Dose-Response Studies with a Pure Tumor-promoting Agent, Phorbol Myristate Acetate1, 2

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SUMMARY

The dose-response relationship of the mouse skin tumor-promoting agent, phorbol myristate acetate (PMA), was determined over a dose range of 0.02 to 25 μg/application. The effect of frequency of application was also examined by three times weekly or once weekly treatments. Administration of PMA three times weekly gave a maximal tumor response with all of the mice at risk in each of the three highest dosage groups, 25, 5, and 2.5 μg, developing papillomas. At the lower dose, 0.5 μg, the tumor incidence was 72% of the population at risk, and no tumors were observed at the two lowest doses, 0.1 and 0.02 μg. The time to 50% incidence of animals with tumors was 7.5 to 8 weeks for each of the three maximal response groups, indicating that the rate of appearance of tumor-bearing mice was similar in each of these groups. However, if the total number of papillomas is compared, a dose-response relationship is clearly apparent. It was found that 2.5 μg of PMA given three times weekly is an optimal dose to obtain 100% of the mice with papillomas, although the total numbers of papillomas and cancers are significantly fewer than at the two higher doses of tumor promoter. While tumor promotion is accompanied by epidermal hyperplasia and inflammation, these manifestations are in all likelihood not related to the mode of action of PMA. A new abbreviated method for the preparation of pure PMA is described.

INTRODUCTION

The phorbol esters derived from Croton tiglium L. have been under extensive study in this laboratory with regard to their chemistry, tumor-promoting activity for mouse skin, and their action in vitro in cell culture (8–10). Our previous investigations have demonstrated that in initiation-promotion experiments on mouse skin with PMA 2 as promoter there is: (a) a high incidence of malignant tumors (40 to 60%); (b) a low incidence of papilloma regressions (< 10%); (c) a rapid appearance of tumors after the beginning of treatment with phorbol ester (31 ± 6 days); (d) a high multiplicity of persisting tumors (usually 10 or more); and (e) low tumorigenicity of phorbol esters applied alone (7.8% papillomas and 1.5% carcinomas).

Earlier studies on dose-response relationships with tumor promoters were carried out with croton oil (1) or unknown fractions derived from it (4) as the promoting agent. Tumor responses at a few doses of PMA have been reported earlier (5, 7, 9). It was, therefore, considered important to carry out an extensive dose-response study with a pure promoting agent using a wide range of dosages and different frequencies of application.

The role of inflammation and cellular proliferation in tumor promotion has been frequently discussed (1, 9) and remains unclear. As a part of the present study acute cytotoxicity, epidermal hyperplasia, and inflammatory cell invasion of the dermis were examined in relation to the dose of tumor promoter applied and the tumor responses observed.

MATERIALS AND METHODS

Animals. Female ICR/Ha Swiss mice (A. R. Schmidt-Millerton Co., Millerton, N. Y.) were used for this experiment. They were vaccinated against ectromelia and tests were started at age 7 weeks. Mice were housed on sterile wood chips in stainless steel cages, 10 to a cage, fed Purina laboratory chow and water ad libitum, and weighed regularly. The animal rooms were maintained at 22–24°.

Bioassay Procedure. The backs of the mice were clipped the day before the initial treatment and then as needed for the duration of the experiment. The initiating agent, DMBA, was applied by micropipet in a single dose, 5 μg in 0.1 ml acetone. This primary treatment was followed 1 month later by 3 times weekly or once weekly applications of PMA, at a range of doses (0.02 to 25.0 μg) in 0.1 ml acetone per application for the duration of the experiments.

1The abbreviations used are: PMA, phorbol myristate acetate (see Footnote 1); DMBA, 7,12-dimethylbenz(a)anthracene; t50, time to 50% incidence of animals with tumors.
Animals were charted for papillomas every 2 weeks and tumors were recorded; tumors greater than 1 mm in diameter were counted and included in the cumulative total only if they persisted for 30 days or more. Animals bearing tumors that appeared grossly to be carcinomas were killed approximately 2 months after the tumors were clinically diagnosed as cancers. All animals were autopsied and specimens from tumors and abnormal-appearing tissues were excised. Specimens were fixed in 4% formalin, blocked in paraffin, and stained with hematoxylin and eosin for histopathological diagnosis.

Included in the protocols were control groups that received initiator once followed by solvent, applied repeatedly; a group receiving promoter only at the various doses, thrice weekly and once weekly; and a group receiving no treatment.

Chemicals. DMBA was freshly recrystallized from an acetone solution and the solutions for bioassay were prepared immediately before use.

Spectroscopic grade acetone was used for preparation of all solutions. The acetone was routinely checked for purity by spectrofluorimetric analysis.

PMA was prepared from croton oil by a simplified procedure compared to the elaborate procedures used earlier by us (10) and by others (2). Because of the widespread need for PMA in research laboratories, the procedure developed by us is described here. Croton oil (Amend Drug and Chemical Co., New York, N. Y.: 453 g) was dissolved in 2.2 liters of hexane previously equilibrated with petroleum ether (b.p., 30-60°). The column was eluted with six 750-ml portions of 90% ethanol. The 90% ethanol extracts were combined and evaporated to dryness. The residue was azeotropically dried by the addition of 500 ml of 100% ethanol which was then evaporated in a vacuum at 35°. About 100 g of a resin were obtained. This resin was chromatographed on neutral Florisil, which was Florisil (60 to 100 mesh; Fisher Scientific Co., New York, N. Y.) packed in a chromatographic column and washed with 5% acetic acid until the eluate was acidic. The Florisil was then washed first with distilled water until the eluate was neutral and finally with methanol. The adsorbant was air dried and activated by heating to 150° for 3 days.

The resin, 100 g, obtained from croton oil as described above was applied to a neutral Florisil column (8.2 x 68 cm) in petroleum ether (b.p., 30-60°). The column was eluted with petroleum ether (2 liters) and petroleum ether:ether (9:1, 2 liters; 6:1, 2 liters; 3:1, 2 liters; 1:1, 8 liters). The fractions were monitored by analysis on Silica Gel F-254 plates with ether as solvent. The majority of the phorbol esters were eluted from the column in petroleum ether:ether, 1:1. Silica Gel F-254 preparative plates were preeluted with methanol and then activated by heating at 100° for 1 hr. The mixture of phorbol esters was applied to plates; 100 mg dissolved in chloroform were applied per plate. Plates were developed with ether. The various phorbol esters appeared as dark bands under UV. The band that was identical in Rf with authentic PMA (purity previously established by mass spectroscopy) was scraped from the plate and eluted with methanol. The methanol extract was filtered and evaporated to dryness in a vacuum (35°); then the residue was extracted with ether, filtered, and evaporated to dryness. The yield was 1.0 g of PMA from 453 g of croton oil. The purity of the material isolated was determined by mass spectroscopy.

RESULTS

Dose Response of Tumor Induction. A summary of the results of the mouse skin initiation-promotion experiments and the appropriate controls is given in Table 1, and the rates of tumor appearance are shown in Charts 1 and 2. Administration of PMA 3 times weekly gave a maximal tumor response with 100% of the mice at risk in each of the 3 highest dosage groups (25, 5, and 2.5 μg) developing papillomas. At 0.5 μg, the tumor incidence was 72% of the population at risk and no tumors were observed at the 2 lower doses. The t50, obtained by probit analysis, was 7.5 to 8 weeks for each of the 3 maximal response groups, indicating that the rate of appearance of tumor-bearing mice was similar in each of these groups (Chart 1A). However, if the total number of papillomas is compared a dose-response relationship is clearly apparent (Chart 1B). The t50 in the 0.5-μg group was 26 weeks, indicating a lower rate of appearance of tumor-bearing mice, which is also apparent from Chart 1A. PMA, 2.5 μg, 3 times weekly, is an optimal dose to obtain 100% of the mice with papillomas, although the total numbers of papillomas and cancers are significantly fewer than at the 2 higher doses of tumor promoter.

In the groups treated once weekly, there was a clear dose-response relationship in both tumor incidence and rate of tumor appearance as shown in Chart 2. This is also reflected by the t50 values in the 3 groups where tumors were induced as a result of the initiation-promotion treatment.

The cancer incidence shows a direct dependence on dose in both treatment groups as does the cumulative numbers of papillomas. Charts 1B and 2B show the rate of appearance of papillomas in the test groups where tumors were observed. Both the cumulative number of tumors and the rate of appearance of tumors increase with increase in dose, in both treatment protocols, i.e., 3 times weekly and once weekly application of PMA.

As we have observed previously, the time to 1st tumor was very similar (~35 days) in the various groups receiving promoting treatment 3 times weekly. In contrast, the time to 1st tumors showed no definite pattern in the groups treated once weekly with PMA, although the tumors generally appeared later and increased at a significantly slower rate than in the groups treated 3 times weekly. As in earlier studies with croton oil and croton resin (1, 9), the frequency of application is more important than total dose of promoter applied. This readily becomes apparent by comparing the experiments in which 0.5 μg was applied 3 times weekly and 2.5 μg were applied once weekly. The total dose for the former was 78 μg compared to 130 μg for the higher dose applied once weekly. Yet the tumor incidences...
Table 1
Dose response: tumor incidences in 2-stage carcinogenesis
Twenty female ICR/Ha Swiss mice were in each group; results at 365 days; median survival time was greater than 365 days, except where noted. Control groups not shown in this table were: (a) those that received PMA only, once weekly; (b) DMBA once, followed by acetone 3 times weekly; (c) acetone only 3 times weekly; and (d) a no-treatment group. No skin tumors were observed in any of these groups. For the test groups, there were 17 to 20 survivors at the times of 1st appearance of tumors.

<table>
<thead>
<tr>
<th>Dose PMA, µg in 0.1 ml acetone</th>
<th>Time to 1st tumor* (days)</th>
<th>Total PMA dose (µg)</th>
<th>Total dose† PMA (µg) at t50</th>
<th>No. of tumor bearers*</th>
<th>Total no. of papillomas</th>
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<tr>
<td>DMBA, 5 µg, once only; PMA, 3 times weekly</td>
<td>25.0*</td>
<td>20 (11)</td>
<td>245</td>
<td>27</td>
<td>7.5</td>
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<td></td>
<td>5.0</td>
<td>20 (9)</td>
<td>155</td>
<td>35</td>
<td>8.0</td>
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<tr>
<td></td>
<td>2.5</td>
<td>19 (4)</td>
<td>127</td>
<td>41</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>13 (3)</td>
<td>54</td>
<td>36</td>
<td>26.0</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
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<td>0 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>DMBA, 5 µg, once only; PMA, 1 time weekly</td>
<td>5.0</td>
<td>15 (8)</td>
<td>70</td>
<td>86</td>
<td>14.5</td>
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<tr>
<td></td>
<td>2.5</td>
<td>11 (3)</td>
<td>32</td>
<td>41</td>
<td>22.5</td>
</tr>
<tr>
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<td>0.5</td>
<td>4 (0)</td>
<td>4</td>
<td>86</td>
<td>31.0</td>
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<td>0</td>
</tr>
<tr>
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<td>0.02</td>
<td>0 (0)</td>
<td>0</td>
<td>0</td>
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<tr>
<td>PMA only, 3 times weekly</td>
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<td>5 (0)</td>
<td>9</td>
<td>53</td>
<td>3800</td>
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<tr>
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<td>5.0</td>
<td>2 (0)</td>
<td>2</td>
<td>170</td>
<td>780</td>
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<td>0</td>
<td>0</td>
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<td>1</td>
<td>237</td>
<td>78</td>
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<td>0</td>
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</table>

* Papillomas; squamous carcinomas are given in parentheses.
† From beginning of treatment with PMA.
‡ Time to 50% animals with papillomas.
§ Dose required for 50% animals with papillomas.
$ Median survival time, 246 days.

for the 2 experiments were similar and even slightly higher for the lower dose applied more frequently. Also the time to 1st tumor is considerably shorter for the latter experiment.

Toxicity of PMA to Mouse Skin. The administration of PMA to the dorsal epithelium of mice induced an acute, dose-related, inflammatory response. The degree of inflammation was graded 1 to 4 as defined in Fig. 1. Maximal inflammation, Grade 4, was observed 24 hr after a single application of 2.5 or 5 µg of PMA. By 72 hr a Grade 2 inflammation was observed with both doses. Doses of 0.5, 0.10, and 0.02 µg resulted in Grade 1 or Grade 2 inflammation in 24 hr and this subsided after 72 hr. The continued application of PMA at 2.5 µg/application 3 times weekly resulted in a continued Grade 1 to 2 inflammation accompanied by a diffuse low-level epidermal hyperplasia persisting up until the time of appearance of the 1st papilloma (~35 days).

Tissue sections examined after 1 year of treatment showed that papillomas can occur in essentially normal skin where neither a general inflammatory response nor epidermal hyperplasia was evident suggesting that the induction of papillomas by PMA may not require the concomitant occurrence of these manifestations of tissue damage.

DISCUSSION

These studies were undertaken to obtain a definitive dose response for the tumor-promoting activity of PMA in initiation-promotion experiments on mouse skin. In addition, the inflammatory response in the skin, induced by the tumor promoter, was examined to determine whether any relationship could be found between this skin response and the tumor-promoting activity of PMA.

Of the several parameters examined in relation to the dose of tumor promoter, the total number of papillomas and cancers most accurately reflects the potency of PMA. The precision of cumulative papilloma measurements is somewhat vitiated by the tendency of papillomas to aggregate and coalesce as the experiments progress. Considering the t50, a dose response is obtained as shown in Chart 3. At weekly doses above 7.5 µg, there is no decrease in t50 with increasing dose levels. Below this value, the t50 increases directly as a function of the weekly dose, regardless of application frequency, reaching an apparent threshold at ~0.3 to 0.5 µg PMA per week. Whether this threshold is apparent or real is unknown, and experiments with increased numbers of test animals will be required to resolve this question.
We have noted on several occasions the remarkable constancy of time to appearance of 1st tumor over a wide range of initiator and promoter doses (9). There appears to be an irreducible minimum of about 4 weeks between the beginning of treatment with promoter and the 1st clinical appearance of a papilloma. The present studies confirm this phenomenon.

The question of whether an inflammatory response and epidermal hyperplasia are necessary prerequisites for tumor development has been argued for many years (3, 7, 9). Frei and Stephens (3), using a series of chemically ill-defined tumor-promoting agents, suggested that stimulation of cellular proliferation is an important feature of the activity of these agents. On the other hand, Raick et al. (7) using PMA as a promoting agent concluded that cell proliferation and hyperplasia are not sufficient to explain the mode of action of tumor-promoting agents. Further, in recent studies by Nebert et al. (6), the offspring of crosses of inbred mice with widely disparate inflammatory response to PMA were examined for their tumor promotion response after a single treatment with DMBA. In F₂ crosses of C57BL and DBA mice, the authors found that, in siblings that have no gross inflammatory reaction to phorbol ester, there is no correlation between inflammation and tumor response. Our results indicate that inflammation and epidermal hyperplasia occur

**Chart 1.** Two-stage carcinogenesis with 5 μg DMBA followed by PMA, applied 3 times weekly. Curve 1, 25 μg; Curve 2, 5.0 μg; Curve 3, 2.5 μg; Curve 4, 0.5 μg.

**Chart 2.** Two-stage carcinogenesis with 5 μg DMBA followed by once weekly application of PMA. Curve 1, 5.0 μg; Curve 2, 2.5 μg; Curve 3, 0.5 μg.

**Chart 3.** Effect of dose on t₅₀: O, 3 times weekly applications, this study; X, once weekly application, this study; ●, twice weekly application (5); △, once weekly application (7). Weeks on test are from beginning of promoting treatment.
concomitantly with tumor promotion by PMA. However, these skin effects are not necessarily related to skin tumor induction. Thus, at low doses of PMA (0.02 and 0.1 μg/ml), an inflammatory response is observed without subsequent tumor induction, even after treatment for 1 year at these doses (see Table 1). Further, one often observes the appearance of papillomas in areas adjacent to skin that exhibits no notable pathological changes.

It is clear that the mode of action of PMA must be much more specific than the general responses of hyperplasia and inflammation. The exact mode of action is as yet unknown; however, experiments in cell culture have strongly implicated the cell membrane as a primary receptor site of the phorbol esters (8, 9). The induction of cell division and preferential outgrowth of neoplastic cells by PMA with in vitro systems (8) suggest that a similar situation may obtain in vivo on mouse skin.

REFERENCES

Fig. 1. Sections of skin illustrative of the varying degrees of inflammation and representative of 4 grades of inflammation. A, Grade 1; B, Grade 2; C, Grade 3; D, Grade 4. Inflammatory response of mouse skin following PMA treatment. In the mildest inflammation (Grade 1), most of the inflammatory cells are lymphocytes. As the inflammatory response increases, an increasingly larger percentage of the cells are neutrophils (Grades 2 and 3). The focal sloughing of epidermis is a feature of the most severe inflammation (Grade 4). x 40.
Fig. 1. C to D.
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