ABSTRACT

The influence of initiator dose and promoter dose, duration, and type on the progression of papillomas to carcinomas was examined in Sencar mice. A good dose-response relationship for promotion of papilloma formation by 12-O-tetradecanoylphorbol-13-acetate (TPA) [following initiation with 6.5 µg of 7,12-dimethylbenz(α)anthracene (DMBA)] was observed in the range of 0.125 to 2.0 µg/mouse. A maximal papilloma response was induced with 2 µg/mouse (24 papillomas/mouse). When adjusted for mortality, the carcinoma incidence after 60 wk of promotion was essentially the same (∼80%) for doses above 0.5 µg/mouse. In a related experiment, mice were given an initiation dose of either 2 or 20 µg of DMBA followed by applications of 2 µg of TPA for 3, 5, 7, or 60 wk. Papilloma formation was proportional to length of treatment, with a maximum of 29 papillomas/mouse (20-µg initiating dose of DMBA) and 10 papillomas/mouse (2-µg initiating dose of DMBA) occurring between 10 and 15 wk of promotion. In this experiment, the carcinoma incidence was clearly proportional to the duration of promoter treatment at the low initiation dose of DMBA. The carcinoma incidence, on the other hand, was similar (∼70%) in groups of mice given an initiation dose of 20 µg of DMBA and promotion treatment for ≥5 wk. Thus, the initiator dose had a dramatic effect on the outcome of these experiments. Additional experiments were performed to compare tumor progression with the transplanted promoter, chrysarobin. At optimal promoting doses, chrysarobin treatment produced a maximum number of papillomas that was approximately 1/2 that produced by TPA (6.4 versus 17.0 papillomas per mouse, respectively). However, the carcinoma response was very similar in these two treatment groups, confirming previous work from this laboratory. In addition, chrysarobin treatment following 10 wk of TPA promotion did not enhance the progression of preexisting papillomas to carcinomas. The data presented in this paper are consistent with a model in which several types or stages of papillomas are initially produced during two-stage carcinogenesis in mouse skin with different probabilities of progressing to carcinomas. However, the data indicate that optimal doses of promoter and initiator exist and can influence interpretation of tumor progression studies in mouse skin.

INTRODUCTION

The standard initiation-promotion protocol of chemical carcinogenesis in Sencar mice utilizing TPA as the promoter results, after a short latency period, in the formation of multiple papillomas, followed later by the development of a relatively few SCC (1, 2). Initiation is an irreversible step in which genetic change occurs, possibly in a gene(s) controlling epidermal differentiation (3, 4). Promotion, on the other hand, appears to operate primarily through epigenetic mechanisms (3, 4). It has been postulated that the conversion from papilloma to SCC is mediated by a second genetic event (6) and that the tumor promoter TPA has little if any effect on the conversion of papillomas to SCC, supporting the concept of epigenetic phenomena in skin tumor promotion. Recently, gross chromosomal alterations have been suggested as candidates for the additional genetic changes that may permit the malignant conversion of papillomas to SCC (7, 8).

The papillomas that initially develop during mouse skin initiation-promotion protocols are considered heterogeneous in that some will persist, some will disappear or regress, and only relatively few (5 to 7%) will "progress" to an invasive carcinoma during the time frame of most experiments (9–14). This information has led to the hypothesis that different subclasses of papillomas exist with different probabilities of malignant progression and, indeed, that some papillomas (referred to as "terminally benign") (10–12) are not at risk for progression to SCC. Support for the latter concept was recently reported by Hennings et al. (13). They reported that only 5 wk of TPA treatment in Sencar mice were required to produce a carcinoma response similar to that in mice receiving continual TPA treatment. Verma and Boutwell (14) reported that 18 wk of twice weekly treatments with TPA were necessary to produce a maximal carcinoma response. The differences between these two studies lie in the observations by Verma and Boutwell (14) that a maximal papilloma response (i.e., 18 wks of promotion in CD-1 mice) was necessary to produce a maximal carcinoma response, whereas in the study of Hennings et al. (13), in Sencar mice a papilloma response only ∼20% (i.e., after 5 wk of promotion in their study) that of the maximal response was sufficient to achieve a maximal carcinoma response. Nevertheless, even in CD-1 mice, it should be noted that the persistent papillomas produced by short-term TPA promotion had a high rate of conversion to carcinomas (13, 14). The implications of these data are that the additional papillomas produced by continual TPA treatment had little or no probability to progress to malignancy. Conversely, Aldaz et al. (15) reported a systematic histological study of papillomas at different times during promotion. In these studies, papillomas were induced using the standard initiation-promotion protocol using TPA promotion. Interestingly, by 16 to 20 wk most of the tumors were moderately to severely dysplastic. These data suggested that most of the tumors generated by initiation-promotion protocols have the potential to become SCC. It is clear, however, from the work of other laboratories (5, 9–14), as well as our own (12, 16), that most of these tumors never convert to SCC during the time frame of these experiments. Such observations suggest that other mechanisms might be involved in limiting the conversion of papillomas to SCC in initiation-promotion protocols.

The present study was designed to provide further evidence for or against the existence of subpopulations of papillomas with different probabilities of progressing to SCC and to attempt to resolve the issue of whether a maximal papilloma response is necessary for a maximal carcinoma response. Through the use of different treatment durations as well as different initiator doses, we conclude that duration of promoter treatment is necessary until a nearly maximal papilloma response is achieved in order to achieve a maximal carcinoma response when using low initiation doses of DMBA. On the other hand, in experiments using continual TPA treatments,
the production of a maximal papilloma response was not necessary to obtain a maximal carcinoma response. Possible mechanisms for the differences between these experimental protocols are discussed.

MATERIALS AND METHODS

Chemicals. DMBA was purchased from the Eastman Kodak Co. (Rochester, NY). Chemicals for Cancer Research (Eden Prairie, MN) supplied the TPA used in these studies. Chrysarobin was purchased from ICN Pharmaceuticals, Inc. (Plainview, NY) and purified prior to use as previously described (17).

Animals. Female Sencar mice were obtained from Research Biogenetics, Inc. (Bastrop, TX) and were used for experimentation at 7 to 9 wk of age. The dorsal skin of each mouse was carefully shaved 2 days prior to initiation; only those mice in the resting phase of the hair growth cycle were utilized. At least 30 mice were used for each experimental group. Mice were weighed biweekly, and the tumor incidence and morbidity/mortality were recorded weekly.

Tumor Induction Experiments. All solutions of DMBA, TPA, and chrysarobin were prepared in reagent-grade acetone and were applied topically in a total volume of 0.2 ml. Control mice were treated with 0.2 ml of acetone. Tumors were initiated in the mice by a single application of DMBA at the concentrations indicated (2, 2.56, 6.5, or 20 µg per mouse). Two wk after initiation twice weekly applications of various doses of TPA (0.125 to 2.0 µg per mouse) were begun and continued throughout the experiment. In a separate experiment, the mice were treated with twice weekly applications of 2 µg of TPA for durations of 3, 5, 7, or 60 wk. In the third experiment, groups of mice were initiated with DMBA followed by continual twice weekly treatments with 2 µg of TPA or weekly treatments with 24 µg of chrysarobin. Additional groups received promotion with 2 µg of TPA for 10 wk followed by continual applications of either acetone or chrysarobin. Another group received acetone treatment for 10 wk followed by weekly applications of chrysarobin. Carcinomas were recorded grossly as downward invading lesions. All suspected carcinomas and lesions with unusual morphology were examined histologically. When potential carcinoma-bearing mice were moribund, they were sacrificed so that all carcinomas could be verified histologically as squamous cell carcinomas and the degree of differentiation noted. In addition, necropsy was performed, and sections of lung and lymph nodes were taken for pathology. The carcinoma incidence and the average number of carcinomas per mouse were expressed as a percentage of the mice at risk at the time of the appearance of the first carcinoma. An adjustment for nontumorous mortality was utilized as described by Peto and coworkers, in that animals lost due to noncarcinoma-related events were not considered at risk (18). All experimental groups were continued for 60 wk after promotion was begun. Statistical significance was determined by the x² test with probability set at P ≤ 0.05.

Histological Evaluation. Tissues for histological evaluation were prepared using conventional paraffin sections and hematoxylin-eosin staining. Squamous cell carcinomas were classified as Grade I, II, or III (GI, GII, or GIII, respectively) according to the degree of differentiation as described previously by our laboratories (16).

RESULTS

Dose-Response Studies with TPA. The effects of TPA dose on the formation of papillomas in female Sencar mice are shown in Fig. 1. In agreement with previous studies (2, 19), there was a very good dose-response relationship for papilloma formation at doses above 0.25 µg per mouse. Interestingly, there were marked differences in the health of the animals in the different TPA dose groups. In this regard, average weight gain per mouse was inversely proportional to the promoter dose (data not shown). The dose of TPA had an even more dramatic effect on the longevity of the animals. Fig. 2 shows total deaths as a function of treatment dose, which includes carcinoma-bearing mice as well as mice that died of unrelated causes. Survival was inversely proportional to the treatment dose, so that TS₅₀ was reached at 36, 41, 45, or >57 wk for doses of 2, 1, 0.75, or 0.5 µg per mouse and below, respectively. The percentage of mice that died due to noncarcinoma-related factors was 35%, 26%, 12%, and 11% for the 2-, 1-, 0.75-, and 0.5-µg per mouse doses, respectively. For this reason to avoid bias, all carcinoma data were corrected for noncarcinoma-related deaths as described in “Materials and Methods” (and see Ref. 18).

The carcinoma response (incidence) was nearly the same for TPA doses above 0.5 µg per mouse, with the first carcinomas appearing in the highest dose groups (i.e., 0.75, 1, and 2 µg per mouse) after about 20 wk of promotion. There were no statistically significant differences in the carcinoma incidences between these 3 treatment groups (Table 1). An intermediate carcinoma response was observed for the 0.5-µg dose, and a very low carcinoma response was observed for doses below 0.5 µg, consistent with the lack of a significant papilloma response. Carcinoma multiplicity did show an upward trend correspond
different with the higher doses giving a single metastasis to the lung, while some animals receiving the lower doses displayed multiple metastases in the lung and in some cases metastasizes to the lymph nodes.

Promoter Duration and Initiator Dose Studies. The effect of promotion duration on the formation of papillomas and carcinomas is shown in Tables 3 and 4 and Figs. 3 and 4. For these experiments, female Sencar mice were given initiation doses of either 2 or 20 |g of DMBA as indicated. Promotion was achieved using twice weekly applications of 2 |g of TPA for 3, 5, 7, or 60 wk. Papilloma yield was proportional to the length of promotion regardless of the dose of DMBA used for initiation (Fig. 3; Table 3). Interestingly, little, if any, papilloma disappearance or regression was observed in the groups receiving limited treatments of TPA for 3, 5, or 7 wk or in the group initiated with 2 |g of DMBA and receiving continual promotion with TPA (Fig. 3A). The only group that showed a rapid loss of papillomas was the group given an initiation dose of 20 |g of DMBA followed by continual promotion with TPA (Fig. 3B).

The carcinoma response in mice given initiation doses of 2 |g of DMBA was found to be related to the length of promotion (Table 3). In this regard, the carcinoma response was highest in the 60-wk treatment group (81%), intermediate in the 7-wk treatment group (48%), and lowest in the 5-wk treatment group (14%). In a related experiment shown in Table 4, mice were given initiation doses of 2.56 |g of DMBA followed by continual promotion (Group 2) or for only 10 wk (Group 3) with TPA. The 10-wk TPA treatment group achieved a maximal papilloma response ~70% that of the continual TPA treatment group and had a very similar carcinoma response compared to the group receiving continual treatment with TPA. In contrast to these results using low initiation doses of DMBA (see Table 3), the carcinoma incidence in mice that had been initiated using 20 |g of DMBA was nearly the same (65 to 77%) in groups receiving TPA treatments for 5 wk or longer. Due to differences in papilloma yield, however, the ratio of carcinomas to papillomas was higher in the limited treatment groups at both initiation doses (Table 3). Although the carcinoma incidence was similar in all the 20-|g DMBA groups, an upward trend was noted in the carcinomas per mouse in that the 10-wk treatment group had the highest total number of carcinomas. No carcinomas were induced by promotion limited treatments of TPA for 3, 5, or 60 wk. Papilloma yield was proportional to the length of promotion regardless of the dose of DMBA used for initiation (Table 3). In this regard, the carcinoma response was highest in the 60-wk treatment group (81%), intermediate in the 7-wk treatment group (48%), and lowest in the 5-wk treatment group (14%). In a related experiment shown in Table 4, mice were given initiation doses of 2.56 |g of DMBA followed by continual promotion (Group 2) or for only 10 wk (Group 3) with TPA. The 10-wk TPA treatment group achieved a maximal papilloma response ~70% that of the continual TPA treatment group and had a very similar carcinoma response compared to the group receiving continual treatment with TPA. In contrast to these results using low initiation doses of DMBA (see Table 3), the carcinoma incidence in mice that had been initiated using 20 |g of DMBA was nearly the same (65 to 77%) in groups receiving TPA treatments for 5 wk or longer. Due to differences in papilloma yield, however, the ratio of carcinomas to papillomas was higher in the limited treatment groups at both initiation doses (Table 3). Although the carcinoma incidence was similar in all the 20-|g DMBA groups, an upward trend was noted in the carcinomas per mouse in that the continual TPA treatment group had the highest total number of carcinomas. No carcinomas were induced by promotion limited treatments of TPA for 3 wk regardless of the initiator dose. The length of promotion also affected the longevity of the mice. Animals receiving continual treatment with TPA had TS50 values of 39 and 32 wk for the 2-|g and 20-|g doses of DMBA, respectively. The limited duration treatment groups in general had 50% survival beyond

Table 1 Dose-response relationship for promotion of papillomas and carcinomas with TPA in Sencar mice

| Initiation | TPA dose (|g) | TS50* | Carcinoma incidence (%) | Carcinomas/mouse | Time to first carcinoma (wk) | Papillomas/mouse | Ratio* |
|------------|------------|-------|-------------------------|-----------------|--------------------------|-----------------|-------|
| DMBA (6.5 | 2.0        | 36    | 83                      | 1.58 ± 0.6      | 20                       | 23.9 ± 2.1      | 0.07  |
|           | 1.0        | 41    | 80                      | 0.92 ± 0.3      | 21                       | 13.5 ± 0.9      | 0.07  |
|           | 0.75       | 45    | 82                      | 1.36 ± 0.5      | 22                       | 11.6 ± 1.0      | 0.12  |
|           | 0.50       | 57*   | 50*                     | 0.75 ± 0.6      | 29                       | 6.8 ± 0.7       | 0.11  |
|           | 0.25       | >60*  | 9*                      | 0.08 ± 0.1      | 30                       | 0.7 ± 0.3       | 0.11  |
|           | 0.125      | >60*  | 4*                      | 0.04 ± 0.1      | 28                       | 0.3 ± 0.2       | 0.13  |
|           | Acetone    |       | 0*                      |                 |                          | 0.3 ± 0.2       |       |

* TS50 is based on the number of animals present at the time the first carcinoma appeared and is defined as the time (in weeks) required to reach 50% survival.

† Derived from data given in Fig. 1.

‡ Ratio of papillomas to carcinomas = av. carcinomas/mouse at 60 wk ± SEM.

§ av. papillomas/mouse at plateau

× Mean ± SEM.

† Significantly different than the 2-, 1.0-, and 0.75-|g/mouse groups (P ≤ 0.01, df = 1). All other groups not significantly different.

‖ Significantly less than incidence for 2, 1, and 0.75 |g of TPA (P < 0.05, df = 1).
52 wk, except in the group initiated with 20 µg of DMBA and promotion for 7 wk, where the T50 was 38 wk.

Tumor Progression during Promotion with a Nonphorbol Ester. As part of the present study, we also examined the progression of papillomas to carcinomas with the anthrone promoter chrysarobin. Compared to TPA, promotion with weekly applications of chrysarobin resulted in the induction of fewer papillomas, but a similar number of carcinomas (Table 4), confirming our previous work (16, 19). Additionally, a 10-wk delay in chrysarobin promotion (Group 5) resulted in an increased papilloma response as previously reported (16). As noted above, when TPA treatment was discontinued after 10 wk a similar number of carcinomas developed compared to the group receiving continual TPA treatment. Group 4 of Table 4 was included to determine whether the high ratio of carcinomas to papillomas produced by chrysarobin could be attributed to an ability of chrysarobin to enhance progression of papillomas to carcinomas similar to that reported for benzoyl peroxide (20). Treatment with once weekly applications of chrysarobin for the duration of the experiment following 10 wk of TPA treatment did not enhance the progression of preexisting papillomas to SCC.

Tumor Classification. Finally, the type of promotion protocol had no obvious correlation with the degree of differentiation found in the carcinomas. The majority of the carcinomas were classified as GI with only a very few tumors classified as GII. Also, there are no statistically significant differences in the incidence of Groups 5, 6, and 7.

In our present study, overall survival was inversely proportional to the dose of TPA as well as to tumor burden. In addition, the greatest percentage of noncarcinoma-related deaths occurred in the two highest TPA dose groups. Thus, in the 1- and 2-µg/mouse TPA dose groups, more animals died without carcinomas than in the lower dose groups. These observations would be expected to reduce the final carcinoma response in the high dose groups, and hence all data were corrected for noncarcinoma deaths (18). Even after this correction for noncarcinoma deaths, the present study has examined the progression of papillomas to SCC in mouse skin using several different promotion protocols. The first experiment examined the dose-response relationship for papilloma and carcinoma formation in Sencar mice using six doses of TPA and a relatively low initiating dose of DMBA. Interestingly, the carcinoma incidence was nearly the same for doses greater than 0.5 µg of TPA/mouse, indicating that, although a 2-µg/mouse dose of TPA produces a maximal papilloma response, it does not lead to a greater carcinoma response compared to lower doses. There are several possible explanations for the results obtained in this dose-response study. (a) Lower doses of TPA may be capable of selecting for subclasses of papillomas with a higher probability of progression to SCC, whereas higher doses may stimulate the development of additional papillomas (11, 13), which have little tendency to progress to SCC. The data in Table 1 do indicate a higher ratio of carcinomas/papillomas in groups treated with lower doses of TPA (≤0.75 µg/mouse) supporting this hypothesis.

(b) An alternative explanation for the similar carcinoma responses in mice receiving TPA doses of >0.5 µg/mouse, which cannot be ruled out at the present time, may involve a combination of factors including local as well as systemic toxicity produced by TPA and high tumor burdens. Klein-Szanto and coworkers (21) reported that chronic treatment of noninitiated Sencar mouse skin with TPA produced systemic toxicity. In particular, chronic administration of TPA alone produced a generalized amyloidosis involving both liver and spleen as well as interstitial nephritis. Furthermore, a 2-µg/mouse dose of TPA led to a significant decrease in survival over a 1-yr period compared to nontreated animals (21). High papilloma burdens may also have adversely affected the conversion of papillomas to carcinomas due to competition for available space and blood supply or alternatively as a result of systemic affects on the host.

In the present study, overall survival was inversely proportional to the dose of TPA as well as to tumor burden. In addition, the greatest percentage of noncarcinoma-related deaths occurred in the two highest TPA dose groups. Thus, in the 1- and 2-µg/mouse TPA dose groups, more animals died without carcinomas than in the lower dose groups. These observations would be expected to reduce the final carcinoma response in the high dose groups, and hence all data were corrected for noncarcinoma deaths (18). Even after this correction

| Group | Initiation | Promotion duration (wk) | T50 | Carcinoma incidence (%) | Carcinomas/mouse | Papillomas/mouse | Ratio
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMBA (2 µg)</td>
<td>60</td>
<td>39</td>
<td>81</td>
<td>1.15 ± 0.4</td>
<td>9.9 ± 0.6</td>
<td>0.12</td>
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<tr>
<td>2</td>
<td>7</td>
<td>57</td>
<td>48</td>
<td>0.56 ± 0.3</td>
<td>1.4 ± 0.4</td>
<td>0.43 ± 0.2</td>
<td>0.33</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>&gt;60</td>
<td>14</td>
<td>0.14 ± 0.2</td>
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<td>0.14 ± 0.2</td>
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</tr>
<tr>
<td>4</td>
<td>3</td>
<td>&gt;60</td>
<td>0</td>
<td>0</td>
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<tr>
<td>5</td>
<td>DMBA (20 µg)</td>
<td>60</td>
<td>32</td>
<td>77</td>
<td>1.50 ± 0.3</td>
<td>29.0 ± 1.5</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>38</td>
<td>70</td>
<td>1.00 ± 0.2</td>
<td>12.1 ± 1.1</td>
<td>0.08</td>
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<tr>
<td>7</td>
<td>5</td>
<td>52</td>
<td>65</td>
<td>0.92 ± 0.2</td>
<td>6.3 ± 0.2</td>
<td>0.15</td>
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<tr>
<td>8</td>
<td>3</td>
<td>&gt;60</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.24 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Effect of TPA dose on SCC metastases in mouse lung and lymph node

DISCUSSION

The present study has examined the progression of papillomas to SCC in mouse skin using several different promotion protocols. The first experiment examined the dose-response relationship for papilloma and carcinoma formation in Sencar mice using six doses of TPA and a relatively low initiating dose of DMBA. Interestingly, the carcinoma incidence was nearly the same for doses greater than 0.5 µg of TPA/mouse, indicating that, although a 2-µg/mouse dose of TPA produces a maximal papilloma response, it does not lead to a greater carcinoma response compared to lower doses. There are several possible explanations for the results obtained in this dose-response study. (a) Lower doses of TPA may be capable of selecting for subclasses of papillomas with a higher probability of progression to SCC, whereas higher doses may stimulate the development of additional papillomas (11, 13), which have little tendency to progress to SCC. The data in Table 1 do indicate a higher ratio of carcinomas/papillomas in groups treated with lower doses of TPA (≤0.75 µg/mouse) supporting this hypothesis.

(b) An alternative explanation for the similar carcinoma responses in mice receiving TPA doses of >0.5 µg/mouse, which cannot be ruled out at the present time, may involve a combination of factors including local as well as systemic toxicity produced by TPA and high tumor burdens. Klein-Szanto and coworkers (21) reported that chronic treatment of noninitiated Sencar mouse skin with TPA produced systemic toxicity. In particular, chronic administration of TPA alone produced a generalized amyloidosis involving both liver and spleen as well as interstitial nephritis. Furthermore, a 2-µg/mouse dose of TPA led to a significant decrease in survival over a 1-yr period compared to nontreated animals (21). High papilloma burdens may also have adversely affected the conversion of papillomas to carcinomas due to competition for available space and blood supply or alternatively as a result of systemic affects on the host.

In the present study, overall survival was inversely proportional to the dose of TPA as well as to tumor burden. In addition, the greatest percentage of noncarcinoma-related deaths occurred in the two highest TPA dose groups. Thus, in the 1- and 2-µg/mouse TPA dose groups, more animals died without carcinomas than in the lower dose groups. These observations would be expected to reduce the final carcinoma response in the high dose groups, and hence all data were corrected for noncarcinoma deaths (18). Even after this correction

Table 3 Influence of duration of promoter treatment on formation of papillomas and carcinomas in Sencar mice

Groups of at least 30 mice were initiated with the dose of DMBA indicated and then received twice weekly treatments with 2 µg of TPA for the durations shown.
Table 4 Conversion of papillomas to carcinomas using a phorbol ester versus an anthrone in Sencar mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Promotion (10 wk)</th>
<th>Progression (50 wk)</th>
<th>T50*</th>
<th>Carcinoma incidence (%)</th>
<th>Carcinomas/mouse*</th>
<th>Papillomas/mouse*</th>
<th>Ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chrysarobin</td>
<td>Chrysarobin</td>
<td>42</td>
<td>65</td>
<td>1.27 ± 0.5</td>
<td>6.4 ± 0.04</td>
<td>0.20</td>
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<tr>
<td>2</td>
<td>TPA</td>
<td>TPA</td>
<td>40</td>
<td>86</td>
<td>1.36 ± 0.3</td>
<td>17.0 ± 0.3</td>
<td>0.08</td>
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<tr>
<td>3</td>
<td>TPA</td>
<td>Acetone</td>
<td>43</td>
<td>74</td>
<td>1.19 ± 0.4</td>
<td>11.9 ± 0.1</td>
<td>0.10</td>
</tr>
<tr>
<td>4</td>
<td>TPA</td>
<td>Chrysarobin</td>
<td>42</td>
<td>87</td>
<td>1.52 ± 0.5</td>
<td>13.3 ± 1.2</td>
<td>0.11</td>
</tr>
<tr>
<td>5</td>
<td>Acetone</td>
<td>Chrysarobin</td>
<td>44</td>
<td>70</td>
<td>1.30 ± 0.1</td>
<td>11.3 ± 1.4</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* T50 is based on the number of animals present at the time of the appearance of the first carcinoma and is defined as the time (in weeks) required to reach 50% survival.

Carcinoma data are given at 60 wk of promotion and corrected as described under "Materials and Methods." There are no statistically significant differences between the carcinoma incidences for Groups 1 and 5; 2 and 3 or 4 (P ≥ 0.05, df = 1).

Papilloma data are given at the plateau of papilloma formation. The papillomas per mouse in Group 5 were significantly (P < 0.05) greater than Group 1 (using Student's t test).

Ratio of papillomas to carcinomas = av. carcinomas/mouse after 60 wk

av. papillomas/mouse at plateau

Mean ± SEM.
slightly higher numbers were present in the 2-¿g/mouse group. Thus, many papillomas were apparently lost in the high TPA dose groups, ultimately reducing the number available for conversion to carcinomas. Possible reasons for the loss of papillomas include local toxicity produced by TPA; the large number of papillomas per unit area of skin, leading to competition for available blood supply and ultimately ischemic necrosis; and coalescence of tumors. These and possibly other factors may have significantly reduced the final carcinoma response in the high TPA dose groups and remain to be explored.

Another interesting finding in our dose-response experiment involved metastases. Only one single metastasis was observed in the higher TPA dose groups (i.e., 2 and 1 ¿g/mouse). In contrast, multiple metastases involving both lungs and lymph nodes were observed in the 0.75- and 0.5-¿g/mouse groups. One of the major differences between the 2-¿g and 0.5-¿g/mouse dose groups was their TS50 (36 versus 57 wk, respectively) (see Table 1). Many of the animals in the 2-¿g and even 1-¿g/mouse dose groups simply did not live as long as the 0.5-¿g/mouse group. However, the TS50 values were quite similar in the 1-¿g and 0.75-¿g/mouse dose groups, even though the profile of metastases was markedly different. These observations suggest that systemic effects of TPA may also be involved in limiting metastases. The relatively high incidence of metastases in the lower TPA dose groups is noteworthy, since there is little evidence in the literature indicating that SCCs produced by the initiation-promotion protocol using TPA have a significant metastatic potential (22-25). Hennings et al. (24) and Patson et al. (25) have demonstrated that the initiation-promotion-initiation regimen and the complete carcinogenesis regimen, respectively, induce SCC with a relatively high metastatic potential. Our present data appear to be the first showing that the promotion protocol may have a significant influence on metastases of SCC in mouse skin. Further work is currently in progress to explore these interesting observations.

Several investigators have explored limited promotion treatments and their effects on progression of papillomas to SCC (13, 14, 26). Verma and Boutwell (14) showed that 18 wk of promotion with TPA in CD-1 mice were necessary for a maximal carcinoma response, although continued TPA treatment beyond 18 wk did not increase the final carcinoma response. On the other hand, Hennings et al. (13) reported that a papilloma response of only ~20% of the maximum, i.e., 5 wk of promotion with TPA, was sufficient for a maximal carcinoma response in Sencar mice. Since high tumor burdens may have adversely affected progression of papillomas to SCC in our dose-response studies (Fig. 1; Table 1), we examined the effect of limited promoter treatments using both a low and a high dose of DMBA as the initiator (i.e., 2 ¿g and 20 ¿g per mouse, respectively). The higher initiator dose (20 ¿g) was similar to the dose used in the study of Hennings et al. (13). Interestingly, with the low dose of initiator, both the papilloma and carcinoma responses were directly proportional to the duration of promoter treatment. In contrast, with the high dose of initiator only the papilloma response was clearly proportional to the duration of treatment. The carcinoma response was very similar in groups treated for 5, 7, or 60 wk of promotion and initiation with 20 ¿g of DMBA. Therefore, it appeared that the initiating dose of DMBA significantly affected the final carcinoma response when using different promotion duration protocols. In a related experiment (Table 4), treatment with TPA for 10 or 60 wk was compared, again using a low initiating dose of DMBA (2.56 ¿g). In this experiment, 10 wk of TPA treatment gave a papilloma response ~70% of the maximum and a nearly maximal carcinoma response.

Based on the promotion duration experiments, we can draw several tentative conclusions. (a) When using low initiating doses of DMBA, TPA treatment appears necessary until a nearly maximal (≥70%) papilloma response is achieved in order to obtain a carcinoma response similar to that observed with continual TPA treatment in Sencar mice (see Tables 3 and 4 and Figs. 3 and 4). However, this relationship appears to be masked when using high initiating doses of DMBA, possibly due to the higher papilloma burdens produced in these animals. From our current experiments, an optimum initiating dose appears to exist for studying the conversion of papillomas to SCC in mouse skin. Burns et al. (11) made a similar observation in studies with different initiator doses using Ha/ICR mice. This conclusion is further supported in our present study by the fact that the carcinoma responses in mice given initiation doses of either 2, 2.56, 6.5, or 20 ¿g of DMBA and receiving continued treatment with TPA (Tables 1, 3, and 4) were all very similar. Thus, in our hands, initiating doses of DMBA greater than 2 ¿g per mouse did not yield greater carcinoma responses despite dramatic differences in papilloma yields.

Finally, our laboratory has been interested in comparing the progression of papillomas to carcinomas using different classes of tumor-promoting agents. Initiation-promotion regimens with chrysarobin as the promoter produced about one-third the number of papillomas induced by promotion using 2 ¿g of TPA (Refs. 16 and 19; Table 4). Despite this difference in papilloma response, the maximum carcinoma response was very similar in both groups (Table 4). Chrysarobin, unlike benzoyl peroxide (20), does not appear to produce this higher carcinoma:papilloma ratio by enhancing the progression of preexisting papillomas (Group 4, Table 4). Rather, chrysarobin appears to be very efficient at selecting papillomas that have a higher probability of progressing to malignancy (15, 16). Several of the treatment protocols with TPA were also clearly more efficient than others for the selection of papillomas with a higher probability of progression to SCC. The most efficient protocols were those using lower doses of TPA (such as 0.5 and 0.75 ¿g per mouse) or limited durations of TPA treatment (e.g., 5 or 7 wk).

In conclusion, our present data are consistent with the hypothesis that different types or stages of papilloma development are initially produced during the standard initiation-promotion protocol on mouse skin (9-11, 14). Some papillomas have a relatively high probability of progressing to SCC during the time frame of the experiment and can be selected for by specific treatment protocols, i.e., low doses of TPA, short treatment durations, or different classes of promoters such as the anthrone derivatives. Our data also suggest, however, that at least some of the papillomas that can be produced on the backs of mice by increasing the dose of TPA or increasing the duration of TPA treatment (Tables 1 and 3, respectively) may also be at risk for conversion to SCC. These latter observations may be consistent with the recent model proposed by Aldaz et al. (15), who suggested, based on their sequential histopathological study, that skin papillomas progress to SCC at different rates and that many of the papillomas have the potential to become SCC. From our present studies, we suggest that optimal initiator doses exist for studies of tumor progression and must be determined for each type of initiator. In addition, optimal promoter doses and treatment protocols exist for experiments to examine the progression of papillomas to SCC. High promoter doses may produce a combination of both local and systemic effects,
whereas high initiator doses combined with high promoter doses may produce inappropriate tumor burdens, thus leading to a significant underestimation of the final carcinoma response. In addition, the frequency of tumor progression should always be considered a low estimate, since the presence of the first carcinoma will clearly limit other conversion events most probably due to early death of the carcinoma-bearing animal. Clearly, more work is necessary to understand the process of tumor progression in mouse skin and how these and other factors play a role.

REFERENCES

Tumor Progression in Sencar Mouse Skin as a Function of Initiator Dose and Promoter Dose, Duration, and Type


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