frozen embryonic tissue served as controls for the cancer studies (Mann, 32).

Active dried tissue was obtained by Gye (28) from fresh sporadic tumors which had developed spontaneously in mice of the R3 and C3H stocks. However, positive results with dried tumors were secured only when the tissue was minced (14) and dried in a Craigie desiccator (15). When the tissues were subjected to the Knox method of drying, only negative results were obtained (29).

Gye (28) postulated that the negative results obtained by other investigators were due to imperfect technic and stated that their experimental data indicated "that cancer has a continuing cause and that this, in mammals as in birds, is a virus."

Mann (33–35) injected frozen spontaneous and transplanted mammary cancers and explained the development of tumors on the basis that the mammary tumor milk agent-virus was liberated in its active form following freezing, since mammary tumors arose at the site of injection of the suspended frozen tumor-mince. The tumors developed as readily in males as in female mice, but only when the frozen tissue was injected into the mammary gland region, because of what the author called "selective infectivity." She found that, within limits, the longer the tissue was frozen, the more tumors developed, and the interval between injection and the appearance of the tumors was related to their rate of growth. Fast growing cancers produced growths within 10 days, while slow growing ones might be delayed for 7–14 weeks following the inoculation of their frozen suspensions. Mann (35) also demonstrated that mammary tumors could be produced by the injection of tumors which had been frozen and dried in vacuo (15), provided no thawing occurred before the material was dry.

Gye, Begg, Mann, and Craigie (29) reported that some tumors could withstand a succession of freezing and thawing, which they claimed completely excluded the persistence of tumor cells, but Mann (34) observed a progressive deterioration of activity of mammary tumors after a second thawing. Individual tumors differed, but in no instance did a tumor inactive after a second thawing produce tumors after a third.

The exact age of the animals at the time of injection was not stated except that "Although all came

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from high cancer lines, they were used before they were old enough to develop sporadic tumors, and in all cases the tumors obtained were at the site of inoculation" (33). The tumor suspension was "inoculated in the right mammary region of mice of the same strain as the tumors" (33).

The studies to be reported here are concerned with the propagation of mammary tumors following the injection of suspensions of frozen mammary carcinoma and the comparative activity of transplanted fresh tissue of the same tumors. Assays were made of the fresh and frozen mammary tumors for the mammary tumor milk agent-virus by the injection of cell-free extracts using different routes of administration. A tumor that resulted after the inoculation of a frozen suspension of tumor-mince was also tested for the agent.

MATERIALS AND METHODS

Mice of several inbred stocks and one group of F1 hybrids were used in the various experiments. All the animals were susceptible for the development of spontaneous mammary cancer, and all possessed the mammary tumor milk agent except the fostered lines of the Z (C3H) and A strains and their hybrids. These are referred to as the Zb and Ax lines; their reciprocal hybrids as either ZbAxF1 (Zb♀ × Ax♂) or AxZbF1 (Ax♀ × Zb♂).

The spontaneous mammary tumor No. 8415A arose in an AZF1 breeder produced by mating an A female with a Z male. This particular tumor was transplanted by the trocar method in F1 hybrids without the milk agent. In eight passages, a total of 65 mice were inoculated, and the grafts grew progressively in all. During the first three passages the tumor was transferred every 4 weeks; after that, because of the increased growth of the transplants, inoculation was made approximately every 2 weeks. Within 6 days some growths attained a size of 0.75 cm. in diameter.

One of the tumors which arose, following the injection of the frozen tumor-mince of AZF1 No. 8415A, was continued as AZF1 No. 8415B. This tumor was maintained for three passages in either AxZbF1, or ZbAxF1 hybrids, and none of the 23 mice which were inoculated was resistant to the grafts.

A mammary tumor from a breeding Z (C3H) female was designated as tumor No. 8044 and was transplanted in F1 hybrids made by reciprocal matings of A and Z mice without the milk agent. This transplant has been continued for 40 passages, and 238 mice have been inoculated. All have shown progressively growing tumors, and the mice usually succumb within 3–4 weeks after they have been inoculated. The tumors may attain the size of 1 cm. in diameter within 1 week.

The frozen suspensions of tumor tissue were prepared according to the technic outlined by Craigie (16). The tumors were removed and minced in a

![Fig. 1. Tissue press used for the preparation of the tumor-mice.](image)

The cylinder of the press may be made to any desired length; the one illustrated is 1.75 inches in length, outside diameter, 1.13 inches, and the diameter of the tube, 0.75 inches. It is made of chromium-plated brass; another in use was made of stainless steel and has a cylinder of 2.75 inches (approximate capacity, 10 gm.). The pressure screw, with plunger attached, operates through the top plate of the press which screws onto the cylinder. The plunger is 0.749 inches, which gives a minimum clearance in the tube. The outer edge of the screen is 0.7 inches, and a "hump" of 0.04 in the center fits tightly into the tube of the cylinder. The openings in one screen are 0.0410, in the other 0.0175 inches. The base plate holds the screen in place.

The tissue may be inserted from either the top or the bottom of the cylinder by the removal of the respective end plate from the cylinder.

A screen with holes 0.0410 inches in diameter (Fig. 1). A screen with openings 0.0175 inches in diameter was substituted for the first screen on the cylinder, and the tissue was forced through the press a second time. Most of the connective tissue had been removed in the former mincing. This press, with the exception of the screen with the smaller openings, has been in use for at least 10 years. Equal volume of 5.3 per cent dextrose was added to the tumor-mince, and the
suspension was stored in glass ampoules, 2 cc. per vial, which were sealed. The ampoules were frozen in cellophane and dry ice and stored in a thermos jug with dry ice at a constant temperature of 

$-79^\circ$ C.

For testing the ability of the frozen tumor-mince to produce tumors, the ampoules were transferred to a water bath kept at 37$^\circ$ C. until the tissue had thawed. Six cc. of saline was added to the contents of each tube, and the suspension was injected so that each animal received an amount equal to 0.05 gm. of the original tumor-mince. The site of injection is given for each experiment.

In addition to testing the frozen tumor-mince, cell-free extracts were also employed. Details will be given for the various studies. Other observations were made on assays of nonfrozen transplants of the same tumors for the milk agent.

**EXPERIMENTAL RESULTS**

Following the first passage of the AZF$_1$ tumor No. 8415A in F$_1$ hybrids without the milk agent, the transplants from several animals were frozen on 1/4/50 and maintained at 

$-79^\circ$ C. for 48 hours. Following thawing, each mouse was injected with 0.05 gm. of the original tumor tissue subcutaneously in the right axillary region. With the exception of four Zb females which were 18 months of age, all the test animals were from 5 weeks to 3 months of age at the time of injection.

All mice, twenty females and fourteen males, of the A and Z (C3H) stocks failed to develop tumors, but tumors appeared in three of the seven females and two of six males of the ZbAxF$_1$ hybrids (Table 1). One of the tumors from a male was continued as No. 8415B.

The thawed mince of tumor No. 8415A from four vials was pooled, 24 cc. of saline was added, and the suspension was centrifuged for 20 minutes at approximately 2,500 r.p.m. The supernatant was removed and recentrifuged for the same time. The final supernatant was injected subcutaneously near the left axillary region of the mice listed in Table 1 and referred to above. All animals, regardless of stock, failed to show tumors at the site of injection during the period they were under observation (1/6/50–2/23/50).

On February 23 (48 days after the injection of either the thawed tumor-mince or the centrifugate supernatant), some of the mice in which no tumors developed (Z, Ax, and F$_1$) were inoculated simultaneously with grafts of tumor No. 8415A (right) and tumor No. 8415B (left). Both tumors grew progressively in the ZbAxF$_1$ mice that had been resistant to the previous injections, and the animals were killed on 3/15/50 with large transplant ed tumors. Mice of the A and Z stocks were all resistant to the grafts from the AZF$_1$ tumors (Table 1).

The AZF$_1$ tumor No. 8415A was inoculated on 12/7/49 in F$_1$ hybrids (mice without the milk agent). The first passage transplants were tested on 1/6/50 for the mammary tumor milk agent by our usual technic (see 11). The tumors were minced with the tissue press, ground with sand, and extracted with distilled water (1:10 by weight). The suspension was centrifuged for 10 minutes, and the supernatant was recentrifuged for the same period (clinical centrifuge). The second supernatant was further diluted, based upon the weight of the wet pressed tissue, and injected either subcutaneously or intraperitoneally into 21–

### Table 1: Development of Mammary Tumors at the Site of Injection of the Suspension of Frozen Tumor-Mince of AZF$_1$ Tumor No. 8415A

<table>
<thead>
<tr>
<th>Stock</th>
<th>Age when injected</th>
<th>No. inoc.</th>
<th>Sex</th>
<th>Tumor Susp.</th>
<th>Inoculation of fresh tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z (C3H)</td>
<td>3 mo.</td>
<td>7</td>
<td>✔</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Z (C3H)</td>
<td>6</td>
<td>2</td>
<td>✔</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zb</td>
<td>18 mo.</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A</td>
<td>6</td>
<td>2</td>
<td>✔</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zb</td>
<td>1 mo.</td>
<td>6</td>
<td>✔</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ax</td>
<td>6</td>
<td>2</td>
<td>✔</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ZbAxF$_1$</td>
<td>6 wk.</td>
<td>7</td>
<td>✔</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>ZbAxF$_1$</td>
<td>6 wk.</td>
<td>6</td>
<td>✔</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

* Frozen 1/4/50, injected 1/6/50.

† Tumors which developed were assayed for the milk agent 8/9/50 (Table 4).

‡ One tumor was inoculated as AZF$_1$, 8415B. Some mice which were negative to the injection of the frozen material were inoculated with fresh tissue grafts of tumors 8415A and 8415B.
25-day-old ZBC females. These are susceptible animals which do not possess the milk agent; the incidence of tumors in controls is less than 2 per cent (see 11). No mammary tumors have developed in any of the injected mice after 185 days (Table 2).

The frozen tumor-mince of the AZF1 tumor No. 8415A, frozen on 1/4/50, was assayed on 1/11/50

<table>
<thead>
<tr>
<th>TABLE 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ASSAY OF THE FIRST PASSAGE TRANSPLANTS (FRESH TISSUE) OF THE AZF TUMOR NO. 8415A FOR THE MAMMARY TUMOR MILK AGENT</strong></td>
</tr>
<tr>
<td>Gm. SUBCUTANEOUS INTRAPERITONEAL</td>
</tr>
<tr>
<td>equiv. injection injection</td>
</tr>
<tr>
<td>injected No. Tumors No. Tumors</td>
</tr>
<tr>
<td>2X10^-2</td>
</tr>
<tr>
<td>10^-3</td>
</tr>
<tr>
<td>* Data tabulated after 185 days.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ASSAY OF THE FROZEN FIRST PASSAGE TRANSPLANTS OF THE AZF TUMOR NO. 8415A FOR THE MAMMARY TUMOR MILK AGENT</strong></td>
</tr>
<tr>
<td>Gm. SUBCUTANEOUS INTRAPERITONEAL</td>
</tr>
<tr>
<td>equiv. injection injection</td>
</tr>
<tr>
<td>injected No. Tumors No. Tumors</td>
</tr>
<tr>
<td>2X10^-2</td>
</tr>
<tr>
<td>10^-3</td>
</tr>
<tr>
<td>* Tissue frozen 1/4/50; tested 1/11/50. Data tabulated after 180 days.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ASSAY OF TUMOR FOR THE MILK AGENT</strong></td>
</tr>
<tr>
<td>Gm. equiv. SUBCUTANEOUS INTRAPERITONEAL</td>
</tr>
<tr>
<td>injection injection</td>
</tr>
<tr>
<td>injected No. Tumors No. Tumors</td>
</tr>
<tr>
<td>Section A 2X10^-2</td>
</tr>
<tr>
<td>10^-3</td>
</tr>
<tr>
<td>Section B 2X10^-2</td>
</tr>
<tr>
<td>10^-3</td>
</tr>
<tr>
<td>* Section A: Assay of tumor for the milk agent which developed following the injection of the frozen tissue of the AZF tumor 8415A. Frozen tissue injected 1/6/50, tumors tested 1/9/50. The data were tabulated 161 days later, first tumor after 138 days.</td>
</tr>
<tr>
<td>* Section B: Assay for the agent in the third passage transplants of tumor 8314B. Data recorded after 76 days.</td>
</tr>
</tbody>
</table>

for the so-called “active” (Mann, 32) milk agent. The second supernatant was injected subcutaneously into 66 and intraperitoneally into 69 young ZBC females (Table 3). To date, after 180 days, two tumors have developed in the animals following the intraperitoneal administration of the extract.

Two tumors which developed following the subcutaneous injection on 1/6/50 of the frozen tumor-mince of the AZF1 tumor No. 8415A were tested on 2/9/50 for the milk agent by using the fresh tissue. Two fractions of the second supernatant were used by different routes of administration. One animal that received the material intraperitoneally developed a tumor after 138 days (Table 4, Section A), and another in the same series had a mammary tumor at a later age.

The AZF1 tumor 8415B, derived from the frozen tissue of No. 8415A, was carried for three passages and assayed for the milk agent (Table 4, Section B). All the test animals have remained free of tumors for 66 days.

The Z (C3H) tumor No. 8044 has been found to possess the mammary tumor milk agent after it has been transplanted for 30 passages in either ZbAxF1 or AxZbF1 hybrids—mice which themselves did not carry the agent. These data are tabulated in Table 5. Only preliminary observations may be given for many of the groups. The earliest tumor appeared in a test animal (ZBC) which was 154 days of age, and the average cancer ages for completed groups ranged from 311 to 364 days of age.

Transplants of the 33d passage of the Z tumor No. 8044 were minced and frozen in dextrose on 2/3/50. The tissue was maintained at —79° C. until 2/20/50, when the contents of several ampoules were tested by injecting mice subcutaneously with 0.05 gm. of the original material.

Some animals of the Z and Zb lines and the AxZbF1 hybrids developed tumors at the site of injection, while all mice of the A and D stocks remained free of tumor (Table 6).

Other vials of tumor No. 8044, frozen on 3/31/50, were used on 3/31/50. In this study, mice of the ZbAxF1 generation, when approximately 8 weeks of age, received both subcutaneous and intraperitoneal injections of 0.05 gm. of the original tissue. As seen from the data (Table 7), there was no significant variation in the number of tumors to be observed at the two sites of inoculation. One male with a large subcutaneous tumor could not undergo autopsy to determine if it might also have an internal tumor.

Weights were taken of twelve of the subcutaneous and all the intraperitoneal tumors. Although the subcutaneous tumors were much more hemorrhagic than the abdominal growths, the fluid was extruded before the tumors were weighed. The tumors from the subcutaneous area varied between 0.9 and 3.6 gm.—average, 2.0 gm.; the range for the internal tumors was 0.15—3.3 gm.—average, 1.5 gm. Two animals had several internal masses.

DISCUSSION

At least three causative factors have been found to play a role in the genesis of spontaneous mam-
mammary cancer in mice (5; see 8, 37, 9—10 for literature). One of these, the mammary tumor milk agent (4), has the characteristics of an infectious agent or virus and will remain active following filtration (7, 1), lyophilization (6), and desiccation (8, 19—23) even after storage for as long as 2 years (Dmochowski, as reported by Gye [26]). When administered subcutaneously in cell-free extracts, mammary tumors developing at the site of injection are no more common than in other regions of the mammary system, and the time of appearance has not been accelerated when compared with intraperitoneal administration of the material. In general, mice of strains with a high incidence of spontaneous mammary tumors show an earlier average cancer age than do mice in the injection experiments.

Our interest in the problem of freezing mammary cancer was stimulated by the reports of Mann (33—35) that an "active" milk agent virus might be liberated from the frozen mammary tumor cells which would be capable of producing mammary cancer in a matter of days, instead of months, following its injection.

To test the ability of a frozen suspension of a mammary tumor-mince to produce cancer following subcutaneous injection, we selected for the first series of studies a spontaneous mammary carcinoma which had developed in a hybrid female derived by crossing a female of the cancerous A stock with a male of the cancerous Z or C3H strain. This was tumor No. 8415A from an AZF1 female. The tumor was transplanted into F1 hybrids which do not possess the milk agent. Tumors of this trans-

**TABLE 5**

ASSAY OF THE SPONTANEOUS Z (C3H) MAMMARY TUMOR No. 8044 AND TRANSPLANTS OF THE TUMOR, CARRIED IN MICE WITHOUT THE MILK AGENT, FOR THE MAMMARY TUMOR MILK AGENT

<table>
<thead>
<tr>
<th>TUMOR PASSAGE</th>
<th>DATE OF INJECTION</th>
<th>GM. EQUIV. INJECTED</th>
<th>AGE 1ST TUMOR</th>
<th>GM. EQUIV. INJECTED</th>
<th>AGE 1ST TUMOR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous tumor</td>
<td>1/17/48</td>
<td>10^2</td>
<td>168</td>
<td>37</td>
<td>76</td>
</tr>
<tr>
<td>1st</td>
<td>2/17/48</td>
<td>10^2</td>
<td>154</td>
<td>33</td>
<td>70</td>
</tr>
<tr>
<td>and</td>
<td>3/8/48</td>
<td>10^3</td>
<td>194</td>
<td>71</td>
<td>75</td>
</tr>
<tr>
<td>7th</td>
<td>7/2/49</td>
<td>10^3</td>
<td>254</td>
<td>47</td>
<td>43</td>
</tr>
<tr>
<td>18th</td>
<td>4/13/49</td>
<td>10^2</td>
<td>256</td>
<td>48</td>
<td>56</td>
</tr>
<tr>
<td>20th</td>
<td>5/9/49</td>
<td>10^2</td>
<td>211</td>
<td>35</td>
<td>60</td>
</tr>
<tr>
<td>24th</td>
<td>8/4/49</td>
<td>10^1</td>
<td>187</td>
<td>25</td>
<td>52</td>
</tr>
<tr>
<td>30th</td>
<td>12/1/49</td>
<td>10^2</td>
<td>172</td>
<td>56</td>
<td>7</td>
</tr>
</tbody>
</table>

* Preliminary data, tabulated 111 days after injection of last series.

**TABLE 6**

TUMORS FOLLOWING THE SUBCUTANEOUS INJECTION OF THE FROZEN TUMOR-MINCE OF Z (C3H) TUMOR, No. 8044, 33D PASSAGE*

<table>
<thead>
<tr>
<th>STOCK No.</th>
<th>SEX</th>
<th>AGE WHEN INJECTED (WEEKS)</th>
<th>TUMORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z (C3H) 6</td>
<td>♀</td>
<td>5-6</td>
<td>5</td>
</tr>
<tr>
<td>Zb 6</td>
<td>♀</td>
<td>5-6</td>
<td>3</td>
</tr>
<tr>
<td>AzZbF1 5</td>
<td>♀</td>
<td>6-0</td>
<td>1</td>
</tr>
<tr>
<td>D 7</td>
<td>♀</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>A 6</td>
<td>♀</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

* Each animal received injections in subcutaneous and intraperitoneal sites. The mice were 8 weeks of age when they were inoculated.
plant generation were frozen to $-79^\circ$ C. with the technic outlined above (16).

When a suspension of the frozen AZF1 tumor was injected subcutaneously into males and females of the A and Z stocks and their F1 hybrids, tumors appeared at the site of injection in some animals of the F1 generation, but all mice of the A and Z parental strains remained free of tumor. One tumor which arose in a male F1 hybrid was transplanted as tumor No. 8415B. A cell-free extract of the frozen tissue failed to produce tumors in any mouse.

Many of the animals which had been resistant to the subcutaneous injection of the frozen material of tumor No. 8415A were reinoculated simultaneously with grafts of fresh tissue of tumors Nos. 8415A and 8415B. All the F1 mice which had shown a negative reaction to the previous injection of the frozen tissue showed progressive growth of the grafts of both tumors and were dead, with large tumors, within a few weeks. Again, mice of the A and Z stocks were resistant.

In selecting a mammary tumor which had developed in a hybrid mouse, we knew from previous studies (2, 3) that such a tumor would grow progressively, when grafts of fresh tissue were transplanted subcutaneously, in mice of the F1 generation, whereas few, if any, of the animals of the parental strains would be susceptible to the transplants. The earlier experiments (2, 3) showed that the genes for susceptibility to a transplantable mammary tumor from a hybrid, as the AZF1, would be transmitted by each parent of the A and Z strains. The mice of the parental strains would all be resistant to grafts of the F1 tumor, since the mice of one strain would not possess the genes necessary for susceptibility that were transferred by the parent of the other stock to the F1 progeny.

The results following the subcutaneous injection of the frozen suspension of the AZF1 tumor were similar to the observations reported by Mann (33) for "mice of the same strain as the tumors," but mice of the parental strains were all resistant. All mice of the F1 generation and the A and Z inbred strains remained free of tumors when a cell-free extract was injected instead of a suspension of tumor-mince. This demonstrated the importance of the suspended tumor-mince in the development of these tumors.

One tumor which arose following the injection of the frozen mince was continued by transplantation. Grafts of this tumor produced transplanted tumors in animals of the F1 generation but not in A and Z animals; thus, its characteristics were similar to the original tumor from which it arose. Both tumors grew when transplanted into mice of the F1 generation which were negative following the injection of the frozen material.

In addition to corroborating the studies of Mann, our data also supported the genetic theory of transplantation advanced by Little and Strong (31), in 1924, and suggested the probability that some tumor cells had survived the low temperature to which they had been subjected. That is, only animals of the same genetic constitution as the mouse which gave rise to the original spontaneous mammary tumor developed mammary tumors following the injection of a frozen tumor-mince; all mice, regardless of their genetic make-up, remained free of tumors when a cell-free extract was tested, although this extract would be expected to possess the mammary tumor milk agent (see 11). As with the usual transplantable mammary tumors, tumors appeared in some mice of the F1 generation which were free of the milk agent (10), but the presence or absence of the agent in the A and Z mice was of no significance.

Histologic examination of the frozen tumor-mince was not made, since it was doubtful if such observations would have contributed anything to what Craigie (17) concluded when he stated: "I, for one, when examining unfixed and unstained tumour cells under the phase microscope am often uncertain whether they are dead or alive." In a recent presentation of their data, Blumenthal, Walsh, and Greiff (12, 13) had made preparations of their frozen tissues, both normal and cancerous, and concluded that some cells appeared to have survived.

The mammary tumor milk agent may be transferred by the injection of cell-free extracts, as mentioned above. Although the agent from one strain of cancerous animals may have a different activity in mice of another genetic susceptibility for spontaneous mammary cancer, no data have been published which would suggest that the agent from any cancerous strain would not produce some tumors, with the other causative factors also active, in other strains. This fact has been acknowledged by Gye (25), who, in discussing the work of Dmochowski on the recovery of the milk agent from dried cancer tissue, wrote: "Thus it can be concluded that the milk factor derived from any high cancer strain of mice can induce breast cancer in susceptible strains of mice, although their genetic constitution differs from that of the strain from which the milk factor originated." Thus, if freezing mammary tumors liberates the "active" milk agent, this agent should produce mammary tumors within a few days at the site of injection (Mann, 33) in mice of strains other than that in which the tumor had developed.
Every mouse inoculated in our studies was susceptible to the development of spontaneous mammary cancer. In the case of the AZF₁ tumor, it would have the milk agent from the female of the A stock. In previous studies it was determined that when females of the Z (C3H) strain were nursed by females of the A stock, so that they obtained the A stock milk agent, the incidence of mammary tumors was higher in the progeny of these fostered mice when they were maintained as nonbreeders than when they possessed the Z milk agent. However, the injection into the mammary gland region of either a suspension of the frozen tumor mince or the cell-free supernatant of the frozen suspension of the AZF₁ mammary tumor did not induce tumors in mice of the Z stock.

One tumor which developed in a male F₁ hybrid without the milk agent, following the inoculation of the frozen AZF₁ tumor, was also tested by using cell-free extracts and was found to be no more active than either extracts of the frozen material or fresh tumor tissue in animals of the A and Z strains. Several hundred test animals have been injected with these extracts, either subcutaneously or intraperitoneally, and the results to date indicate that this material was no more active than the tissue containing the “latent” (to use Mann’s terminology) mammary tumor milk agent. The final results will not be known for many months and may give some interesting data on the comparable activity of the agent in fresh versus frozen tissue as well as different routes of administration.

After 30 passages in mice free of the agent, the agent was found to be present in cell-free centrifugates of the Z (C3H) tumor No. 8044. Transplants of the 33d transfer generation were frozen, and the results following the subcutaneous injection of the frozen tumor suspension were similar to those seen for the AZF₁ tumor, in that tumors arose at the site of injection only in animals of the Z stock and their F₁ hybrids. Again, the genetic relationship between the mice inoculated and the constitution of the tumor inoculated influenced the results—and not an “active virus” liberated from the cancer cells at low temperatures, for mice of other strains were free of tumor after treatment.

As stated above, the mammary tumor milk agent may be demonstrated in filtrates and cell-free extracts (see 8, 9–10, 37), and it seems reasonable to suppose that similar extracts of frozen tissue would likewise contain the virus. Gye and his co-workers (39) centrifuged an extract of a frozen suspension of an induced sarcoma for 15 minutes at 10,000 r.p.m. The sediment was washed with saline and emulsified in 3 cc. of saline. This was injected into six mice, and all developed typical sarcomas. Gye reasoned that “Experiments such as this do not appear to lend any support to the belief that the tumors started by frozen tissues are transplants, i.e., due to division of surviving tumor cells.” If viable tumor cells had been present, they would be expected to be found in the resuspended sediment but not in the supernatant; however, the supernatant should have contained any active virus. The latter was not tested.

Mann (33) discussed in detail what she termed the selective infectivity of the so-called active milk virus from frozen mammary tumors for mammary tissue. So that no personal element will enter into the interpretation of her results and the basis for her hypothesis, it seems appropriate to give quotations from her publications. This will be followed for others, as well.

Mann (33) stated that “If inbred strains of mice are used tumour tissue can be successfully grafted in both subcutaneous and intraperitoneal sites. The new tumours are simple clumps of cells of the original mouse growing in the tissues of the new host. If, however, the active form of the Bittner virus has been obtained from living mouse cells it seems highly probable that only cells of mammary tissue can be infected, and that connective-tissue cells cannot respond to it. Since it is well known that the mammary tissue of the mouse is very widespread, and that sporadic tumours can arise almost anywhere on the surface of the body, subcutaneous inoculation of active virus can never offer evidence for or against selective infectivity.

“On the other hand, sporadic mammary cancer never arises inside the peritoneum, and yet successful intraperitoneal grafts of living tumour tissue can be easily obtained. If, therefore, frozen tumour tissue, which as we have seen readily produces subcutaneous mammary cancers, were inoculated intraperitoneally it should produce tumours if it contained living cells, and should not do so if it contained only the active virus. In the experiments . . . qualitative difference in results exists between subcutaneous and intraperitoneal inoculation of refrigerated mammary tumour. The number of failures is too high to be accounted for by any explanation other than the absence of mammary tissue in the site chosen.

“The technique of intraperitoneal inoculation is difficult because of the ease with which a few cells from the surface may be carried into the deeper tissues by the needle and also because of the difficulty of avoiding contamination of the abdominal wall and subcutaneous tissues by the material in the needle during its withdrawal . . . Again, microscopic examination of subcutaneous and intraperitoneal tumour grafts often reveals the presence
of hair follicles which have been forced in by the grafting-needle. A further source of error is the presence of hair follicles which have been forced in by the grafting-needle. A further source of error is a failure to obtain growths in 90 per cent of the intraperitoneal experiments as against a success in 75 per cent of subcutaneous inoculations (where the chances of the virus encountering a mammary tubule are high but not even there 100 per cent) is strongly in favour of selective infectivity of the virus as against cell survival.

Thus, while the technique of intraperitoneal inoculation is difficult, the chances of the virus encountering a mammary tubule are high but not even 100 per cent following subcutaneous inoculation.

Regarding the distribution of the mammary glands and the development of mammary cancer in males, Mann (33) stated: "In the male mouse, mammary tubules, imperfectly developed and without secreting acini, are present over large areas of the subcutaneous tissue, but even in high cancer strains male mice do not develop mammary cancer unless they are subjected to prolonged treatment with oestrin, when tumours occur as in the females — i.e., the virus changes from the latent to the active form."

These observations on the relative size of the mammary glands in male mice are not supported by details from other published reports. Gardner (24) in 1935, found that "The mammary glands of the normal male mouse persist as rudimentary ducts in the subcutaneous tissue throughout life. They undergo little, if any, growth after the weaning age." When male mice of various strains were from 6 to 9 weeks of age, Richardson and Cloudman (38) made whole mounts of their third glands. In males of the strains which showed the most extensive development, the mammary gland did not involve an area much greater than 1 × 1 cm. in area. Also, a gross dissection of a lactating female showed that intraperitoneal injection could be made without coming in contact with the mammary glands (11).

Twenty-one animals, eighteen males and three females, were tested by subcutaneous and intraperitoneal injections of a frozen suspension of the tumor-mince. Fourteen tumors were found in the subcutaneous region as against thirteen internal tumors. Although only a limited number of mice was tested, no difference was noted based upon the route of administration of the frozen tissue. In most experiments, Mann used only six animals, and her figures represent many series with several different tumors.

In 1949, Mann (33) tested the transplantable carcinoma "63" and determined that the inoculation of a frozen suspension was active in 19 (44 per cent of the mice developed tumors) of 22 experiments, while negative results were obtained in 3 experiments because of technical errors. The year previously, Dmochowski (22) assayed the carcinoma "63" for the mammary tumor milk agent and was unable to demonstrate the presence of the agent in desiccated tissue of tumors from the same laboratory. The tumors were from the 428th passage. No mention was made by Mann of these negative results by an accepted method of testing for the agent.

The usual method of reporting scientific data is to refer in the manuscript to any previous results other investigators have observed in that particular field. As this was not followed by either Gye (28) or Mann (33–35), it seems probable that they proposed that the reader should accept their theories as being original with them.

The interpretation of the experimental results following the use of either frozen and/or dried tumor tissue is dependent upon whether or not some cells may survive. In reporting their preliminary data and advancing the viral theory, Gye (28) made no direct reference to the previous studies of others, but, however, concluded that "We think we are justified in making the contention that the negative results of the past are merely negative and have the value of negative experiment which is the result of imperfect technique."

In a recent publication, Blumenthal and Walsh (19) referred to fifteen manuscripts, published previously to 1949, on the successful transfer of tumor and normal tissues following storage at low temperatures for as long as 2 years. The subject has also been reviewed by Hirschberg and Rusch (30). Only a few of these experiments, selected as pertinent to the discussion, will be considered here.

In 1939, Mider and Morton (36) froze mouse sarcoma and rat carcinoma to a minimum temperature of −74°C by using approximately the same technic as the British workers. When the tissue was frozen en masse, tumors developed following the subcutaneous inoculation of the thawed tissue. Repeated freezing and thawing reduced the number of tumors induced by the material. Among the three tumors they tested, sarcoma was more resistant to cold when frozen en masse than carcinoma, whereas, when frozen in a saline suspension, the opposite was found. If rat skin was maintained for 24 hours at a temperature of −74°C and transplanted subcutaneously, Mider and Morton
Blumenthal and his associates (12, 13) concluded that normal tissue (thyroid and parathyroid of the guinea pig) and mouse Sarcoma 37 would survive freezing to either —70°C. or —190°C. and could later be successfully transplanted. The most accurate indicator of viability, mitotic activity, was noted in the transplants. In referring to the conclusions of Gye et al., they stated that their "findings do not disprove the possibility of a viral transmission of neoplastic disease but rather prove that the criteria used for determining whether or not this mode of transmission obtains must be more strictly defined."

Craigie (16) reviewed the literature in a later report, the first paper in this series to do so, and stated: "Much of the experimental work reported on the production of tumours with frozen and thawed material is concerned with the interpretation of the survival of activity, and it is clear that under certain conditions malignant cells and normal skin . . . will survive freezing temperatures for some time." He tested various solutions to determine the ability of the cells to survive and concluded: "The degree of survival of activity in dextrose preparations at a low temperature is adequate for experimental work requiring the use of transplantable tumours and for some purposes may offer a more convenient source of material than fresh tumour tissue." Elsewhere, Craigie (17) wrote: "It is evident from the results of this experiment that the degree of survival of the different tumours varied considerably. Some lots produced tumours at all sites injected, others only some . . . Complete failure to survive freezing has been observed so far only with two benzpyrene tumours mentioned and an undifferentiated tumour . . . arising near the point of needle entry of intraperitoneal injection of methylcholanthrene."

Mider and Morton (36) dried at least one tumor, while it was in the frozen state, in a lyophilizer apparatus and determined that the subcutaneous injection of the dried material did not produce tumors at the site of inoculation. Although the mammary tumor milk agent will remain active following desiccation (8, 19–23), the subcutaneous injection of fresh, dried tumor tissue has not "induced" mammary cancer at the site of injection within a matter of days or weeks; but after several months the typical spontaneous mammary tumors develop. These tumors appear with the usual random distribution seen for this type of cancer in mice. Similar results have been obtained following oral administration of an extract of lyophilized mammary cancer (frozen to —70°C. and dried in vacuo) (6). Gye (28) reported that when fresh sporadic tumors from animals of the R3 and C3H stocks were dried, with the Craigie desiccator (15), active dry tissue was obtained. The type of tumor which they used was not specified, but these strains have a high incidence of spontaneous mammary cancer. However, until this new apparatus was employed, Gye and his associates (39) had been unsuccessful, in many experiments, in the propagation of dried tumor tissue, or "A long experience of negative results with a different and simpler drying method" (18).

Craigie (17) discussed the survival of cancerous cells following drying in their new apparatus as follows: "Tests of dried material obtained at various times during the development of the drying equipment (Craigie [15']) were negative, although one positive result was obtained with C3H sarcoma and others were obtained by Gye, Begg, Mann, and Craigie (29')." In a later report (18) he concluded: "It would therefore seem to be reasonable to accept the possibility that a few cells (about 1 in 1,000,000) survived the drying process." Presumably, the number of cells to survive drying with their new equipment was sufficient to produce transplantable tumors at the site of inoculation. If this new desiccator did not completely destroy all the tumor cells, the tumors which arose at the site of inoculation of the partially dried material would be due to the propagation of these viable cells and not due to the action of any virus.

In view of the many experiments of others showing that tissues would survive freezing, it is interesting to cite from the 1947–48 report of Gye (27) as Director of the Imperial Cancer Research Fund: "Forty years ago Salvin-Moore successfully transplanted mouse carcinomata and sarcomata after they had been exposed to the temperature of liquid air. Cramer grew sarcomata after freezing them with CO₂ snow, but felt that his results were attributable to the survival of a few cells. Gye and Purdy (1971) studied the matter further and concluded that a few cells were able to remain viable though exposed to freezing temperatures. The subsequent work of Breedis and Furth (1938) and others has produced additional evidence that such survival may occur. Breedis and Furth drew attention to the value of freezing and storing on CO₂ snow as a method of preserving tumors, but although this method has been used by some it has not been generally adopted. Dr. Craigie is studying certain aspects of the problem.

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that do not seem to have been explored, with the immediate objective of further improving this potentially valuable method of preserving certain transplantable tumours.”

Let us now refer to the papers published by Salvin-Moore and his associates (30–40) in 1908 which were mentioned by Gye (27) in 1947–48. Salvin-Moore exposed mouse tumors to the action of liquid air for periods of from 20 minutes to 1 hour. “They were then at once introduced into healthy mice beneath the skin, the presumption being that in these circumstances the tumour cells would be destroyed by the action of the liquid air and consequently that they would multiply no further.” However, in some cases new tumors arose at the site of inoculation of the frozen tissue.

Salvin-Moore and Walker (40) discussed their results as follows: “From these observations it is rendered clear that exposure to liquid air at a temperature of about —195° does not necessarily destroy the potentiality of the substance of a mouse tumour to produce fresh tumours of the same kind in mice into which such frozen tumour substance has been grafted.

“These facts in themselves are somewhat surprising, and they immediately raise a number of questions which it will be desirable to have elucidated in the interests of research concerning the nature of cancer. In the first place, it is rendered clear that exposure to liquid air for a certain period of time does not destroy the principle upon which the vitality of mouse cancer depends. If, as may be the case, the cells composing the mass of the tumour, and constituting the grafts, are killed by exposure to liquid air, then the development of mouse cancer after such exposure indicates not merely that the growth of similar tumours is dependent on the integrity of the ‘cancer cells,’ but also that the new tissues are not necessarily formed from the implanted cells at all, and may arise from the cells of the new host in response to some stimulus introduced along with the frozen material, and quite independent of the integrity of the so-called ‘cancer cells.’”

The authors (40) furthermore stated that their observations suggested “that the production of new tumours in the hosts into which the frozen cancer tissue has been introduced may possibly not be dependent upon the introduction of the ‘cancer cells’ at all, but upon the action of a virus which is independent of these cells, and retains its activity after being subjected to the temperature of liquid air.” Salvin-Moore and Walker recognized the possibility that “it is not certain that the cells from the tumour introduced into a new individual are killed by half an hour’s exposure to the temperature of liquid air, particularly as the seeds of some plants and trypanosomes are said to survive this temperature.”

Earlier in this discussion we quoted a statement by Gye (28) “that the negative results of the past are merely negative and have the value of negative experiment which is the result of imperfect technique.” These positive results were probably obtained, as demonstrated by Craigie (18), because of imperfect technique for the dehydration of cancerous tissue, either fresh or frozen. The tumors produced following the inoculation of suspensions of frozen tumors were likewise the result of the propagation of viable tumor cells. These data were incorrectly interpreted by the authors regardless of the published reports of many workers to the contrary.

Mider and Morton (36) commented upon the virus hypothesis of Salvin-Moore as follows: “This, however, has no bearing on the moot point concerning the possible relationship to transplantable tumors.” There is little doubt but what this remark is as valid today for the inoculation of frozen tumor tissue as when it was made in 1939, and it applies equally well to the 1949 theory of Gye and Mann, since their explanation is practically identical with the one advanced by Salvin-Moore in 1908.

It has often been said that many theories are advanced only for the stimulus they may give to others for further research. Perhaps in some cases this may result in an increase in our knowledge of the problem being investigated, while in other instances it may actually retard progress until the hypothesis has either been refuted or confirmed. This may be necessary, especially when the experiments are completed without adequate controls.

SUMMARY

Salvin-Moore, in 1908, determined that tumors would develop in mice following the subcutaneous implantation of neoplasms which had been exposed to the temperature of liquid air (—195° C.). He proposed a virus theory to explain the results, although he considered the possibility that some cells had remained viable.

In 1949, Gye and his associates submitted a similar theory based upon observations obtained following the freezing and drying of mouse cancers. Furthermore, Mann stated that when mammary cancers (both transplanted and spontaneous) were frozen, the milk agent virus was liberated in its “active” form and when injected subcutaneously, mammary tumors would be produced at the site of injection within a short interval of days or weeks. Few mammary tumors arose at
other sites of inoculation because of the “selective infectivity” of the virus for mammary tissue. To test for the active mammary tumor milk agent virus, transplanted mammary tumors were minced, suspended in dextrose, and frozen to \(-79^\circ\text{C}\). The thawed tumor-mince produced mammary tumors at the site of injection only in mice of stocks which would be susceptible to fresh tissue grafts of the tumor being tested. Negative results were obtained with mice of other strains, although they were susceptible to the development of spontaneous mammary cancer. Intraperitoneal inoculation of the frozen material was as effective as subcutaneous injection in the production of mammary tumors.

Whereas cell-free centrifugates have been found to contain the mammary tumor milk agent, cell-free extracts of the frozen mammary tumor-mince failed to induce tumors, within the specified interval, in any animal regardless of its genetic constitution.

Preliminary data indicate that the milk agent was no more active in cell-free extracts of the frozen than the fresh tissue of the same tumor, regardless of the route of administration. The final results of these tests will not be apparent for months, but the earliest tumors to be observed developed in mice which had received intraperitoneal injections. The revival of this viral theory for mouse cancer by Gye et al. and Mann disregarded the previous finding of many investigators that mouse tissues (normal and cancerous) survived freezing to low temperatures. Evidence was also cited that the newly designed desiccator, used for their studies, did not destroy all cells. The fact that other methods of dehydration, including their own studies, had given negative results which would refute the virus hypothesis was disregarded as due to imperfect technic.

The experimental data submitted by Gye et al. and Mann were, without a doubt, based upon observations which they either incorrectly interpreted, as in the case of the studies with frozen tissues, or secured by using new equipment before it had been adequately tested and which resulted in imperfect technic.

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