Interaction of Dimethylbenzanthracene and Diethylstilbestrol on Mammary Adenocarcinoma Formation in Female ACI Rats

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ABSTRACT

It has been reported that X-irradiation and diethylstilbestrol (DES) act synergistically on mammary adenocarcinoma formation in female ACI rats. The physical carcinogen, X-irradiation, was replaced by a chemical carcinogen, dimethylbenzanthracene (DMBA), and their interaction was studied in this system. Thirty-three female ACI rats were given 13.3 mg of DMBA per 100 grams of body weight. A total of 10 mammary adenocarcinomas were found, 8 in rats with a single mammary adenocarcinoma and 2 in a single rat, over a 266-day study period. Twenty-nine rats were implanted with a cholesterol pellet containing 5 mg of DES, and a total of 47 mammary adenocarcinomas were found, 5 in rats with a single mammary adenocarcinoma and 42 in 5 rats with 2 or more mammary adenocarcinomas. Twenty-four rats were given a combined treatment of both compounds, DES 2 days before DMBA, and a total of 126 mammary adenocarcinomas were found, 2 in rats with a single mammary adenocarcinoma and 124 in 18 rats with 2 or more mammary adenocarcinomas. The interaction between DMBA and DES was interpreted to be synergistic in regard to the proportion of rats with one or more mammary adenocarcinomas, the proportion of rats with two or more mammary adenocarcinomas, and the median times of appearance of both first and second mammary adenocarcinomas. These interactions between DMBA and DES resemble the previously reported synergistic interactions between radiation and DES on mammary adenocarcinoma formation in female ACI rats.

INTRODUCTION

Certain chemicals (5) and ionizing radiation (19) are known to be carcinogenic agents for the mammary gland of rats. The mammary neoplasms found in rats after either chemical carcinogen treatment or radiation appear to be of similar histopathological types and, depending upon the dose of the carcinogenic agents, appear to show similar latent periods between application of the carcinogens and appearance of the mammary neoplasms (17). In the few instances where a chemical carcinogen and ionizing radiation have been studied in combination, these 2 agents appear to show an additive effect on rat mammary carcinogenesis (11, 16, 21). These results suggest that chemical and physical carcinogens may be, in a general sense, interchangeable with regard to their carcinogenic effect on rat mammary gland tissue. It has been demonstrated recently that ionizing radiation and DES act synergistically on adenocarcinoma formation in female ACI rats (15, 20). It thus seemed of interest to replace ionizing radiation with a chemical carcinogen and see if the synergistic interaction with DES would be maintained. Accordingly, the well-studied chemical carcinogen for the rat mammary gland, DMBA, was given to female ACI rats, with and without DES administration, using the same DES treatment (20) that had shown a synergistic interaction with ionizing radiation.

MATERIALS AND METHODS

Female ACI rats were purchased from Microbiological Associates, Bethesda, Md., when they were 28 to 35 days old. Rats were maintained 5/cage in plastic cages on hardwood-chip bedding (Ab-sorb-dr) with Enviro-Guard filter bonnets; both were purchased from Lab Products Inc., Garfield, N. J. The rats were given commercial rat chow and water ad libitum under conditions of 12 hr (7 a.m. to 7 p.m.) of fluorescent light in an animal room maintained at 21 to 23°. Four groups of rats were prepared. One group received a compressed pellet of 25% DES and 75% cholesterol, containing 5 mg of DES, implanted s.c. when the rats were an average of 122 days of age. A second group of rats received DMBA in sesame oil (13.3 mg/ml) by stomach tube at the rate of 13.3 mg of DMBA per 100 g of body weight 2 days later. A third group received the combined treatment of both DES and DMBA, while the fourth group received neither treatment.

Each rat was identified by a numbered ear tag, and each mammary tumor, as it was located by palpation once per week, was recorded as to anatomical location using the nipples as reference points. Mammary tumors were removed under ether anesthesia at a size of approximately 1 cm. If a second tumor was found at the site of a previously removed tumor, it was not recorded as a second tumor unless the site had been palpated as a tumor-free site for 8 weeks. Two hundred sixty-six days after the day of DES pellet implantation, all rats were killed and inspected for gross pathology. All mammary tumors were sectioned, stained with hematoxylin and eosin, and given a pathological classification according to criteria consistent with those of Young and Hallowes (25). Pituitary glands were recorded as pituitary tumors if they were hemorrhagic and fragile and/or if they exceeded 40 mg. All data were reported in terms of days after the day of DES administration.

To determine whether or not DES and DMBA acted synergistically as tumor producers, the following statistical approach was used. We let \( p_1 \) = probability that DES causes a tumor, \( p_2 \) = probability that DMBA causes a tumor, and \( r \) = probability that a tumor is due to the combined effect of DES and DMBA.

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2 To whom requests for reprints should be addressed.

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that the combination of DES and DMBA causes a tumor. Under a model of independent action of the 2 agents, a rat in the combined treatment group would avoid a tumor by avoiding DES- and DMBA-caused tumors; its probability of avoiding a tumor, $1 - p$, would then be equal to the product of the probabilities, $1 - p_1$ and $1 - p_2$, of avoiding DES- and DMBA-caused tumors, by the product rule for probabilities of independent events. This means that the probability that a rat in the combined treatment group would develop a tumor is $p = p_1 + p_2 - p_1 p_2$, if the agents act independently. If, however, the agents act synergistically, then $p$ may be much larger than $p_1 + p_2 - p_1 p_2$. Maximum likelihood estimates for all 3 probabilities [those values of the probabilities which make the data appear most likely (14)] were found under the independence model (subject to the restriction $p = p_1 + p_2 - p_1 p_2$). These estimates were then used to calculate the expected numbers of tumors in the 6 experimental cells (Table 3). To compare these estimates with the observed numbers, a $\chi^2$ goodness of fit test was performed by summing $(O - E)^2/E$, in which $O$ is the observed number and $E$ is the expected number, over all 6 cells; a likelihood ratio test was also done comparing the model of independence to the synergism model. These procedures were used to check for synergism in the production of both first and second tumors.

RESULTS

DMBA and DES treatments alone tended to reduce survival, but the combination of both treatments did not further reduce survival (Table 1). Pituitary tumors were found only in DES-treated rats, but the incidence of rats with pituitary tumors was not different when both DMBA and DES were given as compared to DES given alone (Table 1).

All mammary neoplasms were classified as mammary adenocarcinomas, and these were found only in rats treated with DMBA, DES, or both. The median times (6) to both first and second tumors are much shorter for the combined DES and DMBA treatment group than for the other 2 treatment groups (Table 2). Log rank tests (8, 12, 13) for difference among the 3 groups in distribution across time of both first and second tumors were significant, with $p \ll 0.001$ (Table 2). In both cases, log rank tests of the differences between the combined DES-DMBA treatment group and the 2 single-treatment groups combined and tests of the difference between the 2 single-treatment groups only indicate that the major difference among the 3 groups is the difference between single and combined treatment (Table 2).

The number of mammary adenocarcinomas per rat ranged from 0 to 20 (Table 3). To examine whether the development of second and subsequent mammary adenocarcinomas was an independent process, rats with no previous tumor were tested against those with a previous tumor for difference in time to next tumor. A version of the log rank test was used which reclassifies rats into the previous tumor category as they develop tumors (2, 3, 9). The 2 groups, rats developing a first tumor versus rats developing subsequent tumors, were judged significantly different: $\chi^2 = 52.576$ ($p \ll 0.001$).

The proportion of rats with one or more mammary adenocarcinomas was larger in the combined DES and DMBA treatment group than even the sum of the proportion of rats with mammary adenocarcinomas in the DES- and DMBA-only groups (Table 1). Both tests of whether this was significantly larger than the number of rats with tumors in the combined group that would be expected if DES and DMBA acted independently confirmed the synergism, with $p$ approximately equal to 0.01 (Table 4).

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>No. of rats with mammary adenocarcinomas</th>
<th>% of starting no. of rats with specified no. of mammary adenocarcinomas</th>
<th>Total no. of mammary adenocarcinomas</th>
<th>Mean no. of mammary adenocarcinomas/ rat</th>
</tr>
</thead>
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<tr>
<td>None</td>
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<td>258 ± 34</td>
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<td>DMBA</td>
<td>33</td>
<td>233 ± 49</td>
<td>20</td>
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<tr>
<td>DES</td>
<td>29</td>
<td>224 ± 41</td>
<td>17</td>
<td>17</td>
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<td>224 ± 56</td>
<td>17</td>
<td>26</td>
<td>52</td>
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* Mean ± S.D.


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Table 3

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<tr>
<td>DMBA</td>
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</table>

Table 4

<table>
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<tr>
<th>No. of rats with specified number of mammary adenocarcinomas</th>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>DMBA</td>
</tr>
<tr>
<td>DES</td>
</tr>
<tr>
<td>Both</td>
</tr>
</tbody>
</table>

Results and analysis of rats with one or more mammary adenocarcinomas or rats with 2 or more mammary adenocarcinomas

For 1 or more tumors: goodness of fit, $\chi^2 = \sum (O - E)^2 / E = 5.97 (p = 0.015)$; likelihood ratio, $\chi^2 = 6.52 (p = 0.011)$. For multiple tumors: goodness of fit, $\chi^2 = \sum (O - E)^2 / E = 16.32 (p < 0.001)$; likelihood ratio, $\chi^2 = 17.40 (p < 0.001)$.

In all cases, small $p$ values can be interpreted as strong evidence that synergism exists.

Similarly, both tests found the proportion of rats with 2 or more mammary adenocarcinomas in the combined DMBA- and DES-treated group to be significantly larger than would be expected if DES and DMBA acted independently, with $p$ approximately equal to 0.001 (Table 4).

DISCUSSION

An exact comparison of the interaction of radiation and DES to the interaction of DMBA and DES can best be made if the level of effect of radiation alone is approximately the same as the level of effect of DMBA alone. Further, the level of effect of each agent given alone must be relatively small if additive or synergistic interactions are to be detected. Although these conditions were not exactly fulfilled in the present experiment, the interaction of radiation and DES and the interaction of DMBA and DES on mammary adenocarcinoma formation in ACI female rats appear to resemble each other in some respects.

We have chosen to analyze the measure of rats with a specific number of mammary adenocarcinomas (none, 1, or 2 or more) rather than with the average number of adenocarcinomas per rat. This was done because, in agreement with the report of Clifton and Crowley (2), it was found that multiple mammary adenocarcinomas do not occur independently; that is, a first mammary adenocarcinoma increases the probability of the detection of another mammary adenocarcinoma. This does not necessarily mean that the presence of a mammary adenocarcinoma predisposes the animal to the development of an additional mammary adenocarcinoma. Rather, it may mean that there are inherent differences among rats as to their capacity to develop multiple mammary adenocarcinomas.

Therefore, some rats may have more than the average number of mammary adenocarcinomas because of their inherent capacity to develop multiple adenocarcinomas, while other rats may have fewer than the average number because of their relative lack of capacity to develop multiple adenocarcinomas. It is not likely that differences in mammary adenocarcinoma multiplicity can be attributed to differences in the rate of release of DES from the DES pellet. The rate of DES release has been measured and has been found to be remarkably consistent from pellet to pellet when the pellets were of the same weight and composition (23).

The proportion of rats with either one or more or 2 or more mammary adenocarcinomas was greater in the group that received the combined DMBA and DES treatment than could be accounted for by independent action of the 2 agents. Also, the median times to both first and second mammary adenocarcinomas were less for the combined DMBA and DES treatment group than for the other treatment groups. Similar findings of synergism have been reported previously for X-irradiation and DES (15) and for neutron irradiation and DES (20). Thus, the interaction of DMBA and DES and the interaction of irradiation and DES on mammary adenocarcinoma formation in female ACI rats appears to be approximately the same.

The pituitary tumors found in DES-treated ACI rats in the present study were similar in size, appearance, and time of occurrence to the pituitary tumors found in the previous study (23), in which the same dose of DES and strain of rat were used. In the previous study, the plasma prolactin levels were measured and found to be greatly elevated in those rats that received DES. Thus, we feel justified in assuming that the pituitary tumors found in the present study were releasing large amounts of prolactin. It has been suggested (20) that prolactin plays a significant role in the synergistic interaction of radiation and DES, and it seems likely that prolactin also plays a significant role in the synergistic interaction of DMBA and DES on mammary adenocarcinoma formation in female ACI rats.

It is, of course, too early to state with assurance that chemical carcinogens for the rat mammary gland and radiation can be thought of as being interchangeable carcinogenic agents. However, the list of similarities of these 2 carcinogenic agents for the rat mammary gland is impressive. The similarities include: the histopathological types of mammary neoplasms (25); the latent periods for the development of the mammary neoplasms (18); the strain-related susceptibility (17); the scopal mechanism of induction (1, 22); the enhancement of induction by pituitary hormones (4, 7, 24); an additive interaction between the 2 agents themselves (16, 21); and, here reported, some similarities of interaction with DES on mammary adenocarcinoma formation in female ACI rats.

"Scopal" means local effects as opposed to remote effects. The words scopal and abscopal were first used by Mole (10) to distinguish between radiation effects within an irradiated volume (scopal), and radiation effects outside the irradiated volume (abscopal). New words were needed because direct and indirect were already in use in a chemical sense of radiation damage either within a molecule of interest (direct) or within a molecule produced by the irradiation products of water (indirect). We find the terms scopal and abscopal useful in chemical carcinogenesis as well. For instance, DMBA administered by stomach tube produces mammary gland neoplasia. This fact alone does not tell one whether or not the chemical carcinogen
must reach the mammary gland to produce tumors in the mammary gland. However, the demonstration (22) that DMBA placed directly on a portion of the mammary gland produced mammary neoplasia in the DMBA-treated portion, but not in the non-DMBA-treated portion, clearly demonstrates that the mechanism is scopal. Any DMBA-produced events following systemic DMBA administration outside of the mammary gland are not required for DMBA-induced mammary gland carcinogenesis; thus, the mechanism is not abscopal.

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REFERENCES

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