Accelerated Development of Spontaneous Mammary Tumors in Mice Pretreated with Mammary Tumor Tissue and Adjuvant*

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SUMMARY

C3H and C3H backcross mice were given repeated injections of preparations from strain-specific or strain-related mammary tumors of recent spontaneous origin together with Freund’s adjuvant. The effect of the course of injections on spontaneous mammary tumor development was studied. Pretreatment with mammary tumor tissue did not lead to a significant reduction in spontaneous mammary tumor incidence, did not delay age of tumor onset or prolong survival time following onset of tumors. On the contrary, administration of tumor tissue led to a significant reduction in the age at which spontaneous mammary tumors occurred and to a reduced survival time of the tumorous animals. Freund’s adjuvant alone was without effect on either cancer incidence, cancer age, survival time, or survival time following onset of tumors. The data are discussed in the light of the possibility that auto-immune or enhancement phenomena may contribute significantly to tumor development.

The theoretical framework for experiments in tumor immunity1 is provided by the assumption that tumors contain tumor-specific antigens and that pretreatment of animals with such antigens would induce, to a greater or lesser degree, resistance against subsequent development of tumors containing such antigens. No unequivocal evidence for the presence of tumor-specific antigens in spontaneous tumors has so far been demonstrated, although the presence of tumor-specific antigens in carcinogen-induced tumors (see [26] for the pertinent literature) is now well established.

It has been shown recently (5) that immunization of inbred mice against an isologous transplanted tumor of very recent spontaneous origin did not lead to a reduced tumor incidence following challenge with the same tumor; immunization did, however, result in an increased survival time of the immunized animals. This appeared to be the first instance in which a slight amount of resistance in inbred mice against a strain-specific tumor of recent spontaneous origin was induced; the results gave some support, therefore, to the hypothesis that such tumors might contain tumor-specific antigens. A number of factors other than true tumor immunity were cited which also might have been responsible for the results obtained, and it was pointed out (19, 20) that it would be useful to test the effect of immunization on spontaneous tumor development, rather than to approach the problem through the use of transplanted tumors and other model systems. It was
thought of considerable interest, therefore, to determine whether pretreatment with strain-specific tumor tissues would be of value also in the protection, however slight, against the occurrence of spontaneous tumors. The present report furnishes data dealing with such attempts. Inbred and backcross mice were given repeated injections of preparations from spontaneous mammary tumors specific to the inbred strain, plus Freund's adjuvant, and the effect of the course of injections on spontaneous mammary tumor development was studied.

MATERIALS AND METHODS

Two experiments were run; they are designated as Experiment A and Experiment B, and the methodology used in each will be described separately. Experiment A deals with inbred mice, Experiment B with backcross mice. All mice received Purina Fox Chow and tap water. Following appearance of a mammary tumor, each mouse was housed singly. All animals were inspected once weekly during their complete lifetime for spontaneous tumor formation. The tumorous animals were inspected more frequently, especially during the terminal phase of the disease.

EXPERIMENT A

C3H female mice were employed here; they were from approximately 2½-3½ months of age when the injections were started. Mice of different ages were distributed equally between control and experimental groups. Every animal was given a series of four injections; the experimental group was treated with tumor preparation plus Freund's adjuvant (two injections) and tumor preparation plus saline (two injections); the control group (control #1 in the tables) was given Freund's adjuvant plus saline (two injections); and a control group which received Freund's adjuvant (injections 1 and 4) or saline (injections 2 and 3). Sterile precautions were used throughout, and all procedures were done in the cold. Microscopic examination of the suspensions always revealed the presence of a few cells, all of which were nonviable, as demonstrated by trypan blue staining. No tumors were ever observed at the injection sites.

Injection schedule.—The course of injections was administered in the following manner: injection 1, 0.2 ml., subcutaneously in the back; injection 2, given 13 days later, 0.15 ml., intraperitoneally; injection 3, given 7 days later, 0.2 ml., intramuscularly into one hind leg; injection 4, given 19 days later, 0.1 ml. total, intramuscularly (0.05 ml. into each hind leg).

Preparation of tumor for injection purposes.—The tumors, kept frozen at -45°C until used, were thawed rapidly at 37°C. The tumor tissue was minced with scissors and ground in a mortar and pestle with small amounts of sand and physiological saline for 8-10 minutes. The ground tissue was made up to contain 40 per cent of tissue in saline (by weight) and centrifuged for 4-5 minutes at approximately 2500 r.p.m. The supernate was examined microscopically for the presence of cells. It was measured and diluted 1:1 with Freund's adjuvant (injections 1 and 4) or saline (injections 2 and 3). Sterile precautions were used throughout, and all procedures were done in the cold.

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tion, spontaneous C3H tumors were used, whereas for the second, third, and fourth injections, spontaneous C3H tumors in their first transplant generation in C3H backcross mice were employed; the latter procedure was necessitated by lack of enough spontaneous tumor material. Altogether, material from two different C3H tumors was used for the injections.

Preparation of tumor for injection purposes.—The method was essentially similar to that described for experiment A; microscopic examination of the preparations revealed the presence of some cells in two of the four preparations; all were nonviable, as demonstrated by trypan blue staining. No tumors were ever observed at the injection sites.

Injection schedule.—Injection 1, 0.2 ml., intraperitoneal; injection 2, given 11 days later, 0.15 ml. subcutaneously in the back; injection 3, given 8 days later, 0.2 ml., intramuscularly into one hind leg; injection 4, given 13 days later, 0.1 ml. total (0.05 ml. intramuscularly into one hind leg, and 0.05 ml. subcutaneously in the back).

The Freund’s adjuvant used in both experiments had the following composition: to 1.5 ml. of Arlacel A (Atlas Powder Co., Wilmington, Del.) and 8.5 ml. Bayol F (Penola Oil Co.) were added Mycobacterium butyricum (Difco) to a concentration of 5.0 mg. of bacteria per ml. of adjuvant. Sterile abscesses were frequently found at the site of injection in animals receiving Freund’s adjuvant.

The value of Freund’s adjuvant (13, 14) in enhancing the immunizing effect of vaccine preparations of borderline effectiveness and in prolonging the effect of those that are of established value is well known.

All mice were kept as virgins; the spontaneous mammary tumor incidence in virgin female C3H and C3H backcross mice with the agent is usually high. Tumor incidence is higher in mice kept as breeders; the mice were kept as virgins, however, to reduce the variable of hormonal stimulation, which, in mated mice, varies with the number of litters. The relatively low tumor incidence in the C3H backcross mice observed here (about 20 per cent in the controls) must be ascribed to a preparation containing the mammary tumor agent which was rather low in activity. Barnum et al. (2) have indicated that C3H strain lactating mammary gland is a relatively less potent source of the mammary tumor agent than certain other sources. However, tumor extracts containing the agent were unsuitable in this experiment, for obvious reasons.

All mice were weighed repeatedly; no significant differences in weights between experimental and control groups were observed in either of the experiments.

Histological diagnosis.—The effect of administration of the tumor tissue preparations on survival time following onset of the spontaneous tumors was considered one of the most important variables to be studied in the present experiments. To determine survival time the animals had to be allowed to die of the tumors, thus precluding prior removal of tumors for histological diagnosis. Tumors were collected as soon after death as feasible, but histological examination of all the tumors could not be carried out, owing to frequent post mortem necrotic changes.

Altogether, 29 per cent of the tumors in experiment A and 42 per cent of the tumors in experiment B came to histological diagnosis. All were mammary adenocarcinomas, with the exception of one squamous-cell tumor in experiment B which is not included in the tabulated results. This perianal tumor grossly had already not given the appearance of a mammary tumor. All other tumors had the gross characteristic appearance of mammary tumors and are considered here as such. Since the great majority of all subcutaneous tumors in the mouse are mammary in origin and since the gross appearance of these tumors is fairly characteristic, this was considered quite safe. It is possible that among the tumors which could not be diagnosed and which grossly gave the appearance of mammary tumors, a salivary gland tumor or a spindle-cell sarcoma might inadvertently have been included. Such tumors, according to Dunn (12), are the only tumors which in the gross may closely resemble mammary tumors; they are relatively rare in these mice.

RESULTS

The experiments were designed to test the effect of injection with strain-specific (experiment A) or strain-related (experiment B) mammary tumors on the later development of spontaneous mammary tumors. Such tumors are the only ones which will be considered in the tabulation of results. In assessing the effect of pretreatment with mammary tumor tissue on subsequent spontaneous mammary tumor formation, the following variables were considered in the experimental and control groups: (a) tumor incidence; (b) age at which animals became tumorous (cancer age); (c) survival time of the tumorous animals; (d) survival time of the nontumorous animals; (e) survival times of the tumorous and nontumorous animals combined; (f) survival time of the tumorous animals following onset of the tumor. The last
variable was considered particularly important, since previous experiments (21) had shown it to be the only one affected by prior immunization in a model experiment employing a transplanted tumor of very recent spontaneous origin. In the tables only the mice alive at earliest tumor appearance are included.

**Experiment A**

A summary of the results obtained is given in Table 1. No statistically significant differences in tumor incidence between the experimental and the two control groups were found \((P \approx 0.25)\). The mean survival time following onset of tumors of the experimental group also does not differ significantly from that of the control groups. However, there were considerable differences in cancer ages, the experimental group having a mean cancer age which was 100 days less than that of the control animals treated with Freund’s adjuvant only and 70 days less than that of the saline control. By analysis of variance (see Table 2) it was found that the differences among the three groups were significant \((P \approx 0.05)\).

Two comparisons were of interest: \((a)\) a comparison of the mean cancer ages of the two control groups and \((b)\) a comparison of mean cancer ages of the experimental with the two control groups. These were tested simultaneously. No significant difference between the two control groups was found, but the difference between the experimental and the two control groups was found to be significant \((0.02 < P < 0.05)\).

As far as the survival times of the experimental and control mice with tumors were concerned, it can be seen from the analysis of variance (Table 3) that no significant difference was observed among the three groups \((P > 0.10)\). Again, the two comparisons of interest were \((a)\) a com-
comparison of the mean survival times of the two control groups and (b) a comparison of mean survival times of the experimental with the two control groups. These were tested simultaneously. No significance difference between the two control groups was found. The difference between the mean survival times of the experimental and the two control groups was not so clear-cut as the corresponding difference in mean cancer ages ($P = 0.1$).

However, had we assumed a priori (a reasonable assumption) that the mean survival times for the two control groups would be the same, then the mean survival time of the experimental group would have been found to be significantly different from the mean of the combined control groups ($P = 0.05$).

Of interest was the finding that use of Freund's adjuvant alone did not significantly affect any of the variables studied. On the other hand, the experimental animals (treated with tumor plus adjuvant) as a whole appeared to be less active and less healthy than the animals in either control group and tended to have a shorter survival time, indicating that isologous tumor plus adjuvant had a toxic effect. For this reason, the mean survival time of all animals (regardless of cause of death) was compared in the experimental group and (for reasons of comparison with experiment B) control group 1. This difference was found, by the Mann-Whitney test, to be not significant statistically ($P = 0.25$).

It can be concluded from the present experiment that pretreatment of C3H mice with strain-specific mammary tumors in conjunction with Freund's adjuvant did not result in a decreased tumor incidence or in an increased survival time of the animals following onset of tumors. On the contrary, such a procedure led to earlier tumor onset and lowered survival time in the experimental animals. The use of Freund's adjuvant alone did not affect either tumor incidence, cancer age, or survival time following onset of tumors.

### TABLE 3

**Survival Time (in Days) of Experimental and Control C3H Mice (Experiment A)**

<table>
<thead>
<tr>
<th></th>
<th>Experimental</th>
<th>Control 1</th>
<th>Control 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>322</td>
<td>406</td>
<td>312</td>
<td></td>
</tr>
<tr>
<td>350</td>
<td>406</td>
<td>447</td>
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<td>486</td>
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<td>538</td>
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</tr>
<tr>
<td>546</td>
<td>546</td>
<td>571</td>
<td></td>
</tr>
<tr>
<td>$\bar{x} = 444.5 \pm 21.7$</td>
<td>$\bar{x} = 511.0 \pm 25.0$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$N = 9$</td>
<td>$N = 13$</td>
<td></td>
<td></td>
</tr>
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### ANALYSIS OF VARIANCE*

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<th>Source</th>
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<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
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<tr>
<td>Among groups</td>
<td>2</td>
<td>35916</td>
<td>17963</td>
<td>2.15</td>
<td>N.S.</td>
</tr>
<tr>
<td>Exper. vs. controls 1 vs. control 2</td>
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<td>35014</td>
<td>35014</td>
<td>4.19</td>
<td>=0.05†</td>
</tr>
<tr>
<td>Within groups</td>
<td>32</td>
<td>267352</td>
<td>8360</td>
<td></td>
<td>&lt;1</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>305448</td>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
</tbody>
</table>

* See footnote to Table 2.
† For explanation, see text.

### Experiment B

A summary of the results is given in Table 4. The proportion of animals developing tumors in the experimental group again did not differ significantly from the proportion of animals developing tumors in the control group ($P = 0.11$); mean survival time following onset of tumors of the experimental group was also not significantly different from that of the control group. Even though the proportion of animals developing tumors was smaller in the experimental group than in the control group in both experiments A and B, it was felt that this may be an artifact, since the animals in the experimental groups in both experiments appeared, as pointed out above, as a whole to be less healthy and tended to have a lower

### TABLE 4

**Effect of Pretreatment with Strain-Related Mammary Tumor Tissue on Spontaneous Mammary Tumor Development in C3H Backcross Mice (Experiment B)**

<table>
<thead>
<tr>
<th></th>
<th>Experiments</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. mice used</td>
<td>89</td>
<td>86</td>
</tr>
<tr>
<td>No. with tumor</td>
<td>9</td>
<td>16</td>
</tr>
<tr>
<td>Tumor incidence</td>
<td>10.1%</td>
<td>19.6%</td>
</tr>
<tr>
<td>Mean cancer age (days)</td>
<td>478.7±41.9</td>
<td>568.7±28.4</td>
</tr>
<tr>
<td>Mean survival time following onset of tumor (days)</td>
<td>556.3±41.8</td>
<td>647.9±31.8</td>
</tr>
<tr>
<td>Mean survival time nontumorous mice (days)</td>
<td>77.5±12.6</td>
<td>79.2±12.4</td>
</tr>
<tr>
<td>Mean survival time nontumorous mice (days)</td>
<td>636.0±18.0</td>
<td>650.5±16.1</td>
</tr>
</tbody>
</table>

* For details concerning the mice used, the tumor preparations employed for the injections, number of injections, Freund's adjuvant used, etc., see "Materials and Methods." All means are followed by the standard error of the mean.
survival time than the controls; this tendency may have manifested itself in affording the experimental animals less opportunity for developing tumors.

In this experiment also the experimental ("immunized") group exhibited an earlier cancer age and lower survival time when compared with the control group. A breakdown of the data for tumorous animals as well as the results of the statistical tests are given in Table 5. One-tailed Student’s "t" tests were used to compare mean cancer age and mean survival time of the tumorous animals of the two groups, because experiment A afforded a priori support for a one-sided hypothesis. It was found that mean cancer age for the experimental group was significantly less than that of the control group (0.025 < P < 0.05) and that mean survival time of tumorous animals for the experimental group was significantly less than that of the control group (P = 0.05).

Although experiments A and B were run more or less concurrently, they were completely independent. Hence, Pearson’s P₃ statistic (29) was used to combine the significance probabilities of the two experiments. It was found that the probability of obtaining outcomes as extreme as those observed and in the same direction in the two experiments is less than 0.01 for the differences in mean cancer ages between experimental and control mice, and between 0.01 and 0.02 for the differences in mean survival times of the tumorous animals between the experimental and control series. (For comparative purposes, only experimental and control group 1 from experiment A and corresponding groups in experiment B were used in obtaining these two probabilities.)

It was of interest to test whether the tendency, observed in experiment A, toward lower survival time of the experimental animals (tumorous and nontumorous combined) was maintained in experiment B. It was found, by the Mann-Whitney procedure, that the mean survival time of the experimental animals again was not significantly less than that of the controls (P ≥ 0.19). According to Pearson’s P₃ statistic in testing the combined significance probabilities of the two experiments it was found that, under the hypothesis of no difference, the probability of differences as extreme, and in the same direction, as those observed, is approximately 0.23. Since in both experiments the experimental animals as a whole tended to have lower survival times than the controls and also appeared less healthy, further experimentation would be desirable to investigate interaction, if any, between the "immunizing agent" and Freund’s adjuvant and its effect on survival time of the total population. This is a problem of obvious importance in areas other than the specific one dealt with here.

The conclusions from the present experiment afforded a priori support for a one-sided hypothesis. It was found that mean cancer age for the experimental group was significantly less than that of the control group (0.025 < P < 0.05) and that mean survival time of tumorous animals for the experimental group was significantly less than that of the control group (P = 0.05).

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It was of interest to test whether the tendency, observed in experiment A, toward lower survival time of the experimental animals (tumorous and nontumorous combined) was maintained in experiment B. It was found, by the Mann-Whitney procedure, that the mean survival time of the experimental animals again was not significantly less than that of the controls (P ≥ 0.19). According to Pearson’s P₃ statistic in testing the combined significance probabilities of the two experiments it was found that, under the hypothesis of no difference, the probability of differences as extreme, and in the same direction, as those observed, is approximately 0.23. Since in both experiments the experimental animals as a whole tended to have lower survival times than the controls and also appeared less healthy, further experimentation would be desirable to investigate interaction, if any, between the "immunizing agent" and Freund’s adjuvant and its effect on survival time of the total population. This is a problem of obvious importance in areas other than the specific one dealt with here.

The conclusions from the present experiment
are entirely similar to those from experiment A. Pretreatment of C3H backcross mice with strain-related tissue in conjunction with Freund’s adjuvant did not result in a decreased tumor incidence or an increase in the survival time of the tumorous animals following onset of the disease. Rather, administration of mammary tumor tissue preparations led to earlier spontaneous tumor onset and lowered survival time in the “immunized” animals. The effect of strain-related tissue plus Freund’s adjuvant as an immunizing agent may have had some effect also on survival time of the population as a whole, but a much larger sample would be needed to make sure that this effect was real.

**DISCUSSION**

Experimental data dealing with the important problem of induced resistance against spontaneous tumor development are almost entirely lacking, and only scattered attempts to approach the problem have been reported in the literature. Previous work on the feasibility of inducing resistance in animals against tumors has been done almost exclusively in model experiments employing transplanted tumors. Besides the many objections which can be made to use of the transplanted tumor model experiments and extrapolation of such results to the question of induced resistance against spontaneous tumor development (for discussion, see [19, 20, 25]), it should be realized that immune defense reactions of an animal going through the process of spontaneous tumor formation may be entirely different from those occurring in an animal serving simply as a “test tube” for growing a tumor which has developed spontaneously in a different animal.

The early work on immunization against spontaneous tumors by Haaland, Loeb, Bashford, and Murray and Cramer is reviewed by Hackmann (18). This work was handicapped by the absence of inbred strains of animals with a high tumor incidence, but it did lead to the generalization, expressed in “Haaland’s dictum,” that it was seemingly impossible to induce resistance in an animal against challenge with its own tumor cells. Gross (15) found that inbred C3H mice injected with a methylcholanthrene-induced sarcoma specific to the inbred strain and resistant to that tumor still developed spontaneous mammary tumors. Klein et al. (26) and Prehn (27) have recently shown that immunization with carcinogen-induced tumors was of no avail against subsequent induction of tumors by the same carcinogen, despite the fact that the carcinogen-induced tumors contained tumor-specific antigens. Excision of spontaneous tumors, followed by reinoculation of the spontaneous tumor cells to determine resistance against such cells, has been attempted by a number of authors (1, 16-18, 22). No resistance seemed apparent, although Isojima et al. (22) claimed a prolongation in the survival time in the autoimmunized animals. However, in the experiments of Axelrad and Van der Gaag (1), the tumor cells, on reinoculation, seemed to grow better in the autologous host in which the tumor originated than in isologous, genetically identical but previously tumor-free, hosts. This indicated the possibility that an animal developing a tumor spontaneously actually possesses an internal environment facilitating the growth of its own tumor cells. Similar observations were made by Hackmann (18).

Hackmann (18) attempted to induce active immunity against spontaneous tumor development in an inbred mouse strain; cell-free tumor extracts from spontaneous tumors were used for the immunization procedures. No difference in spontaneous tumor incidence in the control and experimental groups was noted. Baidakova and Avenirova (quoted in [33]) have vaccinated strain A mice at an early age with “a specific antigen” from isologous mammary tumors and found a “lower cancer morbidity.” Isojima, Graham, and Graham (22) reported that active immunization of young virgin C3H female mice with isologous mammary tumor homogenates plus Freund’s adjuvant appeared effective in preventing or delaying tumor development in this strain. Animals receiving Freund’s adjuvant alone also had a low tumor incidence. This may indicate that either the animals used had a low tumor incidence (no untreated, intramural controls were used, and the control spontaneous tumor incidence was taken from the literature) or that administration of adjuvant alone diminished the tumor incidence. In our experiments no effect of adjuvant alone on either tumor incidence or tumor age was apparent.

It has been shown in both of the present experiments that pretreatment with strain-specific or strain-related tumor tissue leads to an apparent earlier tumor development. The possibility must be considered that this phenomenon is not due to some sort of autosensitization toward tumor development but to additional mammary tumor agent introduced with the four injections. It will be remembered that the C3H mice received the agent at birth in their mothers’ milk and that the agent was administered to the C3H backcross mice when they were from 27 to 36 days of age. This explanation is considered unlikely for the following reasons: (a) the additional agent was

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introduced quite late (for the effect of age at injection on tumor development, see [4, 6]); (b) review of the pertinent literature (8–10) reveals no correlation whatever between tumor incidence or tumor age and amount of agent administered, within very wide limits; (c) in both experiments the tumor incidence was actually somewhat lower in the animals receiving the additional agent-containing injections than it was in the controls. As a working hypothesis, then, it can be assumed that injection into mice of strain-specific (isogenic) or strain-related tumor tissue actually lowers the age at which tumors are formed. If this can be further confirmed it might indicate that autoimmune phenomena have some part in mammary tumor development. Pertinent here are a number of recent papers dealing with hypotheses that certain neoplasms such as the leukemias, Hodgkin's disease, and other malignant lymphomas are expressions of an immunologic fault of the host, resulting in autoimmunization (11, 12, 13). An opportunity to test this hypothesis would be afforded by using antibodies against the tumors rather than the tumor preparations themselves for the injections.

It should be pointed out that some recent findings (see Shope [30] for discussion) of earlier tumor development in mice following administration of preparations from human tumor material were mainly in "homogenate" (x-ray or otherwise inactivated) might have given different results. It should also be remembered that in both experiments the experimental animals had a lower tumor incidence than the controls; although the combined probability value (29) for both experiments was not significant (P ≈ 0.1), it is possible that use of a much larger group of animals would reveal some effect. This aspect merits further careful study. If such an effect could be established, the interrelation between a reduced tumor incidence in the animals treated with tumor tissue and an earlier tumor age in those animals developing tumors might prove of considerable interest.

ACKNOWLEDGMENTS

Sincere thanks are due Dr. J. J. Bittner for supplying a nucleus of the CSH mice as well as all the CSH backcross mice used in this study, and to Dr. Martella Frantz for the diagnosis of the tumors. Dr. Byron W. Brown, Jr., kindly read the manuscript and made numerous valuable suggestions.

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Accelerated Development of Spontaneous Mammary Tumors in Mice Pretreated with Mammary Tumor Tissue and Adjuvant

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