SUMMARY—The tumorigenic effect of two chemical carcinogens, 7,12-dimethylbenz[a]anthracene (DMBA) and urethan, and of γ-irradiation were analyzed further on the nodule outgrowth line D1. Nodule outgrowth line D1 treated with DMBA and urethan produced 68 and 83% tumors, respectively, whereas irradiated outgrowths produced only 23%. Prolonged hormone stimulation by a single pituitary isograft enhanced the tumorigenic effect of DMBA, had no effect on urethan-induced tumorigenesis, and inhibited irradiation-induced tumorigenesis. Nodule outgrowths were exposed to various doses of DMBA and urethan. Outgrowths exposed to 1.0 mg and 1.5 mg DMBA produced the same tumor incidence (68%), whereas outgrowths exposed to 0.5 mg produced only 50% tumors. The various doses of urethan produced mammary tumors in the following order: 200 mg > 100 mg = 80 mg > 60 mg = 40 mg > 20 mg. The time of administration of urethan was important in determination of the ultimate tumor incidence and the rate of tumor formation. Outgrowths exposed to urethan between 3 and 5 weeks after transplantation produced tumors earlier and at a higher incidence than outgrowths exposed to urethan at later periods. The implications of these results are discussed as to similar findings of D1 nodule outgrowths treated with 3-methylcholanthrene.—J Nat Cancer Inst 46: 909-916, 1971.

THE D1 nodule outgrowth line is one of a series of BALB/c nodule outgrowth lines. Earlier experiments on the tumor-producing capabilities of the nodule outgrowth line D1 produced the following results:

1) The tumor-producing capability of the nodule outgrowth line D1, free of mammary tumor virus (MTV) and nodule-inducing virus (NIV), was 4% at 404 days (1).

2) The chemical carcinogens, 3-methylcholanthrene (MCA), 7,12-dimethylbenz[a]an-
thracene (DMBA), and urethan, and γ-irradiation increased the tumor-producing capabilities of outgrowth line D1 to 50, 64, 76, and 22%, respectively (2, 3).

3) Prolonged hormone stimulation by pituitary isografts did not significantly increase the tumor-producing capabilities of line D1 (4). However, it did potentiate the effect of MCA-induced tumorigenesis in D1 outgrowths (2).

The purpose of the present studies was to extend these observations on carcinogen-treated nodule outgrowths to determine the following:

1) Does prolonged hormone stimulation potentiate the tumorigenic effect of DMBA, urethan, and irradiation?
2) What doses of DMBA and urethan are most effective in producing mammary tumors?
3) Is the tumorigenic response of outgrowth line D1 to urethan influenced by its degree of proliferation, as has been suggested for liver (5-7), skin (8, 9), in vitro carcinogenesis (10), and MCA-induced mammary gland tumorigenesis (11)?

MATERIALS AND METHODS

Animals.—BALB/c mice, free of detectable MTV and its variant NIV (3, 12), were used in all experiments. All mice were obtained from the Cancer Research Genetics Laboratory colony, University of California, Berkeley, housed 6-10 to a cage in temperature-controlled and light-cycle-controlled rooms, fed Purina Breeder Chow or Berkeley Diet (1), and given water ad libitum.

Transplantation.—The hyperplastic nodule outgrowth line D1 was used in all experiments. The origin and tumor-producing capabilities of outgrowth line D1 have been previously reported (1-4, 12). Samples of the nodule outgrowths were transplanted into the inguinal mammary glandfree fat pads of 3-week-old BALB/c female mice by the standard method of DeOme et al. (13).

All mice were palpated once weekly for mammary tumors. Tumors were removed from mice when they were first palpable, fixed in Tellyesnickzky's solution, embedded in paraffin, sectioned at 7 μ, and stained with hematoxylin and eosin.

The procedures for prolonged hormone stimulation of the host's mammary glands and of the nodule outgrowths were previously reported (2). Similarly, the conditions for administration of the chemical carcinogens and γ-irradiation were previously reported (3). Mice received DMBA between 5 and 7 weeks after transplantation of the outgrowths and urethan between 3 and 14 weeks after transplantation of the outgrowths.

Experiments.—In experiment 1, the effects of different doses of DMBA on the tumor potential of D1 outgrowths were investigated. The mice were divided into 6 groups. Outgrowths were transplanted into both inguinal fat pads of 3-week-old mice in all groups except group 5, which received a single transplant in the right inguinal fat pad only. A single male pituitary gland was transplanted under the kidney capsule in mice of group 6 when the mice were 4 weeks old and remained in place permanently. The DMBA (0.5 mg) was fed to mice in the various groups at the times indicated in table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg)</th>
<th>Time after transplantation when DMBA administered (wk)</th>
<th>Hormone stimulated</th>
<th>Number tumors</th>
<th>% TE50* (days)</th>
<th>Mice with tumors in transplant</th>
<th>Mice without tumors in transplant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>—</td>
<td>—</td>
<td>1/24</td>
<td>4</td>
<td>301</td>
<td>415</td>
</tr>
<tr>
<td>2</td>
<td>0.5 mg</td>
<td>7</td>
<td>—</td>
<td>9/18</td>
<td>50</td>
<td>329</td>
<td>301</td>
</tr>
<tr>
<td>3</td>
<td>1.0 mg</td>
<td>5, 7</td>
<td>—</td>
<td>15/23</td>
<td>65</td>
<td>250</td>
<td>210</td>
</tr>
<tr>
<td>4</td>
<td>1.5 mg</td>
<td>5, 6, 7</td>
<td>—</td>
<td>34/50</td>
<td>68</td>
<td>224</td>
<td>203</td>
</tr>
<tr>
<td>5</td>
<td>1.5 mg</td>
<td>5, 6, 7</td>
<td>—</td>
<td>30/44</td>
<td>68</td>
<td>224</td>
<td>210</td>
</tr>
<tr>
<td>6</td>
<td>1.5 mg</td>
<td>5, 6, 7†</td>
<td>—</td>
<td>22/24</td>
<td>92</td>
<td>168</td>
<td>182</td>
</tr>
</tbody>
</table>

*TE50 refers to 50% tumor endpoint.
†In all groups except group 5, both fat pads received a single transplant. Group 5 received a single transplant in the right #4 fat pad only.
In experiment 2, D1 outgrowths were transplanted into the cleared fat pads of 3-week-old BALB/c mice. Half the mice received a single male pituitary gland under the kidney capsule when the mice were 4 weeks old. The mice received intraperitoneally 20 mg urethan dissolved in distilled water once weekly; a total of 20, 40, 60, 80, 100, or 200 mg. The results are shown in table 2 and text-figure 1.

In experiment 3, D1 outgrowths were transplanted into: a) unstimulated and hormone-stimulated unirradiated mice; b) unstimulated and hormone-stimulated mice irradiated 7 weeks after transplantation; and c) unstimulated and hormone-stimulated mice irradiated 16 weeks after transplantation. A single dose of whole-body irradiation (450 R, 11.4 R/min) was administered. The results are shown in table 3.

Two types of nonparametric statistical tests, the Mann-Whitney U test and the Median test, were used for these data. The 50% tumor endpoint was defined as the time it took 50% of the nodule transplants to produce tumors (4).

RESULTS

Effect of DMBA on Tumor-Producing Capabilities of D1 Outgrowths

The results are shown in table 1. D1 outgrowth maintained in untreated mice produced only 4% tumors (1/24, group 1). D1 outgrowths exposed to 0.5 mg DMBA produced 50% tumors (9/18, group 2) with the 50% tumor endpoint reached at 329 days. D1 outgrowths exposed to 1.0 mg DMBA (group 3) and 1.5 mg DMBA (group 4) produced 65% tumors (15/23) and 68% tumors (34/50), respectively, with the 50% tumor endpoints attained at 259 and 244 days, respectively.

The tumor distributions of outgrowths exposed to 1.0 mg or 1.5 mg DMBA were not significantly different from each other, though they were significantly different from groups 1 and 2 (P<0.05).

The tumor incidence and distribution of outgrowths in group 5 were compared with group 4 to determine whether two outgrowths in the same host might be treated independently as to tumor development. The results in group 5 were not significantly different from those in group 4. Tumor

Table 2.—Summary of effects of urethan and hormone stimulation on the tumor-producing capabilities of D1 nodule outgrowths

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumors</th>
<th>Mean age of death (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tumors</td>
<td>Percent</td>
</tr>
<tr>
<td>Control</td>
<td>3/5</td>
<td>6</td>
</tr>
<tr>
<td>Urethan</td>
<td>99/243</td>
<td>40.7</td>
</tr>
<tr>
<td>Urethan, hormone stimulation</td>
<td>99/248</td>
<td>39.9</td>
</tr>
</tbody>
</table>

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development in one outgrowth did not significantly influence tumor development in the contralateral outgrowth; thus in statistical calculations, each outgrowth was treated independently.

Hormone stimulation enhanced the effects of DMBA. D1 outgrowths transplanted into hormone-stimulated, DMBA-treated BALB/c mice produced 92% tumors (22/24) with 50% tumor endpoint reached at 168 days (group 6). An analysis of the tumor distribution of hormone-stimulated, DMBA-treated outgrowths revealed that most of the tumors were produced within 3 weeks, and though hormone stimulation delayed the initial time of tumor formation, it narrowed the range of tumor appearance.

Effect of Urethan on Tumor-Producing Capabilities of D1 Outgrowths

The data are summarized in table 2. D1 outgrowths exposed to urethan alone produced 40.7% tumors (99/243) with a mean latent period of 254 days, whereas outgrowths exposed to urethan and hormone stimulation produced 39.9% tumors (99/248) with a mean latent period of 248 days. These results were not statistically different (P>0.05).

Since the results indicated hormone stimulation had no significant effect on urethan-induced tumorigenesis, the tumor incidences of the groups of mice treated with urethan at different times were calculated by combination of the number of mice exposed to urethan and urethan plus hormone stimulation for any particular group. The data are presented in text-figure 1. D1 outgrowths exposed to 20 mg urethan (group VIII) produced 20% tumors (28/130), outgrowths exposed to 40 mg (groups VI and VII) or 60 mg (groups IV and V) urethan produced 37% tumors (67/179, 26/67, respectively), outgrowths exposed to 80 mg urethan (group III) produced 68% tumors (23/34), outgrowths exposed to 100 mg urethan (group II) produced 73% tumors (40/55), and outgrowths exposed to 200 mg urethan (group I) produced 78% tumors (73/93). Thus the tumor incidence increased with increasing doses of urethan. However, the time of urethan administration also determined tumor incidence. Outgrowths exposed to 20 mg urethan each at 3 and 5 weeks or 4 and 5 weeks (group VI) produced 56% tumors (40/71), whereas outgrowths exposed to 20 mg urethan each at 5 and 9, 5 and 7, or 7 and 9 weeks (group VII) produced only 25% tumors (27/108). This effect was seen with a total of 60 mg urethan, whereas outgrowths exposed to 20 mg urethan each at 5 and 9, 5 and 7, or 7 and 9 weeks (group VII) produced only 25% tumors (27/108). This effect was seen with a total of 60 mg urethan, whereas outgrowths exposed to 20 mg urethan each at 3, 4, and 5 weeks (group IV) produced 53% tumors (19/37), and outgrowths exposed to 20 mg urethan each at 7, 8, and 9 weeks (group V) produced only 21% tumors (7/30). These results clearly indicate that D1 outgrowths treated with urethan soon after transplantation produced more tumors than outgrowths treated later.

The rate of tumor formation was greater in outgrowths exposed to 200 mg urethan than in those exposed to 80 or 100 mg urethan. D1 outgrowths exposed to 80 and 100 mg reached the 50% tumor endpoint at 294 days and 288 days, respectively, whereas outgrowths exposed to 200 mg reached the 50% tumor endpoint at 224 days.
Effect of Irradiation on Tumor-Producing Capabilities of D1 Outgrowths

The results are shown in table 3. D1 outgrowths in unirradiated, unstimulated mice (group 1) failed to produce tumors (0/16) by 338 days. D1 outgrowths in unirradiated, hormone-stimulated mice (group 2) produced 11% tumors (2/18) with a mean latent period of 354 days. D1 outgrowths in unstimulated mice, irradiated 7 weeks after transplantation (group 3), produced 24% tumors (8/33) with a mean latent period of 234 days, whereas outgrowths in hormone-stimulated mice, irradiated 7 weeks after transplantation (group 4), produced only 9% tumors (3/34) with a mean latent period of 217 days.

D1 outgrowths in unstimulated mice, irradiated 16 weeks after transplantation (group 5), produced 22% tumors (8/36) with a mean latent period of 274 days. Outgrowths in hormone-stimulated mice, irradiated 16 weeks after transplantation (group 6), produced only 8% tumors (3/36) with a mean latent period of 343 days. The results reported herein indicate that γ-irradiation increased the tumor-producing capabilities of D1 outgrowths; however, hormone stimulation inhibited the effects of γ-irradiation.

DISCUSSION

The data reported herein provide additional evidence that the tumor potential of preneoplastic nodule outgrowth cell populations can be increased by exposure to several different chemical carcinogens and γ-irradiation (3). DMBA, urethan, and γ-irradiation increased the tumor potential of D1 outgrowths from 4–68, 83, and 23%, respectively. The poor tumorigenic response from irradiation might be attributed to the permanent atrophy of primordial and graaffian follicles induced by irradiation and gradual loss of corpora lutea (14). The histological changes in urethan and DMBA-treated adult BALB/c mouse ovaries are not well documented, though DMBA does induce granulosa cell tumors in BALB/c mice (15).

The effect of various dosages of the various oncogenic agents used were measured on D1 nodule outgrowths. DMBA and urethan showed a definite correlation between dose and tumor incidence. D1 outgrowths exposed to 1.0 mg DMBA produced as many tumors as outgrowths exposed to 1.5 mg DMBA; however, 0.5 mg DMBA was definitely not as effective as the 2 higher doses (table 1).

Urethan was tested over a larger dose range, 20–200 mg. Outgrowths exposed to 200 mg urethan produced a similar high tumor incidence but a shorter tumor latent period than outgrowths exposed to 100 and 80 mg. Outgrowths exposed to 40 and 60 mg were equally effective, but produced fewer tumors than the higher doses. Outgrowths exposed to 20 mg produced only 20% tumors.

The time of administration also determined the response of outgrowths to the carcinogens. This factor was clearly shown in the urethan experiments (text-fig.1). D1 outgrowths exposed to 40–60 mg of urethan between 3 and 5 weeks after transplantation had a much higher incidence of tumors than D1 outgrowths exposed to urethan at later periods. This correlates with the finding that D1 outgrowths exposed to 1.0 mg MCA between 3 and 5 weeks after transplantation produced a higher incidence of tumors than outgrowths exposed to MCA between 9 and 12 weeks after transplantation (11).

There are two alternative explanations which might account for the different responses of outgrowths to carcinogens administrated at different times. Either the nodule outgrowths were more responsive to carcinogens shortly after transplantation, or the metabolism of the carcinogen varied with the age of the host. There is considerable information regarding the metabolism of urethan in infant and adult mice. This area was recently reviewed by Mirvish (16). Although there is a difference in urethan metabolism between infant and adult mice (17, 18), the adult rate of elimination occurs in mice 3 weeks before the earliest time urethan was administered to mice in the experiments reported herein. In previous experiments, any nonspecific effects of host age that might influence the response of the transplanted outgrowths to MCA were controlled, and no such effects were evident (11).

Apparently, the first explanation is the correct one; i.e., the nature of the cell population exposed to the carcinogen determined the response of the outgrowths. Several authors believe that actively dividing cells are more susceptible to carcinogens.
than nondividing cells. Shinozuka and Ritchie (9) demonstrated that the yield of papillomas induced by a single application of DMBA or urethan followed by repeated applications of croton oil was increased when croton oil also was given 24 hours before, but not 72 hours before, the administration of the carcinogen. They suggested that the carcinogen was exerting its influence on epidermal cells synthesizing DNA.

An analogous situation occurs in urethan-induced liver cancer. Lane et al. (5) suggested that the increased rate of proliferation of liver cells after partial hepatectomy enhanced the susceptibility of the liver to the carcinogenic action of urethan. Similarly, Hollander and Bentvelzen (6) concluded that rapid liver proliferation after partial hepatectomy enhanced urethan carcinogenesis in the mouse liver. Sachs and co-workers have also stressed the importance of cell division in in vitro chemical and irradiation carcinogenesis (10). Their results indicate that the expression of chemically transformed cells requires a process associated with cell division (19).

The in vivo and in vitro experiments discussed above are relevant to these results. It is known that the maximum rate of proliferation of D1 outgrowths occurs between 3 and 5 weeks after transplantation (11), and D1 outgrowths respond maximally to MCA at the time of rapid cell division (11). The experiments reported herein suggest that D1 outgrowths respond maximally to urethan at the time of rapid cell division. In addition, the lesser response of the outgrowths treated after 5 weeks could be overcome by increasing the dose of urethan (text-fig. 1). Similar results were found by Vesselinovitch and Mihailovitch (20), who found that the leukemogenic response of 7-day-old mice could be increased to that of newborn mice by increasing the dose of urethan.

D1 outgrowths exposed to irradiation at 7 weeks (group 3) and 16 weeks (group 5) after transplantation did not show different tumor responses (table 3). The percentage of actively dividing cells is probably not significantly different between the 2 groups (11, 21); thus a differential effect of irradiation was not evident.

The number of cells per se in a nodule cell population was not a contributing factor in determination of the tumor response to any particular carcinogen. D1 outgrowths exposed to urethan at 7 and 9 weeks had roughly 75-100% more cells than outgrowths exposed to urethan at 3 and 5 weeks (17). Similarly, D1 outgrowths exposed to irradiation at 16 weeks (100% of the fat pad filled with outgrowth) had 50% more cells than outgrowths exposed to irradiation at 7 weeks (11).

Chemical carcinogenesis of the mammary gland in BALB/c mice depends on hormonal stimulation of the gland. Biancifiori et al. (15, 22) showed that pseudopregnancy increased to 44% the incidence of mammary tumors in MCA-treated BALB/c mice, while virgin BALB/c mice treated with MCA produced only 11% tumors. Haran-Ghera (23) induced tumors in LAF mice treated with urethan or MCA; however, the concomitant presence of hormone stimulation by pituitary isografts was necessary for a high incidence of mammary tumors.

Faulkin (24) reported the induction of hyperplastic alveolar nodules in BALB/c mice by MCA; however, prolonged hormone stimulation was necessary for nodule development. He has recently shown the same dependency on hormone stimulation for the early appearance of nodules after treatment with DMBA, urethan, or X irradiation (Faulkin, personal communication).

The results reported here indicate that prolonged hormone stimulation is not necessary for the formation of mammary tumors from carcinogen-treated preneoplastic nodule cell populations. Hormone stimulation exerts a synergistic effect on DMBA-induced D1 tumorigenesis as it does on MCA-induced D1 tumorigenesis (2). However, hormone stimulation had no effect on urethan-induced D1 tumorigenesis and inhibited irradiation-induced D1 tumorigenesis.

The effectiveness of chemical carcinogens without prolonged hormone stimulation for the induction of the neoplastic transformations compared to the nodule transformation might be due to several conditions:

1) The effects seen may be quantitative; i.e., a fat pad containing nodule outgrowths contains more parenchyma cells than a fat pad containing normal ductal cells. However, the previously discussed experiments indicated cell number per se was not an important determining factor.

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2) Hormone stimulation, as in pregnancy, leads to an increase in the proliferating cell population, which is more susceptible to chemical or physical carcinogens. The previously discussed experiments stress the importance of cell division for chemical carcinogenesis; thus this factor could be important for noduligenesis. However, the critical experiment of comparison of mitotic rate in a normal ductal cell population undergoing alveolar differentiation to its susceptibility to chemical carcinogenesis has not been done.

3) Finally, continuous hormone stimulation leads to the development of alveoli which may be the cells at risk for chemical carcinogenesis. If alveoli are the cells at risk, it would explain the importance of hormone stimulation of the mammary gland for noduligenesis, and might explain the response of nodule outgrowth lines to chemical carcinogens without prolonged hormonal stimulation, since nodule outgrowth lines retain their alveolar morphology in the hormonal milieu of a nonpregnant mouse.

Apparently, carcinogen-induced neoplastic transformation is related both to the presence of alveolar cells and to the extent of cell division in the alveolar cell population.

REFERENCES


(21) Banerjee MR: Hormonal control of DNA synthesis:

