The Effect of Aging and Interval between Primary and Secondary Treatment in Two-Stage Carcinogenesis on Mouse Skin

B. L. Van Duuren, A. Sivak, C. Katz, I. Seidman, and S. Melchionne

Laboratory of Organic Chemistry and Carcinogenesis, Institute of Environmental Medicine [B. L. V. D., A. S., C. K., S. M.], and Department of Pathology [I. S.], New York University Medical Center, New York, New York, 10016

SUMMARY

Two-stage carcinogenesis experiments on mouse skin (female ICR/Ha Swiss mice) were done by initiating mice at three age levels (6, 44, and 56 weeks) and promoting after a 2-week interval. In another series, mice were initiated at age 6 weeks, and three time intervals (2, 36, and 56 weeks) were used between initiation and promotion. The initiating agent was 7,12-dimethylbenz(a)anthracene and the promoting agent was phorbol myristate acetate in all experiments. The results showed a general decrease in tumor production with increasing age at the time of promotion. However, the initiating effect persisted even when the interval between initiation and promotion was 56 weeks.

INTRODUCTION

Several reports have appeared concerning the effect of aging in chemical carcinogenesis. In these studies, a variety of carcinogenic chemicals were given to mice and rats by different administration routes (2, 3, 5, 6, 8, 12).

The effect of varying intervals between initiation and promotion in 2-stage carcinogenesis has been examined by several workers (1, 4, 7, 9, 10), using croton oil, croton resin, or PMA2 as the promoter on mouse skin, and DMBA or urethane as the initiator, by various administration routes.

This study was undertaken in order to determine the nature of the persistence of the initiating effect of DMBA and the effect of aging in 2-stage carcinogenesis on mouse skin with PMA as promoter. The initiator and promoter were applied at 3 stages during the animals' life-span, and 3 time intervals were used between initiation and promotion. This is the first extensive study of this kind with the pure and potent tumor-promoting agent, PMA.

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 put on test at age 6 weeks. The 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 rooms were maintained at 22-24°.

Bioassay Procedure. The backs of the mice were clipped free of hair the day before the initial treatment and, as needed, for the duration of the experiment. The initiating agent, DMBA, was applied by micropipet in a single dose (20 μg) in 0.1 ml acetone. This primary treatment was followed at various intervals by applications of PMA, 2.5 μg, in 0.1 ml acetone, 3 times weekly for the life-spans of the animals. Included in the protocol were control groups given initiator or promoter alone, or solvent alone, and groups receiving no treatment.

All experiments were continued until there were no survivors. Secondary treatment, i.e., PMA or acetone, was continued until there were no survivors. The specific details of the experimental protocol are depicted in Chart I.

The mice were observed regularly; tumors were recorded,

[Table]

<table>
<thead>
<tr>
<th>EXPERIMENT AND NUMBER</th>
<th>TREATMENT AND AGE (WEEKS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>INITIATION ONLY</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
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<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>11</td>
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</table>

Chart 1. Protocol for determining effect of aging and interval between initiation and promotion in 2-stage carcinogenesis.

1 This work was supported by USPHS Grants CA-15095 from the National Cancer Institute and ES-00260 from the National Institutes of Environmental Health Sciences.

2 The abbreviations used are: PMA, phorbol myristate acetate (trivial name for Chemical Abstracts Registry No. 20839-11-6); DMBA, 7,12-dimethylbenz(a)anthracene.

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and those greater than 1 cu mm were counted and charted twice monthly. Only tumors persisting for 30 days or more were counted in the cumulative totals.

Mice bearing tumors appearing grossly as carcinomas were killed approximately 2 months after the tumors were clinically classified as cancers. Animals in moribund condition were also killed for necropsy. All mice were necropsied, and specimens from tumors and all abnormal tissues and organs were excised, fixed in 4% formalin, blocked in paraffin, and stained with hematoxylin and eosin for histopathological diagnosis.

**Chemicals.** PMA was prepared from croton oil by the procedure developed in our laboratory (11). DMBA was freshly recrystallized in acetone and the solutions were prepared immediately before use. Spectroscopic-grade acetone was used for preparation of all solutions. The acetone was routinely checked for purity from one batch to the next by spectrofluorimetry.

**RESULTS**

The experiments described in this paper were undertaken with a 2-fold purpose: to determine the effect of aging in 2-stage mouse skin carcinogenesis and to determine the effect of interval between initiation and promotion in the same test system. The protocol for the experiments is best shown in diagrammatic form (Chart 1). The results of all the experiments are given in Table 1.

In spite of the wide variation in group sizes, which resulted from the operational complexities involved, it is instructive to compare the findings. In Groups 1, 2, and 3, early, middle, and late in terms of life-span, experiments were carried out, while keeping the interval between initiation and promotion constant, i.e., 14 days. In all 3 experiments, the time to 1st tumor was between 31 and 47 days from the beginning of promoting treatment. However, there was a decreased rate of appearance of papillomas in Groups 2 and 3, i.e., groups in which initiation and promotion were started in older animals, compared with Group 1. This decrease is depicted in Chart 2. The final percentage of animals with papillomas was not markedly affected (Chart 2); however, there was a notable decrease in the average number of tumors per tumor bearer in Groups 1, 2, and 3, i.e., 9.4, 8.7, and 4.2, respectively. The carcinoma incidence also showed a marked decline in the 3 groups (Chart 3).

It is next instructive to compare Groups 1, 4, and 5. In these 3 groups the intervals between initiation and promotion were 2, 36, and 56 weeks, respectively. There was a decreased rate of tumor appearance as the time interval between initiation and promotion was increased (Chart 4), and the final percentage of animals with papillomas was not much affected. The average number of tumors per tumor

**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Primary treatment</th>
<th>Secondary treatment</th>
<th>Interval between primary and secondary treatment (wk)</th>
<th>Age of mice at secondary treatment (wk)</th>
<th>No. of mice in test group</th>
<th>Days to 1st papilloma after secondary treatment</th>
<th>No. of mice with papillomas/total no. of papillomas</th>
<th>No. of mice with squamous carcinoma</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>DMBA</td>
<td>PMA</td>
<td>2</td>
<td>8</td>
<td>119</td>
<td>31–57</td>
<td>118/1107</td>
<td>61</td>
</tr>
<tr>
<td>2</td>
<td>DMBA</td>
<td>PMA</td>
<td>2</td>
<td>46</td>
<td>19</td>
<td>41</td>
<td>17/148</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>DMBA</td>
<td>PMA</td>
<td>2</td>
<td>58</td>
<td>51</td>
<td>47–50</td>
<td>28/117</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>DMBA</td>
<td>PMA</td>
<td>2</td>
<td>35</td>
<td>35</td>
<td>18–40</td>
<td>31/157</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>DMBA</td>
<td>PMA</td>
<td>2</td>
<td>35</td>
<td>35</td>
<td>28–48</td>
<td>20/63</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>DMBA</td>
<td>Acetone</td>
<td>2</td>
<td>35</td>
<td>35</td>
<td>268</td>
<td>2/3</td>
<td>1</td>
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<tr>
<td>7</td>
<td>DMBA</td>
<td>None</td>
<td>2</td>
<td>25</td>
<td>209</td>
<td>1/1</td>
<td>0</td>
<td>0</td>
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<tr>
<td>8</td>
<td>DMBA</td>
<td>None</td>
<td>2</td>
<td>25</td>
<td>217</td>
<td>1/2</td>
<td>1</td>
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<td>9</td>
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<td>PMA</td>
<td>8</td>
<td>123</td>
<td>107–347</td>
<td>32/45</td>
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<tr>
<td>10</td>
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<td>8</td>
<td>39</td>
<td>214–288</td>
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<tr>
<td>11</td>
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<td>PMA</td>
<td>62</td>
<td>55</td>
<td>137</td>
<td>1/1</td>
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</tbody>
</table>

* Given only once (20 μg in 0.1 ml acetone).
* PMA, 2.5 μg in 0.1 ml acetone, was given 3 times weekly.
* The results presented in this Table for Groups 1 to 11 were from several groups of animals run concurrently, but as separate tests; the days to 1st tumor varied from test to test, and the days given are from the shortest to the longest intervals to 1st tumor, taken from the different tests. All experiments were continued until there were no survivors.
bears decreased from Groups 1 to 4 to 5, and the percentage of animals with carcinoma decreased markedly (Chart 3).

The patterns of tumor induction, in a comparison of groups 1, 2, and 3 with groups 1, 4, and 5, are thus very similar and suggest that the age of the animals at the time of promotion is an overriding factor. The persistence of the initiating effect of a single dose of DMBA is nonetheless clear.

Groups 6 through 11 are the necessary control experiments for Groups 1 to 5. These control groups show the low but expected tumor incidences listed in Table 1.

DISCUSSION

Although a number of earlier studies have been carried out on the effect of aging in chemical carcinogenesis (2, 3, 5, 6, 8, 12), only a few of these have a direct bearing on the present studies. Some of the earlier studies (6, 8, 12) dealt with comparisons of newborn and older animals, and these do not bear on the present studies. Forbes (3) studied the effect of DMBA-induced mouse skin carcinogenesis in Rhino mice and found that the younger mice were more susceptible to skin carcinogenesis than older mice. However, the interpretation of that study is complicated by the fact that Rhino mice show marked and unusual pathological changes in the skin with aging. Ebbesen (2) also found that aging increases the susceptibility of mouse skin to DMBA-induced carcinogenesis in which skin grafting was used in syngeneic hosts of various ages.

Several earlier studies demonstrated the persistence of the initiating effect in 2-stage carcinogenesis (1, 4, 9, 10). This is again demonstrated in a more extensive experiment in this report. However, the earlier studies were not specifically designed to examine also the effect of aging in 2-stage carcinogenesis. The latter is directly interrelated with experiments in which there is a prolonged interval between initiation and promotion.

The experiments described in this work were designed to take both of the above-mentioned factors into consideration. We have demonstrated in these experiments that there is a decrease in tumor yield when there is a long time interval between initiation and promotion (Group 5) or when 56-week-old animals are initiated with DMBA, followed by continued promotion for their life-spans (Group 3). Therefore, the age of the animals at the beginning of the secondary treatment is important and is the overriding factor in determining the final tumor yields. The tumor yields are considerably higher when the initiating and/or promoting treatments are started at age 42 or 44 weeks (Groups 2 and 4), compared with Groups 3 and 5.

There is 1 report in the literature (7) which is at variance with the present work and with that referred to above (1, 4, 9, 10) concerning the persistence of the initiating effect. Roe et al. (7) suggest that they have obtained evidence for the reversibility of initiation” in 2-stage carcinogenesis. In their experiments, mice were given 100 μg DMBA, followed 50 weeks later by twice weekly applications of 3.5 μg PMA for 20 weeks. They observed virtually no tumors. In our experiments reported here, mice were given 20 μg DMBA, followed 56 weeks later by 3 weekly applications of 2.5 μg PMA for their life-spans. Their experiment was complicated by (a) twice weekly i.p. injections of distilled water and (b) termination of the test at 20 weeks after the beginning of promoting treatment. Thus, their conclusions cannot be reconciled with the overwhelming evidence for the persistence of the initiating effect of a single application of DMBA in 2-stage carcinogenesis provided by earlier studies (1, 3, 4, 9, 10) and that presented in this report.

The most likely explanation for the present observation and those described earlier (1, 4, 9, 10) lies in the shorter life expectancy of mice given carcinogenic chemical treatment later during their life-spans.

REFERENCES


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