Soon after the mammary tumor agent was found to be a causative factor in the genesis of mammary cancer in mice (9), it was determined that the agent could be transferred by grafts of normal tissues (13—14) from young mice of cancerous stocks and by the injection of extracts of either normal mammary glands (15) or mammary tumors (5, 17, 26). However, when mice which had been free of the agent obtained it by inoculation of an extract after they became adults, a low incidence of mammary tumors was observed (1, 4, 18, 21, 35). In 1945, Dmochowski (29) reported that, when 16-week-old BAF hybrids received weekly injections containing large amounts of desiccated mammary tumor tissue, the "age resistance" of the mice to the development of mammary cancer could be overcome. Each animal received by subcutaneous injection the amount of the agent from 1.5 gm. of dried tissue.

In view of past reports (5—8, 22—25, 38) that mice from our colony would have a high incidence of mammary cancer following the injection of the amount of the mammary tumor agent from either 10−4 or 10−4 gm. equivalents of tissue, the same technic was used to assay extracts of transplanted mammary tumors in adult mice. Young animals were injected for control purposes in each experiment. Additional data will be presented on the transmission of the agent by the experimental mice.

**MATERIALS AND METHODS**

The transplanted tumors, when used as source material for the agent, had been grown in mice which themselves did not possess the agent. The tissue was macerated by means of a tissue press (28), ground with sand, and extracted with distilled water to make a 20 percent suspension. This was spun for 10 minutes in a clinical centrifuge, at approximately 2,500 r.p.m., following which procedure the supernatant fluid was removed and recenterfuged for the same time. The final supernatant fluid was diluted so that the injection of 1 cc. of the extract contained the amount of the agent, based upon the weight of the original tissue, specified in the tables. The extracts were given intraperitoneally.

The test animals were Ax (fostered A), Zb (fostered Z or C3H), and their reciprocal F1 hybrids, and in no group was the incidence of mammary cancer higher than 0.4 per cent. The ZBC hybrids were produced by mating the F1 females (AxZbF1 or ZbAxF1) with males of the fostered Zb stock, except in a few instances where Z males had to be used. The incidence in ZBC mice, kept as breeders, was less than 2 percent (23, 25). Details regarding the age of the mice at the time of the experiment, dose, etc., are given in the tables for the respective studies.

**Experiment 1.**—This series was started on 7/30/48, and all the experimental mice, with the exception of one group of young ZBC mice, were injected with an extract of mammary tumor which arose in a mouse of the Z or C3H stock. The tumor had been transplanted for seven passages. The mice that received other injections were inoculated on 7/81, 8/2, 8/8, and 8/4. Another group of young mice was tested with the extract used on 8/3 to make certain that it contained the agent. When young mice were included, they were the last group to be inoculated with the respective fractions.

The results observed on the development of mammary cancer in the experimental animals are tabulated in Table 1 with
the incidence, average age at the time of appearance of the tumors, average age at death of the noncancerous mice, etc. Only noncancerous mice which survived for longer than 300 days were included. The two groups of young mice, used to assay the extracts for the first and fourth injections of some mice, have been combined, since no significant difference was noted.

In general, mice of the various age groups which received the amount of the agent from $10^{-4}$ gm. equivalents of the tumor showed not only the highest incidence but, with one exception, had the earliest average cancer age. Conversely, the lowest incidence and latest average cancer age was found, for mice of the oldest groups, in those having five injections of the extract with the highest concentration of the agent. The younger the mice were at the time of injection, the higher was the percentage of mammary tumors. This was evident when the data for the 22- to 27-day-old mice were compared to observations from older animals.

Experiment 2.—Preliminary data may be cited for another study where Ax, Zb, their reciprocal F1 hybrids, and ZBC females, 132–135 days of age, received a single injection of an extract of another transplanted mammary tumor. This arose in an A2F1 female and had been carried in agent-free mice for nine passages. Extraction of tumor A2F1 No. 8416 was such that the test animals received the amount of the agent from $5 \times 10^{-4}$, $10^{-4}$, or $10^{-5}$ gm. equivalents of tissue. Among 102 Ax, Zb, and the AxZbF1 or ZbAxF1 hybrids injected with the various fractions, six tumors have been observed after 30 months, while the incidence for the ZBC females (181 mice) had been 84 per cent (Table 3). However, females of the inbred stocks and the F1 hybrids became infected because mammary tumors have developed in their progeny. In these experiments, only males without the agent were mated with the injected females. Young ZBC females, 22–24 days of age, were tested with the same fractions to make certain that they contained the mammary tumor agent. To date, 120 (90 per cent of the total) have developed mammary tumors, at average ages from 325 to 345 days. Three mice are under observation in the control series.

TABLE 2

<table>
<thead>
<tr>
<th>MOOTHERS</th>
<th>TREATMENT OF MOTHERS</th>
<th>BORN 1ST LITTERS</th>
<th>OBSERVATIONS FOR ALL PROGENY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gm. equiv.</td>
<td>No. of injections</td>
<td>Ca/nonca Cancer</td>
</tr>
<tr>
<td>2 ca. 9</td>
<td>$10^{-4}$</td>
<td>1</td>
<td>3/0</td>
</tr>
<tr>
<td>3 nonca.</td>
<td>$10^{-4}$</td>
<td>1</td>
<td>6/1</td>
</tr>
<tr>
<td>Total</td>
<td>$10^{-4}$</td>
<td>1</td>
<td>9/1</td>
</tr>
<tr>
<td>4 nonca.</td>
<td>$2 \times 10^{-4}$</td>
<td>1</td>
<td>4/6</td>
</tr>
<tr>
<td>5 nonca.</td>
<td>$2 \times 10^{-4}$</td>
<td>5</td>
<td>1/12</td>
</tr>
</tbody>
</table>

Fourteen females from three of the injected groups were mated with males of the susceptible D4 line (28), and the observations for the progeny are recorded in Table 2. All mothers had been injected when they were 126 days of age and were mated the day after some had received their fifth injection.

Two of the mothers, which received the smallest amount of the agent, developed mammary cancer at an average age of 512 days, while the others died free of tumors when they were from 382 to 784 days of age (average, 676 days). The noncancerous females had their first litters from 41 to 61 days (average, 44 days) after their first injection, and the two cancerous females had their first litters on the 48th and 58th days. Only the first litters born to the five females which received five injections of the extract diluted 50-fold are available to give data for this group, as the male in the breeding pen died and was not replaced. For comparative purposes, the progeny born to females of the other series are listed according to their first litters and the total number of offspring. These nine females had from five to eight litters each.

As seen in Table 2, the offspring born in the first litters to females which had been injected with multiple doses of the more concentrated fraction showed the lowest incidence, while the offspring born to mothers receiving the smallest amount of the agent had the highest incidence. These females obtained a 100-fold difference in the amount of the agent. Based upon small numbers, where comparisons could be made, there was no significant difference in either the incidence or the average cancer age between mice born in the first litters and the total number of young. The three females which died noncancerous after they obtained the agent from $10^{-4}$ gm. equivalents of tissue had offspring with an incidence of 81 per cent at an average age of 385 days. Considering only the first litters born to all females of the three groups, as the concentration of the extracts administered to mothers increased, the incidence in their progeny decreased, to 90, 44, and 8 per cent, respectively.

TABLE 3

<table>
<thead>
<tr>
<th>TEST animals</th>
<th>Gm. equiv.</th>
<th>No. injected</th>
<th>No. injected</th>
<th>No. cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ax</td>
<td>$10^{-4}$</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Zb</td>
<td>$10^{-4}$</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>F1</td>
<td>$10^{-4}$</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>ZBC</td>
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<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Ax</td>
<td>$10^{-4}$</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Zb</td>
<td>$10^{-4}$</td>
<td>10</td>
<td>10</td>
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<tr>
<td>F1</td>
<td>$10^{-4}$</td>
<td>10</td>
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</tr>
<tr>
<td>ZBC</td>
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<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Ax</td>
<td>$5 \times 10^{-4}$</td>
<td>14</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Zb</td>
<td>$5 \times 10^{-4}$</td>
<td>12</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>$5 \times 10^{-4}$</td>
<td>16</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>ZBC</td>
<td>$5 \times 10^{-4}$</td>
<td>40</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>
In numerous investigations, it has been found that, following the administration of the agent to mice of different ages, adult mice become more resistant to the induction of mammary cancer with increasing age. It was suggested that possibly mammary tumors did not appear because the agent was needed before the mammary glands had developed (18). This theory was advanced, since others (36, 37) had previously reported that a difference in the architecture of the glands could be detected in mice of the same stock, with and without the mammary tumor agent. This morphological difference could not be found when females of other inbred strains and their hybrids were examined (34).

Andervont, Shimkin, and Bryan (4) showed that the oral administration of milk containing the agent produced few tumors in mice 3.5—4 months of age, whereas a high incidence was found when young animals were tested. They thought it was possible the agent either was destroyed or could not penetrate through the gastrointestinal tract of the older animals. Females which had been kept as either virgins or breeders for 30—70 days were also studied, and a low incidence was observed after the intraperitoneal injection of an extract with the agent (21). In another study, Andervont (1) used 4—5-month-old females which had had one litter, and the introduction of the agent into these adult animals produced tumors in two of 25.

Further interest in the problem was stimulated by the publication of Dmochowski (29), who showed that the repeated injection of the mammary tumor agent present in dried tumor tissue would induce mammary cancer in 4-month-old females which had previously littered. The amount of material obtained from 0.1—5 gm. of tissue was resuspended for each injection. Although four weekly injections did not cause a marked increase in the incidence, a high incidence was seen after twelve weekly inoculations. In the latter group,
the mice would obtain the amount of the agent from 1.5 gm. of tumor.

Dmochowski explained his results on the basis of a difference in the action of the agent and the hormones in the development of mammary cancer in mice. He suggested that, while the hormones may induce mammary cancer in susceptible mice only when the mammary glands have developed in the presence of the agent, the agent could exert its action in mice even after their glands had been stimulated by hormones, in the absence of the mammary tumor agent.

The data considered here would appear to refute the interpretation offered by Dmochowski in that we observed the highest incidence in the oldest mice following the administration of extracts with the lowest concentration of the agent. Several suggestions may be afforded to interpret the differences between the two studies.

By the use of supernatant fluids from centrifugation at low speeds of either normal or tumor tissues, the results for our colony have been quite consistent in that the amount of the agent obtained from $10^{-2}$, or $10^{-4}$, gm. equivalents of the tissue may be as active in producing mammary tumors as $10^{-1}$ gm. equivalents. In fresh tissue, there may be an inhibitor which might be diluted out faster than the agent, and the inhibitor could be destroyed during the process of desiccation. It is also probable that the amount of the agent present in the same amount of fresh tissue would be considerably less following desiccation. Thus, the injection of the agent from 1 gm. of dried tissue might be comparable to the use of a much smaller amount of fresh material. Whereas Dmochowski (29) gave twelve weekly injections, we used five injections over a period of 6 days.\(^1\)

As indicated by one study in this report (Table 3), the introduction of the same fraction of a tumor need not induce the same incidence of mammary cancer in mice of different groups, even though the mice are closely related. Primary consideration must be given to possible differences in the genetic susceptibilities of the test animals, including end-organ sensitivity and hormonal mechanisms.

Males of the C3H stock, without the mammary tumor agent, have been used to test the association between the state of development of the mammary glands at the time of the administration of the mammary tumor agent and the later development of mammary cancer (85). The males that were castrated when 4 weeks old and grafted with ovaries from females of the same stock and age, followed by the injection of the agent the following day, gave an incidence of mammary tumors of 74 per cent. In other groups, the mice either were castrated and received the ovarian grafts at 1 month and the agent when 4 months of age, or were injected with the agent when 4 weeks of age and were castrated at 4 months when they also obtained implanted ovaries. In mice of these latter groups, the incidence was approximately 25 per cent, yet the average latent period of tumor development after the injection of the agent was approximately the same in all groups. This would suggest that the relative resistance of older mice may not be due to the degree of mammary development of the time of infection with the agent.

The use of repeated injections of large amounts of dried tumor tissue by Dmochowski (29) induced a high incidence of mammary cancer in females of the BAF1 generation. When females of the reciprocal generation (ABF1 hybrids) were maintained as virgins, a low incidence of mammary tumors was noted (12). The ABF1 females would obtain the mammary tumor agent and the inherited susceptibility from their mothers of the cancerous A stock, but neither of the parental strains would transmit the inherited hormonal influence (27) associated with the development of mammary cancer in virgins. In the absence of this hormonal mechanism possessed by the Z or C3H stock, there may exist some difference in the mammary glands which would make them sensitive to the development of mammary cancer following the injection of the mammary tumor agent when they became adults.

The mammary tumor agent transferred by females of cancerous strains does not induce the same incidence of mammary cancer in hybrids with the same genetic constitutions (10). This subject has recently been reviewed (23), and the data would indicate that we may be dealing with different agents. To complicate the problem even more, extracts of spontaneous mammary tumors from females of the same cancerous inbred strains may not show the same activity when injected into comparable test animals (unpublished data). The factors that may be involved are not understood at this time.

In the first series of the foster-nursing studies which demonstrated the mammary tumor agent, it was observed that, while some of the fostered fe-
males died without mammary cancer, cancer appeared among their progeny and descendants (9, 11). Likewise, females of a low susceptible B (C57 black) line were found to have a low incidence after they were nursed by females of cancerous stocks; but, when these B females with the agent were used to nurse susceptible mice, these susceptible animals gave rise to mammary cancer (14). The observations demonstrated that infected mice of either susceptible or relatively nonsusceptible strains may die noncancerous, but they will transfer the agent to their progeny.

In other cases, the agent may "appear" in mice born of parents neither of which possessed the agent (16, 23). If progeny have been continued when this occurs, they give rise to a cancerous line (18), or the agent may be recovered from the mammary tumors that appear, as shown by biological assay (23, 25).

Evidence has accumulated that the transmission of the agent in the milk of females which obtained the agent by the grafting of normal tissues, the injection of an extract, or infection from the male, is comparable to that seen when the mice secure the agent by nursing. Following the grafting of normal tissues (spleen, thymus, mammary glands), even noncancerous females transferred the agent to their progeny (18, 14). Some of these experimental mice had had one litter before they had been inoculated with the normal tissue from young donors.

Andervont (1) used females from 4 to 5 months of age, after they had given birth to one litter; and, following the injection of a tumor extract, only two of 25 had mammary tumors. When the injected females were mated with males of the cancerous CSH stock, seven of the females had cancerous progeny, but the offspring born to fourteen other females remained free of mammary tumors. He concluded that, while the adult mice might be resistant to the agent, adults may become infected and pass the agent to their progeny.

In 1948 (28) data were reported on the development of a higher incidence in young mice which received the amount of the agent from $10^{-1}$ than from $10^{-1}$ gm. equivalents of tumor tissue, and the experimental females which obtained the smaller amount of the agent also had progeny with the higher incidence. Injected mice which died noncancerous but had cancerous progeny (Tables 4 and 5) were included.

It was observed in several experiments that mice developed mammary cancer following the administration of the agent in dilutions representing $10^{-4}$ (5, 23) or $10^{-7}$ (33) gm. of either normal mammary tissue or mammary cancer. Andervont (2) found that mice which obtain comparable amounts of the agent may have cancerous progeny, showing that they were infected.

When females of various stocks become infected with the agent when it is transferred by males of cancerous strains (8, 24, 25, 32), strain differences in the sensitivity of the females to infection, as well as the ability of the males to infect, may be seen by studying various crosses. Whereas Andervont and Dunn (3) found that less than 5 per cent of the C females developed mammary cancer when mated with males of the Andervont CSH stock, if males of our subline of the CSH strain were used, 59 per cent of the C females developed mammary cancer, and others became infected. The incidence in C females mated with males of the A strain was 38 per cent (24), and only 3 per cent of the C3H females without the agent gave rise to mammary cancer when crossed with males of the C3H cancerous line (19, 25). In our studies, the progeny born to females prior to infection from the male showed a low incidence; but, following infection, the progeny had a high incidence. Infection may not take place until the females have eight litters. It would be difficult to interpret the appearance of the high incidence in the C female and their progeny as resulting from the introduction of a large amount of the agent.

Further studies are indicated to determine the possible relationship between the induction of mammary cancer following the introduction of the mammary tumor agent into adult mice and the amount of the agent injected, genetic factors, possible hormonal mechanisms, the transmission of the agent, etc.

**SUMMARY**

The mammary tumor-inducing activity of the mammary tumor agent was studied by the administration of either one or five injections of fractions of transplanted mammary cancer into mice ranging in age from 22 to 126 days.

The concentration of the fractions represented the amount of the agent derived from either $2 \times 10^{-2}$ or $10^{-2}$ gm. equivalents of fresh tissue. In general, the incidences observed in adult mice could not be correlated with the amount of the agent that was injected.

The highest incidence occurred in the progeny of the mice which received a single injection of the fraction with the lowest concentration of the agent.

The transmission of the agent was observed following the administration of the mammary tumor agent into both young and adult females.

No significant difference was to be seen in the
transmission of the agent in the milk of either cancerous or noncancerous experimental mice.

Data were presented suggesting that the age resistance to the tumor-inducing action of the mammary tumor agent may be determined, in part, by the genetic constitution of the test animals. Other possibilities were discussed.

REFERENCES

Tumor-inducing Properties of the Mammary Tumor Agent in Young and Adult Mice

John J. Bittner


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