Apparent Rat Strain-related Sensitivity to Phorbol Promotion of Mammary Carcinogenesis

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ABSTRACT

It has been reported that twice-weekly i.p. injections of 4 mg phorbol for 10 weeks, after a single feeding of 6 mg dimethylbenz(a)anthracene (DMBA) in female Wistar rats, led to a significant augmentation of mammary adenocarcinoma incidence and of lymphatic leukemia incidence as compared to 6 mg DMBA alone. In an experiment reported here, in female Sprague-Dawley rats, using the same doses of DMBA and phorbol and the same injection schedule, phorbol given after DMBA did not augment mammary adenocarcinoma incidence or lymphatic leukemia incidence as compared to DMBA given alone. It thus appears that there is a strain-related sensitivity between Wistar and Sprague-Dawley rats with regard to the promoting activity of phorbol when phorbol treatment follows DMBA treatment, and mammary adenocarcinoma incidence and lymphatic leukemia incidence are studied. Further, in Sprague-Dawley rats, phorbol did not promote mammary fibroadenoma incidence in DMBA-treated rats, mammary adenocarcinoma incidence in procarbazine-treated rats, and mammary adenocarcinoma incidence or mammary fibroadenoma incidence in X-ray-treated rats. DMBA and procarbazine, with or without phorbol, tended to induce more mammary neoplasms in the anterior (thoracic) than in the posterior (abdominal) mammary glands. X-irradiation tended to induce mammary neoplasms in approximately equal numbers in the anterior and posterior mammary glands. It was suggested that regional differences in chemically induced mammary carcinogenesis were due to a difference in the transport and delivery of the chemical carcinogens to the regions rather than a difference in the amount of mammary gland tissue in the regions. An analysis of the numbers of Sprague-Dawley rats that developed either no mammary neoplasms, or only mammary adenocarcinomas, or only mammary fibroadenomas, or both mammary adenocarcinomas and mammary fibroadenomas in response to DMBA, procarbazine, and X-ray, suggested that the development of a mammary adenocarcinoma or the development of a mammary fibroadenoma are independent processes.

INTRODUCTION

Mammary carcinogenesis in the rat is relatively easy to induce by several different chemical carcinogens and by various types of ionizing radiation (7). The rat mammary carcinogenic response to either chemical or physical carcinogenic agents may be enhanced by various hormonal treatments (12) or special diets (4). However, in the sense of the initiation-promotion hypothesis of carcinogenesis, there were no reports of promotion of chemical or radiation mammary carcinogenesis in the rat, in which the promoting agent was a true, nonhormonal, promoting agent, until the publication of Armuth and Berenblum (2). These investigators reported the promotion of mammary carcinogenesis after DMBA3 in female Wistar rats by the i.p. injection of phorbol (phorbol is the unesterified parent alcohol of the cocarcinogenically active 12-O-tetradecanoylphorbol-13-acetate). Because of the great theoretical importance of the finding of Armuth and Berenblum, it was decided to see if the promotion of DMBA-induced mammary carcinogenesis, as observed by them, could be extended to another strain of rat, Sprague-Dawley, and extended in this strain of rat to the induction of mammary fibroadenomas and to an additional chemical carcinogen, procarbazine (5), and to ionizing radiation.

While the current experiment was in progress, Torgersen (10) reported that DMBA administered to female Sprague-Dawley rats induced more mammary adenocarcinomas in the anterior (thoracic) than in the posterior (abdominal) mammary glands. Since the protocol of the current experiment allowed a regional, anatomical analysis of the distribution of mammary neoplasia within individual rats, the various effects of phorbol, DMBA, procarbazine, and X-irradiation on the regional, anatomical distribution of mammary adenocarcinomas and mammary fibroadenomas were examined.

During the course of the experiment, it became apparent that of the rats that developed mammary neoplasia, some rats developed only mammary adenocarcinomas, some developed only mammary fibroadenomas, and some rats developed both mammary adenocarcinomas and fibroadenomas. These results were analyzed in an attempt to determine if the development of the 2 histologically different types of mammary neoplasms were independent processes or if the development of one type of mammary neoplasm was correlated with the development of the other type of mammary neoplasm in the same rat.

Since Armuth and Berenblum (2) also reported that phorbol promoted the leukemogenic action of DMBA, the thymus, liver, spleen, and lymph nodes of all rats were examined grossly, and leukocyte counts were performed on rats that received DMBA, DMBA and phorbol, phorbol, and nontreated controls.

MATERIALS AND METHODS

The protocol of Armuth and Berenblum (2) was followed generally except that female rats of the Sprague-Dawley strain were used in the current experiment rather than those of the Wistar strain. The Sprague-Dawley rats were purchased from Taconic Farms, Germantown, N. Y., and delivered to this laboratory on their 22nd day of age. They were maintained on

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3 The abbreviation used is: DMBA, 7,12-dimethylbenz(a)anthracene.
commercial rat chow and water ad libitum in metal cages with corn cob bedding, 5 rats/cage, under conditions of fluorescent light from 8 a.m. to 8 p.m. at 22 ± 2°C. Each rat was given a numbered ear tag so that individual records could be kept for each rat. The experiment was started when the rats were 60 days of age with the administration of DMBA, procarbazine, or X-radiation. The first phorbol injection was given 1 week later. Beginning 1 week later, each rat was palpated weekly for the presence of mammary tumors. When mammary tumors were found, they were recorded as to location, using the nipples as reference points, counting in days after the 60th day of age. Mammary neoplasms were removed under ether anesthesia at a size of approximately 2 cm, and all mammary neoplasms were sectioned, stained, and given a classification of either mammary adenocarcinoma or mammary fibroadenoma according to criteria consistent with those of Young and Hallowes (13). If a second mammary neoplasm, of the same pathological classification, was found at the site of a previously removed neoplasm, it was not recorded as a second neoplasm unless a 10-week period had elapsed between removal of the first neoplasm and detection of the subsequent neoplasm. All rats were killed 304 days after starting the experiment and examined for gross pathology, including a visual examination of lymph nodes, thymus, liver, and spleen. Leukocyte counts were done on rats that received DMBA, DMBA plus phorbol, phorbol, and control, nontreated rats.

DMBA, 3 mg in 1 ml of sesame oil, was given by stomach tube at the rate of 3 mg/100 g of body weight (to the nearest 0.1 ml and 10 g of body weight) to rats with an average weight of 199 g.

Procarbazine (procarbazine hydrochloride, a gift from Roche Laboratories, Division of Hoffman-LaRoche Inc., Nutley, N. J.), 16.6 mg/ml of water, was given by stomach tube at the rate of 16.6 mg/100 g of body weight (to the nearest 0.1 ml and 10 g of body weight) to rats with an average weight of 200 g.

Total-body X-irradiation, either 100 or 300 R, was delivered at a rate of approximately 35 R/min, 250 kVp, 30 ma, 0.5 mm Cu, 1.0 mm Al, and a target-skin distance of 100 cm; and dosimetry was done with a 100 R Victoreen chamber on the 60th day of age.

Phorbol was purchased from the same source as used by Armuth and Berenblum (Dr. Theodor Schuchardt GmbH and Co., Munich, Germany). Each phorbol-injected rat received a total dose of 80 mg of phorbol i.p. beginning 1 week after carcinogen application and continuing for 10 weeks of twice-per-week injections. Each individual phorbol injection contained 4 mg of phorbol in 0.5 ml of phosphosaline buffer (0.01 M phosphate, pH 7.6, plus 0.15 M NaCl). The phorbol solution was mixed using a 1-min pulse of low-energy ultrasonication immediately before use. After the phorbol injections were completed, a sample of the phorbol used in the present experiment was examined with UV spectra and thin-layer chromatography, and the phorbol used in the present experiment was shown to be authentic phorbol.4

As a control for the phorbol injections, phosphosaline buffer was given in the same volume and on the same schedule as the phorbol injections, and these groups were designated phosphosaline treated.

Differences between incidence of rats with mammary neoplasia were evaluated for statistical significance by the χ² test (6), and the mean time of appearance of mammary neoplasia was evaluated by the t test (8). The independence of the appearance of mammary fibroadenomas and mammary adenocarcinomas was evaluated by the χ² test for independence (3).

RESULTS

The survival rate of all groups was excellent and was not different between groups (Table 1). For this reason, no corrections for intercurrent mortality were made.

Of the 236 carcinogen-treated rats, 92 developed no mammary neoplasms, 58 developed only mammary adenocarcinomas, 47 developed only mammary fibroadenomas, and 39 developed both mammary adenocarcinomas and fibroadenomas. Analysis of these data, as well as the data for and within each carcinogen-treated group, suggested that the development of mammary adenocarcinomas and mammary fibroadenomas appeared to be independent processes. Thus, we have chosen to analyze the following 3 measures of mammary carcinogenesis: rats with mammary neoplasia; rats with mammary adenocarcinomas with or without mammary fibroadenomas; and rats with fibroadenomas with or without mammary adenocarcinomas.

Phorbol treatment, after DMBA, procarbazine, or X-radiation, did not increase the mean number of mammary neoplasms, mammary adenocarcinomas, or mammary fibroadenomas per rat above the values for DMBA, procarbazine, or X-radiation, respectively (Table 1). No further analysis of the mean number of mammary neoplasms per rat was done.

DMBA treatment, with and without phosphosaline injection, was followed by an increased incidence of rats with mammary neoplasia, mammary adenocarcinomas, and mammary fibroadenomas as compared to nontreated controls (Table 1). Phorbol treatment after DMBA treatment did not modify any of these 3 measures of mammary neoplasia incidence, or the mean time of appearance of the neoplasms.

Procarbazine treatment, with or without phosphosaline injection, was followed by an increased incidence of rats with mammary neoplasia and mammary adenocarcinomas as compared to nontreated controls (Table 1). Phorbol treatment after procarbazine did not modify any measure of mammary neoplasia incidence or the mean time of appearance of the neoplasms.

X-irradiation, at a dose of 300 R with and without phosphosaline injection, was followed by increased incidences of rats with mammary neoplasia, mammary adenocarcinomas, and mammary fibroadenomas as compared to nontreated controls (Table 1). Phorbol treatment after 300 R did not modify any measure of mammary neoplasia incidence or the mean time of appearance of the neoplasms.

X-irradiation, at a dose of 300 R with and without phosphosaline injection, was followed by increased incidences of rats with mammary neoplasia, mammary adenocarcinomas, and mammary fibroadenomas as compared to nontreated controls (Table 1). Phorbol treatment after 300 R did not modify any measure of mammary neoplasia incidence or the mean time of appearance of the neoplasms.

Phorbol, by itself, had no influence on any measure of mammary neoplasia incidence, and phosphosaline injections were also without effect (Table 1).

Mammary neoplasia tended to occur more often in the anterior half than the posterior half of the rats given DMBA or

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4 The authors wish to thank Dr. Walter Trost for these examinations.
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discerning, but occurred randomly in the irradiated rats. The actual anterior-posterior number of mammary neoplasms was: DMBA: anterior, 81 ; posterior, 44. Procarbazine: anterior, 42; posterior, 25. X-ray: anterior, 63; posterior, 57.

No indication of leukemia, as judged by the enlargement of the lymph nodes, thymus, spleen, and liver, was found in any animal. The leukocyte count was not different among groups that received DMBA, DMBA and phorbol, phorbol, or no treatment.

**DISCUSSION**

It seems clear that the promoting effect of phorbol on DMBA-induced mammary adenocarcinoma formation, as observed by Armuth and Berenblum (2) in female Wistar rats, was not found in female Sprague-Dawley rats in the present experiment. Also, the promoting effect of phorbol on DMBA-induced leukemia reported by Armuth and Berenblum in Wistar rats was not found in the present experiment with Sprague-Dawley rats. The longer follow-up period in the present experiment was adequate to disclose that there was no promoting effect of phorbol on mammary fibroadenoma formation in Sprague-Dawley rats. The experimental protocol of the 2 experiments was almost the same with the obvious exception of rat strain difference. The most probable, and the most obvious, explanation for the positive promoting effect of phorbol on DMBA-induced mammary adenocarcinoma formation in Sprague-Dawley rats, noted in the current experiment, has to do with inherent differences in the 2 strains themselves. Support for the strain difference explanation has been provided by Armuth (1), since he reported that phorbol induced leukemia in only 1 of 7 strains of mice, accelerated the appearance of reticulum cell sarcomas in another of the 7 strains, and, combined with thymectomy, induced leukemia in yet another strain. Other aspects of mammary carcinogenesis (7) and leukemogenesis (9) in the rat are known to exhibit strain-related differences; therefore, it should not be surprising if promotion also exhibits strain-related sensitivity. The biological and biochemical mechanisms that would allow phorbol to exhibit promoting activity in Wistar rats but not in Sprague-Dawley rats are poorly understood but might involve whether or not a particular strain has the capacity to esterify phorbol, thus transforming the weakly active phorbol to the strongly active esterified phorbol.

The failure of phorbol to promote procarbazine-induced or X-ray-induced mammary carcinogenesis in Sprague-Dawley rats cannot be taken as proof that phorbol has no promoting activity for these 2 agents because of the lack of a "positive control" (11). The promoting activity of phorbol on DMBA-induced mammary carcinogenesis was demonstrated (2) in Wistar rats. In the absence of demonstrated phorbol-promoting activity on DMBA-induced mammary carcinogenesis in Sprague-Dawley rats, it is not clear whether the lack of phorbol-promoting activity in regard to procarbazine and X-irradiation is due to an intrinsic lack of promoting activity of phorbol itself or due to the incapability of Sprague-Dawley rats to exhibit the phenomenon of promotion with this agent.

In the present experiment, the analysis of mammary carcinogenesis has been divided into 3 separate analyses: mammary adenocarcinoma formation; mammary fibroadenoma formation; and mammary neoplasia formation of either type. This was done in part because the 2 types of mammary neoplasms display different latent periods as well as different histological characteristics (13) and because a statistical test for independence suggested that the 2 types of mammary neoplasms oc-

### Table 1

**Survival rate and incidence of mammary neoplasia**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Rats with mammary neoplasia</th>
<th>Rats with mammary adenocarcinomas</th>
<th>Rats with mammary fibroadenomas</th>
<th>All mammary neoplasmas</th>
<th>All mammary fibroadenomas</th>
<th>All mammary adenocarcinomas</th>
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<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
<td>Days of study</td>
<td>Total %</td>
<td>Total %</td>
<td>Total %</td>
<td>Total %</td>
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<tr>
<td>DMBA</td>
<td>20</td>
<td>18</td>
<td>292 ± 46a</td>
<td>15±5</td>
<td>75±10</td>
<td>50±12</td>
<td>60±4</td>
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<tr>
<td>DMBA + phosphosaline</td>
<td>20</td>
<td>19</td>
<td>292 ± 53</td>
<td>15±5</td>
<td>75±10</td>
<td>50±10</td>
<td>50±4</td>
</tr>
<tr>
<td>Procarbazine</td>
<td>18</td>
<td>18</td>
<td>304</td>
<td>12±5</td>
<td>67±11</td>
<td>61±3</td>
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<td>20</td>
<td>16</td>
<td>278 ± 62</td>
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<td>18</td>
<td>301 ± 11</td>
<td>10±6</td>
<td>50±8</td>
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<td>15±1</td>
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<td>19</td>
<td>19</td>
<td>304</td>
<td>12±5</td>
<td>95±11</td>
<td>58±13</td>
<td>68±4</td>
</tr>
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<td>19</td>
<td>303 ± 2</td>
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<td>65±8</td>
<td>40±11</td>
<td>55±4</td>
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<td>18</td>
<td>291 ± 54</td>
<td>14±4</td>
<td>70±10</td>
<td>50±7</td>
<td>35±4</td>
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<tr>
<td>100 R</td>
<td>20</td>
<td>19</td>
<td>303 ± 6</td>
<td>6±4</td>
<td>40±4</td>
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<tr>
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<td>19</td>
<td>303 ± 6</td>
<td>5±2</td>
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<tr>
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<td>304</td>
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</tr>
<tr>
<td>Phorbol</td>
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<td>304</td>
<td>1±5</td>
<td>5±0</td>
<td>1±5</td>
<td>1±0.1</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

* Different from no treatment: $\chi^2$, $p < 0.01$.

* Different from phosphosaline treatment: $\chi^2$, $p < 0.01$.

* Different from phorbol treatment: $\chi^2$, $p < 0.01$.

* Different from phorbol treatment: $\chi^2$, $p < 0.05$.

* Different from no treatment: $\chi^2$, $p < 0.05$.

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curred independently. Presumably, this means, in the Sprague-Dawley rats at least, that there are some rats which are at risk in response to DMBA, procarbazine, and X-irradiation for the development of mammary adenocarcinomas and some other rats which are at risk for the development of mammary fibroadenomas, and some other rats which are, presumably by chance, at risk for developing both types of mammary neoplasms. It would seem profitable to profile and compare, in an endocrinological and an immunological sense, the rats that develop only mammary adenocarcinomas to the rats that develop only mammary fibroadenomas in order to gain additional insight of the factors that modify the mammary carcinogenic response to chemical carcinogens and to radiation.

It has been reported (10) that more mammary neoplasms appear in the anterior than in the posterior mammary glands of Sprague-Dawley rats following DMBA administered by the i.v. route. The general tendency in the present experiment was to find about twice as many mammary adenocarcinomas and mammary fibroadenomas in the anterior than in the posterior mammary glands following DMBA given p.o. Procarbazine also had the tendency to produce more mammary adenocarcinomas in the anterior than in the posterior mammary glands but too few mammary fibroadenomas for analysis. In contrast, X-irradiation tended to produce approximately equal numbers of both types of mammary neoplasms in anterior and posterior mammary glands. It seems reasonable to assume that the magnitude of a carcinogenic response depends directly upon both the size of the carcinogenic stimulus and the amount of tissue undergoing interaction with the carcinogenic stimulus. Since total-body irradiation with X-rays must deliver the same amount of carcinogenic stimulus to both the anterior and the posterior mammary glands and since the carcinogenic response of the anterior and posterior mammary glands to X-irradiation was approximately equal, it is possible to conclude that the relative amount of mammary tissue in the anterior and posterior portions of the rats must be approximately equal. If the equality of the carcinogenic response in the anterior and posterior mammary glands in response to X-irradiation can be taken to indicate an equal amount of mammary tissue in both regions, then the larger response to DMBA and to procarbazine of the anterior mammary glands must be taken to mean that there was a larger carcinogenic interaction with the chemical carcinogens in the anterior than in the posterior mammary glands. This conclusion implies that a larger carcinogenic stimulus from DMBA p.o. and procarbazine p.o. was delivered to the anterior than to the posterior mammary glands, perhaps because of different clearance rates or other vascular differences of the 2 areas. It would appear relatively easy to compare the distribution of chemical carcinogens to the anterior than to the posterior mammary glands by use of isotope-labeled carcinogens to verify the suggestion that regional differences in carcinogenesis in response to chemical carcinogens are due to a difference in delivery of the carcinogens to the regions rather than a difference in amounts of mammary tissue in the regions.

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REFERENCES

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