Promotion of Mammary Carcinogenesis and Leukemogenic Action by Phorbol in Virgin Female Wistar Rats

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SUMMARY

Twice-weekly i.p. injections of 4 mg phorbol for 10 weeks, after a single feeding of 6 mg 7,12-dimethylbenz(a)anthracene (DMBA), in virgin female Wistar rats led to a significant augmentation of mammary tumor induction (78%) as compared to that resulting from 6 mg DMBA alone (21%). A slight effect, of doubtful significance, was observed when such phorbol treatment was given after feeding 2 mg DMBA and there was no effect after 0.25 mg DMBA. No mammary tumors developed in the rats receiving phorbol alone for 10 weeks, and 18% of the untreated controls (after a very long latent period) developed tumors.

Continuation of the phorbol treatment in the phorbol control group for a total period of 9 months resulted in a very high incidence of lymphatic leukemia (94%, as compared to 2% in the untreated controls). However, the latent period was more than twice as long as in 2 other groups of rats that received a single dose of DMBA p.o., followed by only 10 weeks of phorbol injections. The thymus was involved in every case, in contrast to the previous findings in SWR mice similarly treated with phorbol, in which the induced leukemia was predominantly nonthymic.

The results are discussed in relation to (a) the specific problem of promotion in mammary carcinogenesis, (b) the more general problem of systemic promoting action by phorbol, and (c) the possible meaning of the differences in pattern of leukemogenic response according to the species of animal used.

INTRODUCTION

Unlike 12-O-tetradecanoyl-phorbol-13-acetate, identified as one of the major constituents of croton oil responsible for its promoting action in mouse skin carcinogenesis (9), unesterified phorbol was found to be inactive in this respect (8, 9). However, when administered systemically, phorbol proved to be leukemogenic in SWR mice (3) and also to act as a promoter for liver and lung carcinogenesis in AKR mice (1).

This communication deals with further examples of promoting action and of leukemogenesis, this time in virgin female Wistar rats.

The initial purpose was to explore the possibility of demonstrating systemic promoting action by phorbol in relation to mammary carcinogenesis, using single feedings of DMBA as initiator, based on the previous findings of Shay et al. (19) and of Huggins et al. (10) that single feedings of large doses of carcinogenic hydrocarbons, acting alone, were effective in inducing mammary tumors in rats after relatively short latent periods. The intention was to use subeffective doses to serve as initiating stimulus, followed by prolonged treatment with phorbol as promoter.

After completion of the experiments with respect to mammary carcinogenesis, 2 of the control groups, those receiving phorbol alone and the untreated control group, were kept under further observation; and phorbol injections were continued in the former group in order to detect any excess of leukemia in the treated animals.

MATERIALS AND METHODS

Virgin female rats of the Wistar strain, raised in the Institute Breeding Center under specific-pathogen-free conditions, were used for this investigation, and the experiment started when the animals were 40 to 60 days old. They were kept in an air-conditioned room at 21–25°C, 5/large plastic cage, and fed Purina laboratory chow and tap water ad libitum.

The DMBA was purchased from Sigma Chemical Co., St. Louis, Mo., and the phorbol (the unesterified parent alcohol of the cocarcinogenically active 12-O-tetradecanoyl-phorbol-13-acetate) was from Dr. Theodor Schuchardt GmbH and Co., Munich, Germany.

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The 1st experiment included: Group A, 45 rats, kept as untreated controls; Group B, 35 rats, as phorbol controls, receiving twice-weekly i.p. injections of 2 ml of 0.2% phorbol in phosphate-buffered saline; Group C, 35 rats, as DMBA controls, receiving a single feeding by stomach tube of 0.25 mg DMBA in 1 ml sesame oil; Group D, 35 rats, for the experiment proper, receiving a single feeding of DMBA, followed 1 week later by twice-weekly injections of phorbol.

When it became evident that the dose of DMBA used (0.25 mg) was too low to act as initiator, a 2nd experiment was begun, including the following groups: Group E, 35 rats, receiving 2 mg DMBA alone; Group F, 34 rats, receiving 2 mg DMBA, followed by twice-weekly injections of phorbol; Group G, 39 rats, receiving 6 mg DMBA alone; Group H, 36
rats, receiving 6 mg DMBA, followed by twice-weekly injections of phorbol; Group I; 10 rats, receiving a single feeding of 15 mg DMBA.

The phorbol injections were continued for 10 weeks (cumulative dose per animal, 80 mg), except that in the phorbol control (Group B) the injections were continued for a total of 9 months, as a supplementary (3rd) experiment, concerned with the possibility that phorbol alone might prove to be leukemogenic.

The untreated rats (Group A) and those receiving phorbol alone (Group B), from the 1st experiment, also served as controls for the 2nd; while the additional small group of 10 rats (Group I), receiving 15 mg DMBA, was added to check the responsiveness of our rats to the agent when acting alone at higher dose levels.

Since no tumors developed in Groups C and D by 7 months after the beginning of treatment, these animals were killed, and the 2nd experiment was begun. All the animals were observed daily and examined more systematically once weekly. Those moribund or with palpable tumors or enlarged thymuses or spleens were killed and autopsied, and mammary tissues and tumors, thymuses, spleens, livers, and kidneys were fixed in Bouin's solution. Paraffin sections of the tissues were stained with hematoxylin and eosin for histological examination.

RESULTS

The results of the 2 experiments, with respect to mammary carcinogenesis, are combined in Table 1, showing (a) that 0.25 mg DMBA failed to induce mammary tumors, whether acting alone or followed by phorbol injections; (b) that the difference in incidence between those receiving 2 mg DMBA alone (8%) and those treated with 2 mg DMBA followed by phorbol (21%) did not seem significant; but (c) that the difference in incidence between 6 mg DMBA acting alone (21%) and followed by phorbol (78%) was highly significant (p < 0.001).

The untreated control rats naturally survived longer than most of the treated animals. The majority of the former are still alive after 20 months since birth, with a spontaneous mammary tumor incidence to date of 8 out of 43 (18%). [By comparison, incidences of 6.7% (18) and 8.3% (4) have been reported in the literature for spontaneous mammary tumors in Wistar rats, after approximately the same latent periods.]

Histologically, the mammary tumors in the present series were all well-differentiated adenocarcinomas, exhibiting a marked increase in the number of acini, mostly single-layered, although occasionally composed of 2 or 3 layers of cuboidal epithelial cells. Mitoses were common. Papilliform outgrowths into the surrounding stroma were also noted in some of the tumors.

Lymphatic leukemia developed in some of the experimental animals and in only 1 untreated control, as summarized in Table 2. The highest incidence was in the phorbol control group, in which the phorbol injections were continued for 9 months, with an incidence of 94%, compared to 2% in the untreated controls. The latent period of leukemia development was rather long (299 ± 22 days) from the start of treatment, in striking contrast to the relatively short average latent period (although with lower incidences) in Groups F and H (152 ± 21 and 127 ± 16 days, respectively), in which the phorbol injections were given for only 10 weeks, but the treatment was preceded by p.o. administration of 2 and 6 mg DMBA, respectively (see Table 2 and Chart 1). (The actual incidences of leukemia in these 3 groups cannot, of course, be compared, since the duration of phorbol treatment was 9 months in the 1 case and 10 weeks in the other 2 cases.) There was no leukemia in Group E, receiving 2 mg DMBA alone, and only 1 of 38 in Group G, receiving 6 mg DMBA alone. [In a recent report in the literature (17), in which Sprague-Dawley rats were given 3 i.v. injections of DMBA, totalling 6 mg, 7 of 215 (3%) developed leukemia.]

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals at start of experiment</th>
<th>Mammary tumor incidence (per effective total)</th>
<th>Av. latent period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>45</td>
<td>8/43 = 18%</td>
<td>567 ± 27b,c(489)</td>
</tr>
<tr>
<td>Phorbol alone</td>
<td>35</td>
<td>0/35 = 0%</td>
<td></td>
</tr>
<tr>
<td>0.25 mg DMBA</td>
<td>35</td>
<td>0/35 = 0%</td>
<td>112 ± 28</td>
</tr>
<tr>
<td>0.25 mg DMBA + phorbol</td>
<td>35</td>
<td>0/35 = 0%</td>
<td>130 ± 44</td>
</tr>
<tr>
<td>2 mg DMBA</td>
<td>35</td>
<td>3/35 = 8%</td>
<td>95 ± 20</td>
</tr>
<tr>
<td>2 mg DMBA + phorbol</td>
<td>34</td>
<td>0.05 &lt; p &lt; 0.20</td>
<td>98 ± 29</td>
</tr>
<tr>
<td>6 mg DMBA</td>
<td>39</td>
<td>7/34 = 21%</td>
<td>103 ± 13</td>
</tr>
<tr>
<td>6 mg DMBA + phorbol</td>
<td>38</td>
<td>28/36 = 78%</td>
<td>98 ± 29</td>
</tr>
<tr>
<td>15 mg DMBA</td>
<td>10</td>
<td>9/9 = 100%</td>
<td></td>
</tr>
</tbody>
</table>

- **a** Effective total = number of survivors at time of 1st tumor in group.
- **b** Calculated from birth. The figure in parentheses corresponds to latent period for treated animals, in which latency was calculated from the date of DMBA feeding.
- **c** Mean ± S.D.
- **d** 4 mg phorbol, injected i.p., twice weekly for 39 weeks.
- **e** 4 mg phorbol, injected i.p., twice weekly for 10 weeks. (Interval between the single feeding of DMBA and the start of phorbol injections = 1 week.)
- **f** Statistical evaluation = \( \chi^2 \) test.
DISCUSSION

The fact that phorbol caused a pronounced promoting effect in mammary carcinogenesis after a single feeding of 6 mg DMBA while only a slight effect, of questionable significance, was noted after 2 mg and no effect at all was seen after 0.25 mg DMBA is an indication of how critical the conditions must be in order to demonstrate an initiation-promotion system for this particular type of carcinogenesis. Yet the results are of interest, from the viewpoints of both phorbol action and mammary tissue response.

Phorbol is a systemic promoter for liver and lung carcinogenesis in mice (1), although not for skin carcinogenesis (8, 9), in striking contrast to some of the phorbol diesters, which are among the most potent promoters for skin (9). (Because of their insolubility in water, the phorbol diesters could not effectively be tested for systemic promoting action with respect to other organs in the body.) With the present evidence that unesterified phorbol is a promoter for mammary carcinogenesis as well, the compound acquires special importance as a useful reagent for the general study of systemic promoting action. Further testing of the compound, in relation to other target tissues, is therefore indicated.

From the viewpoint of mammary tissue response, the present results confirm and extend, by a different procedure, the previous indications of a possible initiation-promotion system in mammary carcinogenesis (in which hormonal factors had served as potential promoters). In the experiments of Dao and Sunderland (5), mammary tumor induction following a single feeding of 3-methylcholanthrene was greatly augmented by subsequent pregnancy or pseudopregnancy, but not when the sequence was reversed or when the 2 overlapped in time. Progesterone (possibly associated with excess of estrone) was presumably responsible for the promoting effect. Some other claims of hormonal promoting effects on mammary carcinogenesis are subject to criticism on the grounds that the postulated initiating and promoting actions were allowed to overlap in time. This applied, for instance, to the mouse experiments of Kim and Furth (15), in which pituitary grafts served as potential promoter. Where this complication of overlap was avoided, as in the experiment of Haran-Ghara (7), in which a pituitary mammatrophic tumor (implanted s.c. for limited periods and then excised) served as the added factor and tested at different stages in relation to the initiating stimulus, augmentation did occur in some of the groups, but not when the hormonal action came after the initiating stimulus, thus indicating a cocarcinogenic effect other than that of initiation-promotion.

In view of the evidence presented here that mammary carcinogenesis can be promoted by other (i.e., nonhormonal) means, namely, by phorbol, some of the earlier attempts at hormonal promotion of mammary carcinogenesis should now be repeated under more varied and more controlled conditions.

Spontaneous leukemia in Wistar rats occurs late in life, generally after 17 months of age, with a peak at 28 months...
The spontaneous incidence of lymphatic leukemia is reported to be low [2.8% (21); 9% (14)]. The incidence in our untreated controls was 2%. Earlier reports of chemically induced leukemia in rats by repeated administration of DMBA, 3-methylcholanthrene, etc., include erythroblastic (20), stem-cell (11), acute mononuclear (16), or myeloblastic (chloroma) types (13), while induced lymphatic leukemia in rats seems to have been produced thus far only by viral action (6, 12).

It is, therefore, all the more significant that the high incidence of leukemia in our present experiment, resulting from repeated phorbol injections, should have been exclusively lymphatic, involving the thymus. [The leukemias induced by phorbol in SWR mice were also lymphatic in type, but predominantly nonthymic (3).] Two questions come up for consideration: (a) whether the rat leukemias were of T-cell origin and the mouse leukemias of B-cell origin; and (b) whether a latent oncogenic virus was involved in the rat leukemias. These possibilities are now being followed up by appropriate immunological and other methods.

Another interesting feature was the fact that the latent period of leukemogenesis with phorbol alone was more than twice as long as with phorbol preceded by a single p.o. administration of DMBA, in spite of the fact that the dose of phorbol, in terms of number of administrations, was much lower in the latter case. Actually, all the leukemias in the phorbol control group began to appear more than 1 month after the last of the leukemias in the other 2 groups. DMBA alone was practically free from leukemogenic action. Some kind of cocarcinogenic action (2) is clearly implicated, but whether a true initiation-promotion mechanism is involved here cannot be decided from the results thus far available.

There is the remote possibility to consider that the leukemias in Groups F and H were detected earlier because so many of the animals in these groups were killed as soon as mammary tumors were found to be present. That this could account for the shortening in the latent period of leukemogenesis can be discarded (a) because of the enormous difference in average latent period (about 6 months) and (b) because the cases in which both diseases occurred in the same animal (in 11 of 14 rats) were confined to Group H with no such cases at all in Group F.

REFERENCES

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