Systemic Promoting Action of Phorbol in Liver and Lung Carcinogenesis in AKR Mice

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SUMMARY

Newborn male and female AKR mice receiving a single s.c. injection of dimethylnitrosamine (DMN) and, after a 2-week interval, repeated i.p. injections of phorbol, developed lung adenomas and hepatomas in a high percentage of survivors. When a higher dose of DMN was injected into 10-day-old AKR mice with subsequent phorbol treatment, the incidence of lung adenomas and hepatomas was low; while no such tumors appeared in the untreated mice or in those receiving phorbol alone, and very few tumors appeared in the DMN control mice. The results thus demonstrate a pronounced promoting action by phorbol on lung and liver carcinogenesis provided that the initiating stimulus (by DMN) was given soon after birth. Phorbol, whether administered alone or after initial treatment with DMN, failed to increase the spontaneous incidence of thymic leukemia.

INTRODUCTION

With the isolation from croton oil of certain phorbol diesters (cf. review in Ref. 10), responsible for the promoting action of the oil for skin carcinogenesis in mice, an important, refined, new tool was made available for the study of cocarcinogenesis (2, 10, 20). The unesterified phorbol itself—a tetracyclic diterpene of complicated structure—was found to be inactive as promoting agent for skin, whether applied topically (10) or administered systemically (3). Repeated i.p. injections of phorbol did, however, lead to the unexpected development of (nonthymic) lymphatic leukemia in SWR mice (3).

The question then arose as to whether or not, under appropriate experimental conditions, phorbol might also possess carcinogenic and/or cocarcinogenic activity in other tissues. It was thought possible to test both these possibilities at the same time, with the phorbol control groups serving as tests for carcinogenic activity and the experimental groups (receiving prior initiating action) serving as tests for cocarcinogenic (or, more specifically, promoting) activity. The availability of a suitable initiator was critical, as was the choice of the right mouse strain; and it seemed likely at first that different combinations might be needed according to the intended site of action (i.e., for leukemogenesis, liver carcinogenesis, lung carcinogenesis, etc.).

MATERIALS AND METHODS

Newborn and 10-day-old AKR mice, from the Institute Animal Breeding Center, foster-nursed for the 1st 3 weeks of life by ICR female mice, were used for these investigations. (AKR mothers tend to eat their young; hence the need for foster-nursing.) The animals were kept in an air-conditioned room at 21—25°, 1 litter per metal cage till weaning and then 10 per cage, separated according to sex, and fed Purina laboratory chow and tap water ad libitum.

The DMN was purchased from Eastman Organic Chemicals, Rochester, N. Y., and the phorbol was from Dr. Theodor Schuchardt GmbH and Co., München, Germany. The DMN was dissolved in 0.9% NaCl solution (15 and 30 µg in 0.05 ml, respectively) and injected s.c. The phorbol was made up as an 0.2% solution in phosphate buffer solution and 0.1 ml was injected i.p. twice weekly during the 1st 2 months and 0.2 ml thereafter.

One group of males and females received 1 s.c. injection of 15 µg DMN at birth; another group received 30 µg DMN 10 days after birth, followed in both cases, after an interval of 2 weeks, by repeated i.p. injections of phorbol. The control groups comprised 1 receiving no treatment whatever, 1 with phorbol alone, and 2 with 15 µg DMN at birth and 30 µg at 10 days of age without subsequent phorbol treatment (see Tables 1 to 3).

All the animals were observed daily and systematically examined twice weekly. Those with palpable tumors were killed, and these, as well as those killed at the end of the experiment (after 9 months), were autopsied, the various organs were fixed in Bouin’s solution, and paraffin sections were stained with hematoxylin and eosin.

1 Referred to in a previous communication (3) as Vlasta Lonai.
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2 The abbreviation used is: DMN, dimethylnitrosamine.
### Table 1

Incidence of leukemia in AKR mice killed at 9 months of age

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per total no. of mice</td>
<td>Per effective total</td>
<td>Per total no. of mice</td>
<td>Per effective total</td>
</tr>
<tr>
<td>DMN (μg)</td>
<td>Phorbol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>3/20 (15%)</td>
<td>3/20 (15%)</td>
<td>5/20 (25%)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4/20 (20%)</td>
<td>4/19 (21%)</td>
<td>4/18 (22%)</td>
</tr>
<tr>
<td>15c</td>
<td>–</td>
<td>1/18 (5.5%)</td>
<td>1/7 (14%)</td>
<td>5/21 (24%)</td>
</tr>
<tr>
<td>15c</td>
<td>+</td>
<td>1/25 (4%)</td>
<td>1/25 (4%)</td>
<td>2/20 (10%)</td>
</tr>
<tr>
<td>30d</td>
<td>–</td>
<td>0/20 (0%)</td>
<td>0/5 (0%)</td>
<td>1/19 (5%)</td>
</tr>
<tr>
<td>30d</td>
<td>+</td>
<td>0/15 (0%)</td>
<td>0/16 (0%)</td>
<td>2/18 (11%)</td>
</tr>
</tbody>
</table>

Table 2

Incidence of lung adenomas in AKR mice killed at 9 months of age

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per total no. of mice</td>
<td>Per effective total</td>
<td>Per total no. of mice</td>
<td>Per effective total</td>
</tr>
<tr>
<td>DMN (μg)</td>
<td>Phorbol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>0/20 (0%)</td>
<td>0/20 (0%)</td>
<td>0/20 (0%)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0/20 (0%)</td>
<td>0/19 (0%)</td>
<td>0/20 (0%)</td>
</tr>
<tr>
<td>15c</td>
<td>–</td>
<td>0/18 (0%)</td>
<td>0/7 (0%)</td>
<td>0/21 (0%)</td>
</tr>
<tr>
<td>15c</td>
<td>+</td>
<td>14/25 (56%)</td>
<td>14/25 (56%)</td>
<td>7/20 (35%)</td>
</tr>
<tr>
<td>30c</td>
<td>–</td>
<td>1/20 (5%)</td>
<td>1/16 (5%)</td>
<td>2/19 (10.5%)</td>
</tr>
<tr>
<td>30c</td>
<td>+</td>
<td>1/15 (7%)</td>
<td>1/5 (20%)</td>
<td>1/18 (5.5%)</td>
</tr>
</tbody>
</table>

#### RESULTS

Since there is a high spontaneous incidence of thymic lymphatic leukemia in AKR mice, developing from about the 6th month of age and continuing to appear long after the 9th month (when the present experiment was terminated), the recorded incidences in the various groups (see Table 1) did not reach the potential maximum (over 80% in untreated AKR mice allowed to live their full life-span). No significant difference in incidence was observed between the untreated control group and that receiving phorbol alone (average latent periods, 239 ± 15 and 200 ± 38 days, respectively). The somewhat lower incidences in some of the experimental groups (receiving DMN alone or followed by phorbol) may possibly be accounted for by (a) higher mortality rates due to the toxicity of DMN administered at or soon after birth and (b) the fact that some of the animals (bearing hepatomas) were sacrificed before the time for leukemia to occur. There was certainly no significant evidence of promoting action by phorbol, with respect to leukemogenesis.

No pulmonary tumors were seen in the untreated or phorbol control group and very few were seen in the DMN control groups (see Table 2); a high incidence of such tumors was found in the group receiving an initial dose of 15 μg DMN at birth followed by phorbol treatment and a much lower incidence was seen in the group receiving, at 10 days of age, 30 μg DMN followed by phorbol treatment. Evidence of promoting action by phorbol for lung carcinogenesis was thus
confined to the situation where the DMN as initiator was administered at birth.

The pulmonary tumors were all sharply circumscribed, pearl-white nodules, mostly under the pleura, characterized histologically as adenomas with papillary formation, and with no mitotic figures discernible. There was no significant difference in incidence between males and females (56 and 58%, respectively) in contrast to the results of Toth and Shubik (19) with a higher dose of DMN alone, who found about twice the incidence in females than in males.

There were no liver tumors in the untreated control group or in the phorbol control group (see Table 3) and very few in the 2 DMN control groups and in the experimental group in which 30 μg DMN were injected 10 days after birth followed by phorbol treatment. In contrast to this, there was a significantly high incidence in the group receiving 15 μg DMN at birth followed by phorbol treatment, thus indicating pronounced promoting action under these specific conditions.

According to the modified Stewart and Snell classification (11, 16), all the hepatic tumors were liver adenomas, clearly differentiated from the surrounding liver tissue. The rather large tumor cells had acidophilic cytoplasm, with a single large nucleus. The cells were arranged in cords or sheets, with occasional appearance of endothelium-lined sinuses. In some instances, regenerating hepatic tissue existed in the lobes bearing a neoplasm. There was no indication of malignancy in any of the tumors.

The incidence of hepatic tumors in the experimental group was higher in males than in females (14/25 as against 4/12 per effective total) which differs from the results of Toth and Shubik (19) who found no sex difference with a higher dose of DMN at birth without other treatment.

**DISCUSSION**

Most of the available data about promoting action are derived from experiments on mouse skin (cf. reviews in Refs. 1, 2, 10, and 20); and, although many attempts have been made to demonstrate such action in organs such as the liver (6, 7), thyroid (5, 8), mammary tissue (4, 9), kidney (14, 18), etc. (15), the evidence has never been altogether convincing. Two explanations may be offered for this: (a) the difficulty of finding appropriate promoting agents suitable for systemic carcinogenesis for internal organs which could be considered at all comparable to croton oil for skin, and (b) the fact that, in many of the attempts to demonstrate a 2-stage mechanism operating in systemic carcinogenesis, the action of the 2 agents (as potential initiator and promoter) were allowed to overlap in time rendering it difficult to distinguish between true initiation-promotion and other forms of cocarcinogenesis (cf. Ref. 2). [In 1 case (9) where the suspected promoting agent (mammotropic hormone) was tested separately before, during, and after the initiating stimulus (methylcholanthrene) in relation to mammary carcinogenesis, augmentation only occurred when the 2 agents were administered at the same time, thus indicating cocarcinogenesis other than initiation-promotion (cf. Ref. 2).]

The present results, with phorbol as a promoting agent for liver and lung carcinogenesis (and DMN as initiator) in AKR mice would seem to be the 1st unequivocal example of systemic promoting action, and thus provide a new approach for the study of systemic 2-stage carcinogenesis. (Attempts are now being made to explore the possibility that phorbol is a promoter for other kinds of systemic carcinogenesis.)

The failure of phorbol to raise the leukemia incidence in AKR mice seems, at first sight, to conflict with the previous observation (3) that phorbol is leukemogenic when tested in SWR mice. However, spontaneous leukemia in AKR mice is predominantly thymic in origin, whereas that induced in SWR mice is predominantly nonthymic in origin: but this leaves unexplained why nonthymic leukemia did not develop in the phorbol-treated AKR mice. It is conceivable that these might have appeared if the animals had been kept alive longer. (The experiment was terminated after 9 months in order that the pulmonary tumors would seem to be the 1st unequivocal example of systemic promoting action, and thus provide a new approach for the study of systemic 2-stage carcinogenesis.)

**Table 3**

*Incidence of hepatic tumors in AKR mice killed at 9 months of age*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Males</th>
<th></th>
<th>Females</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMN (μg)</td>
<td>Per total no. of mice</td>
<td>Per effective total</td>
<td>Per total no. of mice</td>
<td>Per effective total</td>
<td>Per total no. of mice</td>
</tr>
<tr>
<td>Phorbol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>0/20 (0%)</td>
<td>0/20 (0%)</td>
<td>0/20 (0%)</td>
<td>0/20 (0%)</td>
<td>0/40 (0%)</td>
</tr>
<tr>
<td>+</td>
<td>1/18 (5.5%)</td>
<td>1/7 (14%)</td>
<td>0/20 (0%)</td>
<td>0/18 (0%)</td>
<td>1/21 (5%)</td>
</tr>
<tr>
<td>15c</td>
<td>1/18 (5.5%)</td>
<td>1/7 (14%)</td>
<td>0/20 (0%)</td>
<td>0/18 (0%)</td>
<td>1/21 (5%)</td>
</tr>
<tr>
<td>15c +</td>
<td>14/25 (56%)</td>
<td>14/25 (56%)</td>
<td>4/20 (20%)</td>
<td>4/12 (33%)</td>
<td>18/45 (40%)</td>
</tr>
<tr>
<td>30c</td>
<td>3/20 (15%)</td>
<td>3/16 (19%)</td>
<td>0/19 (0%)</td>
<td>0/14 (0%)</td>
<td>3/39 (8%)</td>
</tr>
<tr>
<td>30c +</td>
<td>0/15 (0%)</td>
<td>0/5 (0%)</td>
<td>1/18 (5%)</td>
<td>1/7 (14%)</td>
<td>1/33 (3%)</td>
</tr>
</tbody>
</table>

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a Interval between DMN and phorbol treatment, 2 weeks.

b Effective total, number of survivors at the time of the first recognized hepatic tumor in the group.

c Administered on day of birth.

d The p values refer to the figures immediately above and below.

e Administered at 10 days of age.
The experimental conditions required for systemic promoting action with phorbol, in relation to liver and lung carcinogenesis, seem to be fairly critical, i.e., demonstrable when the initiator (DMN) is administered at birth, but not when it is given (even at a higher dose level) 10 days later. Two alternative explanations for this may be considered: (a) the possibility, by analogy with urethan (13), that newborn mice might be incapable of catabolizing DMN, whereas 10-day-old mice might be able to do so (for data in rats, cf. Ref. 17), and (b) that the defective immunocompetence of newborn mice (cf. Ref. 12) might be responsible for the difference in response. Further information is needed to decide which of these 2 explanations is correct.

REFERENCES

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