The Leukemogenic Action of Phorbol

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SUMMARY

The unesterified, water-soluble compound phorbol, which does not act as a promoting agent for skin carcinogenesis when applied topically (although phorbol esters extracted from croton seed oil display pronounced activity), was tested in female SWR mice for promoting action by the systemic route, in the hope of overcoming a suspected solubility barrier in the skin. The results were negative.

A high incidence of leukemias developed, however, both in the group receiving preliminary skin painting with 7,12-dimethylbenz(a)anthracene followed by phorbol injections and in the phorbol control group. The tumors were of lymphoid origin, mostly nonthymic. There were also many reticulum cell sarcomas in the phorbol-treated mice, but the incidence did not appear to be higher than in the control groups.

The potential value of phorbol as a chemical leukemogenic agent for the study of the mechanism of leukemogenesis is discussed.

INTRODUCTION

This investigation was first conceived as an attempt to explain an apparent anomaly concerning the active principle of croton oil [recently isolated and identified by Hecker et al. (9); see also Van Duuren and Orris (13)], namely, that its promoting action for mouse skin carcinogenesis is dependent on the attachment of two fatty acid chains to the complex parent alcohol, phorbol, while the unesterified phorbol is itself inactive (9). A simple physicochemical explanation of this would be that phorbol, being water soluble, is incapable of penetrating the skin epithelium; attaching fatty acid chains renders the compound also lipid soluble, thereby permitting penetration and enabling the substance to express its (?) potential promoting property. On the basis of this working hypothesis, one could imagine that the water-soluble phorbol might act as an effective promoting agent for skin carcinogenesis if administered systemically (in conjunction with prior surface application of an initiating agent to the skin).

In order to test this possibility, a solution of DMBA in paraffin oil was applied to the skin to serve as initiating stimulus, and an aqueous solution of phorbol was then given by injection i.p. twice weekly to provide (systemic) promoting action. Additional controls included a group receiving phorbol injections alone, as well as a group of untreated mice, to detect any other biological activity which phorbol might possess.

The results were negative as far as skin tumor development was concerned. However, leukemia developed in a high proportion of animals after a relatively short average latent period, both in the experimental group receiving DMBA plus phorbol and in the phorbol control group.

MATERIALS AND METHODS

Female SWR mice, inbred in the Institute Animal Breeding Center under specific pathogen-free conditions, were kept 10 to a metal cage in an air conditioned room at 21—25° throughout the experiment, and they were fed Purina laboratory chow and tap water ad libitum.

The DMBA, from Sigma Chemical Co., St. Louis, Mo., was made up as a 0.5% solution in paraffin oil (medicinal liquid paraffin) and applied, once only, with a glass rod to a small area of clipped skin (about 1.5 x 1.5 cm) in the dorsal region of the back. The amount applied was estimated to be approximately 0.05 ml, containing 0.25 mg of DMBA.

The phorbol, from Dr. Theodor Schuchardt GMBH & Co., München, Germany, was made up as a 0.1% solution in phosphate-buffered saline, and 0.2 ml was given by injection i.p. twice weekly for 20 to 30 weeks (i.e., 0.2 mg of phorbol per injection, totalling 8 to 12 mg).

After an initial test on 2 groups of 3 mice (3 months old) each, which received a single i.p. injection of 1.0 and 0.5 mg of phorbol, respectively, for evidence of toxicity, the following groups of animals were set up for the long-term experiment (for numbers and ages, see Table 1).

Group 1 received a single application of DMBA to the skin, followed 2 weeks later by twice weekly i.p. injections of phorbol.

Group 2 received twice weekly injections of phorbol i.p., without prior DMBA treatment.

Group 3 received a single application of DMBA to the skin, without subsequent phorbol injections.

Group 4 were untreated controls.

Since the duration of the experiment in Group 1 could not be calculated in advance, we did not include the reversal control (3) of testing promoting action prior to initiating action. (Since, in fact, no skin tumors developed in Group 1, the omission of the reversal control was immaterial.)
The 6 mice used in the initial trial test for toxicity were killed 1 month after the injection of phorbol. Autopsies were performed on them, and the following organs and tissues were kept for histological examination: liver, kidneys, lungs, spleen, several lymph nodes, and a piece of skin, including the underlying muscle down to the peritoneum, at the site of the injection. The sections were stained with hematoxylin and eosin. In the long-term experiment, the animals were observed daily and were more carefully examined twice weekly. In order to prevent cannibalism or decomposition, animals that appeared to be in poor health or which showed evidence of a palpable tumor were killed for autopsy, and various organs were kept for histological examination, as above.

After all the animals of Groups 1 and 2 had died or had been killed, those of Group 3 were also sacrificed, while the untreated mice (Group 4) were divided into 2 groups; one-half was killed at that time, to serve as comparable controls for the first 2 groups. The remainder were left until their natural deaths to provide information about the true, spontaneous incidence of tumors in the strain.

RESULTS

The initially tested mice, examined for toxicity after single injections of 1.0 and 0.5 mg of phorbol, respectively, showed no abnormalities during the 1-month period of observation or at autopsy. Neither were any abnormalities detected in the tissues examined histologically, i.e., in the liver, kidneys, lungs, and lymph nodes, or at the site of injection. In short, phorbol injected at these 2 dose levels appeared to be entirely free from acute toxicity.

In the long-term experiment, no skin tumors developed in the animals receiving either DMBA skin painting alone (Group 3) or DMBA skin painting followed by twice weekly i.p. injections of phorbol (Group 1). However, in the 2 groups receiving phorbol injections (Groups 1 and 2), 86 and 75%, respectively, developed lymphoreticular tumors within 8 months after the 1st phorbol injection. (The survival rate of the animals until the development of the tumors was excellent, with no deaths from intercurrent infection or from any effects attributable to the treatment given, until the actual development of tumors.)

The results of tumor development are summarized in Table 1, with a further breakdown of the data according to lengths of treatment given in Table 2.

Most of the tumors were of lymphoblastic origin (see Figs. 1 to 3); the others could be classified as reticulum cell sarcoma, type B (Fig. 4), according to Dunn's classification (7). The incidences of lymphoid tumors in the phorbol-treated mice (Groups 1 and 2) were 70 and 57%, respectively, as compared to 0 and 6% in the controls (Groups 3 and 4). No such striking differences between phorbol-treated and control groups with reticulum cell sarcoma were found, the incidences being 5 and 29% as against 4 and 20%. The latency of appearance of the tumors seemed to be influenced to some extent, by the age of the animals at the start of the experiment, late tumors (i.e., those appearing after the 30th week of treatment) occurring only among those in which treatment was begun at 16 weeks of age (Table 2). There was little difference in average latent period for lymphoid leukemia and reticulum cell sarcomas. This was a surprising result in that the latter type, especially when of spontaneous origin, generally arises much later than lymphoid leukemia in mice.

Macroscopically, the tumor-bearing animals showed evidence of enlargement of the lymph nodes, spleen, liver, and kidneys. Histologically, most of the leukemias (i.e., except those with very early lesions) showed advanced infiltration of the affected organs. The criteria used for defining and

Table 1

Tumor development in female SWR mice after skin painting with DMBA and i.p. injections of phorbol

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Treatment</th>
<th>Age started (wk)</th>
<th>Leukemia incidence</th>
<th>Latent period (days)a</th>
<th>Reticulum cell sarcoma incidence</th>
<th>Latent period (days)a</th>
<th>Total incidence</th>
<th>Average cumulative dose (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>DMBA + phorbol</td>
<td>9</td>
<td>13/18 (72%)</td>
<td>193 ± 8</td>
<td>1/18 (5%)</td>
<td>105</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td></td>
<td>13</td>
<td>13/19 (68%)</td>
<td>150 ± 12</td>
<td>2/19 (10%)</td>
<td>132 ± 27</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td></td>
<td>13</td>
<td>13/19 (68%)</td>
<td>163 ± 31</td>
<td>0/19 (0%)</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>39/56 (70%)</td>
<td>168 ± 17</td>
<td>3/56 (5%)</td>
<td>118 ± 13</td>
<td>42/56 (75%)</td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>Phorbol</td>
<td>9</td>
<td>18/19 (94%)</td>
<td>169 ± 25</td>
<td>0/19 (0%)</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>b</td>
<td></td>
<td>16</td>
<td>8/18 (44%)</td>
<td>210 ± 24</td>
<td>7/18 (38%)</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>c</td>
<td></td>
<td>16</td>
<td>6/19 (31%)</td>
<td>211 ± 29</td>
<td>9/19 (47%)</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>32/56 (57%)</td>
<td>196 ± 26</td>
<td>16/56 (29%)</td>
<td>207 ± 32</td>
<td>48/56 (86%)</td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>DMBA</td>
<td>8</td>
<td>0/25 (0%)</td>
<td>1/25 (4%)</td>
<td>187</td>
<td>1/25 (4%)</td>
<td>12/45 (27%)</td>
<td></td>
</tr>
<tr>
<td>Group 4</td>
<td>Untreated</td>
<td>3/45 (6%)</td>
<td>291 ± 49</td>
<td>9/45 (20%)</td>
<td>297 ± 51</td>
<td>12/45 (27%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aFrom the start of phorbol treatment, except in Group 4, where it refers to age from birth.
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Table 2

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Age started (wk)</th>
<th>No. of lymphoreticular tumors appearing after start of phorbol treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before 15 wk</td>
<td>16–20 wk</td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>9</td>
<td>1 (0 + 1)*</td>
</tr>
<tr>
<td>b</td>
<td>13</td>
<td>1 (0 + 1)</td>
</tr>
<tr>
<td>c</td>
<td>13</td>
<td>1 (1 + 0)</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>9</td>
<td>1 (1 + 0)</td>
</tr>
<tr>
<td>b</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>c</td>
<td>16</td>
<td>0</td>
</tr>
</tbody>
</table>

*First figure in parentheses refers to lymphocytic leukemia; second figure refers to reticulum cell sarcoma.

classifying the lymphoreticular tumors were based on Dunn's detailed studies (7). The spleen and lymph nodes in the tumor-bearing animals contained large, lymphoblast-like cells, many of them in mitosis, filling the pulp and cortex of the respective organs. In the sinuses of the liver and in the intertubular spaces of the renal cortex, similar malignant infiltration was evident, with considerable distortion of the normal architecture of the organs. Although no transplantation studies were carried out, the malignant nature of the lesions was evident on general histopathological grounds and by comparison with the many radiation-induced malignant lymphomas in this laboratory. Lymphoid infiltration of the peritoneum, and of the skin and musculature at the site of phorbol injections, was also a common feature (Fig. 5).

Thymic involvement was noted in only 15 cases (20%) of lymphoid leukemia and in none of the cases of reticulum cell sarcoma.

Among the 22 animals given injections of phorbol and bearing no tumors, circumscribed lymphocytic infiltration of the peritoneum and skin was observed in 6 cases (27%). There were also 4 cases of lung adenomas, 1 case of hepatic tumor, 1 case of nephrosis, and 1 case of pulmonary abscess. In the DMBA control Group 3 and the untreated control Group 4 (among those killed at the 30th week), 57 animals (82%) were free from any demonstrable pathological changes in the organs examined. The remaining 13 animals (18%) included a few leukemias (see Table 1), 1 lung adenoma, and 2 cases of pyelonephritis. (The animals of the untreated control Group 4 that had been left to live their full life-span are still all alive.) There was no significant difference in average latent periods between the leukemias induced by phorbol in Groups 1 and 2 and those that developed spontaneously in Groups 3 and 4. (The differences recorded in Table 1 are deceptive, in that the latent periods for induced tumors are calculated from the first injection of phorbol, whereas those of the controls are calculated from birth.)

DISCUSSION

The original purpose of the investigation was to determine whether unesterified, water-soluble phorbol could act as a systemic promoting agent for skin carcinogenesis, i.e., when injected i.p. over a long period, following a single local application of DMBA to the skin. The fact that when applied locally to the skin phorbol was inactive (9), in contrast to the strong promoting effects obtained with the esterified compounds (containing attachment of 2 fatty acid chains) in the products isolated from croton oil, might have been due to failure of the water-soluble phorbol to penetrate the skin epithelium when applied from without.

The present results fail to support this simple physicochemical explanation. No skin tumors appeared in Group 1, in which local application of DMBA was followed by repeated i.p. injections of phorbol.

The objection could be raised that the dose of phorbol used was insufficient to elicit a positive effect. The dose actually chosen (0.2 mg/injection, constituting a cumulative dose of 8 to 11 mg) represents about 100 times that found to be effective for the esterified compound applied locally (9). Even allowing for the dilution effect when a substance is administered systemically, some evidence of promoting action might have been expected if the compound possessed latent promoting capacity. A higher dose of phorbol than that actually given could have been tolerated by the animals, in the light of the low toxicity demonstrated in the initial tests. Unfortunately, the amount the suppliers were able to provide at the time rendered this impossible.

Among the other possible explanations of the negative skin responses are failure of the phorbol to reach the skin from its site of injection and its rapid metabolic breakdown before reaching the skin. Neither explanation seems probable in view of the water solubility and ready diffusibility of the substance. (As a more critical test, experiments, as yet incomplete, are under way involving the direct application of phorbol to the skin with Polyethylene Glycol-400 as solvent, in order to facilitate the penetration of the water-soluble phorbol through the lipid barrier in the skin. The results thus far are completely negative.)

While the skin response was entirely negative, the lymphoid tissues responded dramatically to the injections of phorbol, resulting in a high incidence of lymphoid leukemias, predominantly nonthymic, after a relatively short average latent
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period. The effects were about the same in Group 2, which received phorbol alone, as in Group 1, which received DMBA plus phorbol (57 as against 70%). The phorbol must therefore be credited with responsibility for the lymphoid tumors. The considerable number of reticulum cell sarcomas in the phorbol-treated animals (5 and 29% in Groups 1 and 2) would seem to be of spontaneous origin, in view of a similar incidence in the nonphorbol controls (4 and 20% in Groups 3 and 4).

Leukemia induction in mice can be readily achieved by whole-body X-irradiation (7-11), as well as by chemical carcinogens (1, 3, 6), although the mechanisms involved may not be identical (4, 5). In the previous comparative studies between chemical and radiation leukemogenesis, complications arose from the fact that the chemical leukemogenic agents also elicited other carcinogenic activities. The use of phorbol may, therefore, be of special value, since its tumor-inducing capacity seems to be restricted to leukemogenesis (at least, under the present experimental conditions) with an incidence of induced leukemia and a short latent period comparable to that which occurs in whole-body X-irradiation.

An additional point of interest about the leukemogenic action of phorbol is that phorbol is a hydrolytic product of a naturally occurring substance (or group of substances) extracted from croton oil; it is, therefore, comparable to other naturally occurring carcinogens such as aflatoxin (2), or rather to carcinogenic hydrolytic products of naturally occurring substances such as cycasin, which is derived from cycad nuts (12).

ADDENDUM

Of the 40 mice from the untreated control Group 4, which were left for further observation, 32 are still alive; of the 8 that died, 1 had a large s.c. lipoma, 3 had ovarian cysts, 3 had bronchopneumonia, and 1 had nonthymic lymphatic leukemia (at the age of 400 days).

REFERENCES

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