Dose and Frequency Effect in Mouse Skin Tumor Promotion


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ABSTRACT

In order to study the influence of both dose and application frequency of tumor-promoting agents on tumor development, we conducted a large-scale mouse skin two-stage carcinogenesis experiment. The back skins of 1110 CD-1 mice were painted once with 50 ng benzo(a)pyrene. These mice were divided into 24 groups according to subsequent schedules of 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment. Mice were treated with TPA at five different frequencies, i.e., daily, or every second, fourth, eighth, or sixteenth day, and six different TPA doses per application were used (0.1, 0.2, 0.4, 0.8, 1.6, or 3.2 µg), which allowed groups to be established with the same total dose of TPA applied per time unit. Six of the 30 frequency/dose combinations at extreme low or high frequency and dose were excluded. At each fixed frequency of TPA application, there was a good dose-response of TPA in mouse skin papilloma incidence. There was also a good application frequency-response relationship at fixed doses of TPA application.

Within the set of groups in which animals received the same total dose of TPA per time unit, some variation was observed with respect to frequency of application. In general, TPA applications every 4th and 8th day tended to yield a small number of tumors.

INTRODUCTION

Although dose-response studies of carcinogens are an important element for risk estimation of particular risk factors, there is little information concerning dose-response relationships in terms of multistage carcinogenesis. Multistage carcinogenesis probably involves several risk factors and the incidence of tumors may therefore not be predictable from a single dose-response study of each factor. Thus, in mouse skin two-stage carcinogenesis, skin tumor incidence can reach 100% when both initiating and promoting agents are administered, whereas most mice will show no tumor when only one of these agents is used (1, 2).

Mouse skin and rat liver models are the most widely used animal two-stage models of carcinogenesis and the mechanisms involved have been studied extensively (for reviews, see Refs. 3–5). From these studies, it appears that the promotion stage is to some extent reversible, whereas initiation is an irreversible event. These biological observations are consistent with the molecular mechanisms of action involved in each stage. Mouse skin tumor-initiating agents appear to be able to cause a specific mutation in cellular oncogenes; for example, many tumors induced by DMBA2 initiation and TPA promotion contain an 3-5). From these studies, it appears that the promotion stage is to some extent reversible, whereas initiation is an irreversible event. These biological observations are consistent with the molecular mechanisms of action involved in each stage. Mouse skin tumor-initiating agents appear to be able to cause a specific mutation in cellular oncogenes; for example, many tumors induced by DMBA2 initiation and TPA promotion contain an activated c-Ha-ras gene in which a A to T transversion has occurred at the 61st codon. Such a specific mutation was, however, not detected in tumors induced when N-methyl-N'-nitro-N'-nitrosoguanidine or B(a)P was used as an initiating agent, suggesting that the mutation of Ha-ras was induced by DMBA and not by TPA (6–8). Tumor-promoting phorbol esters are, in fact, not mutagenic. Differences in the reversibility of the action of promoters may be due to their different abilities to cause irreversible DNA damage.

The reversibility of various effects of tumor-promoting phorbol esters has been well documented (9, 10). Because of such reversible actions of phorbol esters and the apparent reversibility of the tumor promotion process, it has been assumed that there should be a threshold in tumor promotion (11, 12). However, this argument has often been misinterpreted to imply that tumor-promoting agents exhibit a measurable threshold dose (13, 14). The threshold concept, derived from the reversible action of tumor-promoting agents, is based on application frequency of promoting agents rather than doses; because of this reversibility, the promotion effect may not be cumulated unless the interval between applications is short enough (12, 15, 16).

The present study was undertaken to obtain more detailed information on the effect of doses and application frequency of tumor-promoting agents on tumor production. We used mouse skin two-stage carcinogenesis as a model, since the kinetics of appearance of tumors can be obtained without sacrificing animals.

MATERIALS AND METHODS

Animals. Female CD-1 mice (Charles River-France) at age 40–44 days were purchased. On the following day, all mice were vaccinated against ectromelia.

Chemicals. TPA, from LC Service Co., Woburn, MA, was dissolved in acetone (Prolabo-France). The solutions were freshly prepared every 2 weeks and kept in sealed dark vials at −21°C. Each day, new vials were opened. The remaining quantity was discarded. B(a)P was purchased from Jacquignon, Git-sur-Yvette, France.

Treatment with TPA. Doses per application were 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 µg in 0.1 ml of acetone.

Two-Stage Mouse Skin Carcinogenesis Experiment. At the age of 45–49 days, the back skin of mice was shaved, and 3 days later 50 µg of B(a)P in 0.1 ml of acetone were painted once. A total of 1110 B(a)P-treated mice were then assigned to the different TPA treatments. TPA (0.1, 0.2, 0.4, 0.8, 1.6, or 3.2 µg in 0.1 ml acetone) was painted at frequencies of once a day (group 1/1), or every second (group 1/2), fourth (group 1/4), eighth (group 1/8), or sixteenth day (group 1/16) for the duration of the experiment. An additional 90 mice were used for treatment groups given TPA alone without initiation by B(a)P.

The experimental design and the number of mice used for each group can be seen in Table 1. Since we know that high TPA doses and high frequency of application induce skin damage and ulceration and that low TPA doses and low frequency are almost ineffective, we excluded six groups, three from each extreme. More animals were allocated to groups where we expected to see fewer tumors (Table 1).

Mice were randomized and two animals were caged together. Serial numbers were assigned to individual animals. TPA painting was performed with a semiautomatic high-precision dispenser in such a manner that the same skin area was treated each time. Each animal was individually examined and recorded for papilloma appearance or regression.

Statistical Analysis. Time to first tumor (papilloma) was considered as a basic end point in the statistical analysis. For descriptive purposes, the survival functions for all groups were estimated using product-limit estimation (17). Weibull models which have been shown to be very suitable for the development of skin tumors (18, 19) were used and fitted with a computer program developed by Aitkin and Clayton (20).
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Table 1 Experimental design of two-stage mouse skin carcinogenesis experiment with varied application schedules of TPA dose and frequency

Number of animals with papilloma/number of animals treated (percentage of tumor-bearing animals), and number of papillomas per animal, by dose and frequency of TPA application, after 737 days of promotion, are presented.

<table>
<thead>
<tr>
<th>Dose (µg)/application</th>
<th>0.1</th>
<th>0.2</th>
<th>0.4</th>
<th>0.8</th>
<th>1.6</th>
<th>3.2</th>
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<tbody>
<tr>
<td><strong>Freqency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/1*</td>
<td>11/50</td>
<td>15/40</td>
<td>24/30</td>
<td>29/30</td>
<td></td>
<td></td>
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<tr>
<td>No. of animals with papilloma/no. of animals treated (%)</td>
<td>22</td>
<td>38</td>
<td>80</td>
<td>97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of papillomas/animal</td>
<td>0.40</td>
<td>0.72</td>
<td>2.70</td>
<td>4.70</td>
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<td></td>
</tr>
<tr>
<td>1/*</td>
<td>5/60</td>
<td>4/50</td>
<td>21/40</td>
<td>33/30</td>
<td>26/30</td>
<td></td>
</tr>
<tr>
<td>No. of animals with papilloma/no. of animals treated (%)</td>
<td>8</td>
<td>8</td>
<td>53</td>
<td>77</td>
<td>87</td>
<td></td>
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<tr>
<td>No. of papillomas/animal</td>
<td>0.03</td>
<td>0.08</td>
<td>1.07</td>
<td>1.60</td>
<td>2.97</td>
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</tr>
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<td>1/4*</td>
<td>3/70</td>
<td>3/60</td>
<td>5/50</td>
<td>7/40</td>
<td>16/30</td>
<td>21/30</td>
</tr>
<tr>
<td>No. of animals with papilloma/no. of animals treated (%)</td>
<td>4</td>
<td>5</td>
<td>10</td>
<td>18</td>
<td>53</td>
<td>70</td>
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<td>No. of papillomas/animal</td>
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<td>0.05</td>
<td>0.12</td>
<td>0.37</td>
<td>0.90</td>
<td>2.30</td>
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<td>1/6*</td>
<td>0/70</td>
<td>1/60</td>
<td>13/50</td>
<td>10/40</td>
<td>18/30</td>
<td></td>
</tr>
<tr>
<td>No. of animals with papilloma/no. of animals treated (%)</td>
<td>0</td>
<td>2</td>
<td>26</td>
<td>25</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>No. of papillomas/animal</td>
<td>0.00</td>
<td>0.02</td>
<td>0.46</td>
<td>0.32</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td>1/8*</td>
<td>6/70</td>
<td>7/60</td>
<td>10/50</td>
<td>12/40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of animals with papilloma/no. of animals treated (%)</td>
<td>9</td>
<td>12</td>
<td>20</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of papillomas/animal</td>
<td>0.20</td>
<td>0.10</td>
<td>0.24</td>
<td>0.24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Daily.
* In these three combinations, an additional 30 noninitiated mice were treated only with TPA at the corresponding doses and frequencies.
* Every second day.
* Every fourth day.
* Every 8th day.
* Every 16th day.

Using the statistical software package GLIM (21). Maximum likelihood estimates of model parameters and their standard errors are derived together with a term called deviance which serves as a goodness-of-fit statistic. In particular, the significance of the inclusion of new factors or variables into a model can be judged by a likelihood ratio test based on the differences of the deviances (22).

The essential aspect of such analyses (23) is to model certain parameters of the Weibull distribution which determine the age-specific risk of developing a tumor as a simple function of the design parameters (dose, frequency of application, or combinations of these two). Under the Weibull model, the age-specific incidence of tumors at age t is expressed as $\exp(-\beta(t-w)^k)$ where $\beta$, $k$, and $w$ are parameters. The exponent $k$ represents the shape parameter where $\beta$ a delay parameter, and both are considered to be equal for all experimental groups. Parameter $\beta$ determines the force of the tumor incidence and is considered to vary between the experimental groups. In the various regression models $\beta$ is modeled to depend on the features of the design (dose, frequency of application or combinations of these two) such that the derived parameter estimates are the logarithms of the relative risks compared to the baseline category (Ref. 23, p. 135).

For dose six levels (0.1 to 3.2 µg) are considered in the design and they are numbered 1 to 6 in the statistical analysis, corresponding to the five levels of frequency (groups 1/1-1/16) 1 to 5, and the six diagonals representing the factor total dose are numbered 1 to 6 starting with the bottom left diagonal which represents the smallest dose per time unit. In this framework, the differences in tumor incidence as they relate to the factors dose, frequency, or total dose are investigated by fitting the respective models to all experimental groups simultaneously. Here, dose is used to express “dose per application” and total dose means “dose per 15 days,” i.e., dose rate. Considering a factor with its different levels results in parameter estimates which are logarithms of relative risk estimates, treating the factor as a variable results in estimates of the average change in relative risk between adjacent factor levels.

RESULTS

Papilloma Development under Various Schedules of TPA Treatment. The time course of papilloma development on B(a)P-initiated CD-1 mouse skin after various schedules of TPA-promotion is presented in Fig. 1. Fig. 1 contains results from mice treated at the same frequency but with a different dose of TPA and presents the proportions of tumor-free survivors in each group. There is a clear dose-response relationship at each fixed application frequency interval.

The numbers and crude percentages of papilloma-bearing animals in each group at the end of the experiment (737 days after the first TPA treatment) are also presented in Table 1. From Fig. 1 and Table 1, it is also clear that, at a given dose of TPA per application, the tumor incidence decreases with decreasing frequency, with the exception of 0.8 µg/application, where 18% of the animals treated every 8th day developed a papilloma compared to 12% treated every 4th day.

In order to examine the effect of the frequency of TPA application at a given total cumulative TPA dose, results presented in Fig. 1 are rearranged so that each display contains experimental groups which received the same total dose of TPA (Fig. 2). This is achieved by gathering different groups presented along diagonals in Table 1. Some effect of application frequency is evident from Fig. 2 and a detailed statistical analysis was carried out to obtain clear quantitative estimates of this effect (see below).

TPA can induce some papillomas on noninitiated mouse skin (24, 25). TPA treatment on noninitiated mouse skin was also carried out to investigate application frequency effect. Thus, three groups of noninitiated mice received 1.6 µg equivalent of TPA per 4 days. The application frequency for each group was daily (0.4 µg/application), every second day (0.8 µg/application), or every fourth day (1.6 µg/application). The papilloma development in these groups is presented in Fig. 3. Although the cumulative dose of TPA applied in each group was the same, fewer papillomas developed when the interval between TPA applications was longer.

Statistical Analysis of Data. The results of fitting statistical Weibull models are summarized in Table 2. In all models, the shape parameter of the Weibull model is not very different to one, with estimates ranging from 1.18 to 1.24, indicating that the hazard function is almost constant over time. The derived parameter estimates represent logarithms of relative risks, and

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Fig. 1. Kaplan-Meier estimates of the survival function. Equal frequency groups per display.

their antilogs are subsequently used for descriptive purposes.

In the first model, dose and frequency are considered as factors with 6 and 5 levels, respectively, according to the experimental design (Table 1). For example, factor level 3 for frequency corresponds to the category “every 4th day.” The parameter estimates for level 1 of a factor are 0.00 by default and therefore not listed. For both factors, the parameter estimates of the various factor levels are listed with their standard errors.

For the factor dose the increasing risk of developing a papilloma can be seen in the increasing sequence of parameter estimates [by definition the parameter for level 1 (0.1 ng) is set zero]. The corresponding relative risk of developing a papilloma at dose 0.2 μg [level 2] is 1.41-fold [1.41 = exp(0.342)] compared to 0.1 μg [level 1], while at the 0.8-μg dose (level 4) it is 18.6-fold compared to level 1. Correspondingly, the decreasing parameter estimates for the factor frequency illustrate the decreasing risk with decreasing frequency of application. The apparent linear pattern of the parameter estimates for both dose and frequency allows us to consider both as continuous variables. The values of the above factor levels, 1 to 6 and 1 to 5, respectively, which correspond to the logarithms of dose and frequency, were used. Model 2 shows that doubling the dose increases the risk by a factor of 2.34 [= exp(0.852)] whereas doubling the time between application reduces the risk by a factor of 0.35 [= exp(−1.047)].

The effect of total dose per time unit can be investigated in these experiments by comparing the diagonal experimental groups in the two-factorial experimental design (Table 1). The lowest total dose is given when 0.1 μg is applied every 4th day, 0.2 μg every 8th day, or 0.4 μg every 16th day; this is considered as level 1 of this factor. Correspondingly, 0.1 μg every 2nd day, 0.2 μg every 4th day, 0.4 μg every 8th day, or 0.8 μg every 16th day comprises level 2, and so on. There are altogether six levels in this factor. Again, the parameter estimate for level 1 is set at zero and model 3 gives the estimates for the remaining levels. Again, there is a linear pattern in the parameter estimates which can easily be summarized by considering total dose as a continuous variable. Model 4 shows that doubling the total dose per time unit, either by doubling the dose per application or doubling the frequency of application, increases the timespecific risk of developing a papilloma by a factor of 2.46.

Whether there remains, given this effect of total dose per time unit, an effect of the frequency of application is investigated in the next model (model 5). Frequency was added as factor with 5 levels again. The estimate for total dose remains stable when compared with the previous model but the considerable reduction in deviance indicates a significant effect of frequency. The parameter estimates indicate that application every second day (level 2) does not lead to a risk greatly different from daily application (level 1); however, application every fourth day (level 3) reduces risk by a factor of one-third, whereas the less frequent applications (levels 4 and 5) reduce the risk by factors of only one-half and two-thirds, respectively, in comparison with level 1.

In model 6, some adjacent factor levels for frequency showing similar effects in model 5 were merged. The applications every
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Fig. 2. Kaplan-Meier estimates of the survival function. Equal total dose groups per display.

Fig. 3. Kaplan-Meier estimates of the survival function. Noninitiated groups (average total dose, 0.4 µg/day).

day and every second day are combined (parameter set zero) and compared to application every fourth day (level 3), and applications every 8th or 16th day combined (levels 4 and 5). There is only a slight increase in the deviance of model 6 compared to model 5 with two more degrees of freedom, indicating that the effect of frequency at a given total dose is sufficiently described by model 6. The effect for total dose remains unchanged, as well as the risk estimate for frequency level 3 and levels 4 and 5 combined. Thus, at a given level of total dose per time unit, application every day or every second day does not show different time-specific risks of developing a papilloma. However, application every fourth day reduces this risk by a factor of about 40%, whereas applications every 8th or 16th day do so by a factor of two-thirds.

Halving the frequency of application at a given total dose per time unit is equivalent to doubling the dose per application. Thus, in the results discussed above, the variable frequency could be replaced by dose accordingly. However, due to the special diagonal character of the design, the results for fitting model 7, which comprises total dose and dose, have to be interpreted in terms of the absolute values of the individual doses.

At a fixed total dose per time unit the use of 0.1 µg as single dose whenever used in that series leads to the highest risk, and use of 0.2 µg leads to a reduction in risk by one-half. From 0.4 to 3.2 µg as single dose, the reduction in risk increases monotonically, i.e., greater reduction in risk with larger single doses. However, these differences do not achieve statistical significance.

The results of the fitted Weibull model 6 (Table 2) can also be reconstructed in the raw data of Table 1. The average tumor frequency along the diagonals representing the same total dose per unit time increases gradually from the diagonal in the lower left corner to the one in the upper right corner. However, in each of these diagonals the group receiving the promoter application every fourth day (group 1/4) is among those with the lowest diagonal specific tumor frequency.

DISCUSSION

Our results presented here indicate that effects of TPA treatment on mouse skin papilloma depend not only on dose but also on frequency of application of the tumor promoter, TPA. Analysis of results based on Weibull regression models suggests that, if the application interval is halved at a given total dose, the result would be similar to that obtained with the application...
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Table 2 Parameter estimates with standard errors (SE), relative risks (RR), and deviances for different regression models fitted under the assumption of a Weibull distribution

<table>
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<th>Model</th>
<th>Deviance</th>
<th>Degrees of freedom</th>
<th>Type</th>
<th>Parameter ± SE</th>
<th>RR</th>
<th>Type</th>
<th>Parameter ± SE</th>
<th>RR</th>
<th>Type</th>
<th>Parameter ± SE</th>
<th>RR</th>
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<td>1099</td>
<td>2</td>
<td>0.342 ± 0.313</td>
<td>1.41</td>
<td>2</td>
<td>-1.107 ± 0.170</td>
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<td>3</td>
<td>-2.760 ± 0.201</td>
<td>0.06</td>
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<td>3</td>
<td>1.923 ± 0.267</td>
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<td>-3.323 ± 0.219</td>
<td>0.04</td>
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<td>18.58</td>
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<td>5</td>
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* Numbers indicate factor levels; V, continuous variable with values according to the factor levels is listed.

of double TPA dose per application without changing the application frequency. These results confirmed that papilloma production is dependent on the dose of TPA. However, our results also showed that the application frequency also influences tumor production, suggesting that the effect of tumor-promoting agents cannot be simply additive (15).

The importance of treatment frequency of tumor-promoting agents on mouse skin tumor development was previously suggested by Boutwell (26). He applied a total dose of 1500 µg croton oil to mouse skin in single doses of 125 µg at intervals of 1, 2, or 4 weeks after initiation with a single application of 75 µg DMBA. He found that, although the total dose of croton oil was the same, no papilloma was produced at the longest application interval. He also observed that, when he applied croton oil once a week, 1.6 papillomas were induced per mouse, whereas only 1.1 papillomas per mouse were obtained when the same amount was applied once every 2 weeks. The effect of the application interval of tumor-promoting agents was also confirmed in an experiment conducted by Van Duuren et al. (27). Mouse skin was given a single initiating painting with DMBA, and papillomas were produced by painting TPA at different doses, either once a week or three times a week. By analyzing the tumor yield, the authors concluded that the frequency of application is more important than the total dose of TPA. The results obtained from our large-scale experiment confirm and extend the importance of frequency of TPA application on tumor development.

The effect of frequency of TPA application seen above is in contrast to the lack of dependence of tumor production on initiator application frequency. Boutwell (26) fractionated the doses of the initiating agent (DMBA), applying a total of 1 µg either as a single dose, as four doses at intervals of twice a week, or as four doses once every 2 weeks; then, all mice were painted with croton oil for 32 weeks. He found that the tumor yields were very similar (26). These results are consistent with the idea that the action of the initiating agent involves genetic damage which is not readily reversible; thus the effect can be cumulated.

Although our present results and those from others suggest that the frequency of application of a tumor-promoting agent can alter the yield of tumors on mouse skin, when the effect of different doses of TPA at a fixed interval of application on tumor induction was examined, there was generally a good dose-response effect (this paper and Refs. 27–29). This suggests that there is no dose-related threshold and no drastic difference in the dose-response relationship of tumor promoters in comparison to that of initiating agents.

Because the animals must be killed in order to count internal tumor yield accurately, there are fewer studies on dose- and frequency-response relationships of tumor-promoting agents in internal organ carcinogenesis. However, good experimental data suggest that the frequency of application of tumor-promoting agents can influence the yield of rat liver enzyme-altered foci. Pitot et al. (30) have shown that when phenobarbital administration is interrupted, there is a decrease in the yield of foci in comparison to a group in which phenobarbital administration is not interrupted, although the total doses of administered phenobarbital were approximately the same in the two groups. These results are consistent with the idea that the promoting effect of a chemical is not simply cumulative when the administration interval is long.

Although TPA is known as a tumor promoter, it also has complete carcinogenicity (24, 25). As in previous studies, we demonstrated here that TPA can produce skin papillomas even when applied to noninitiated mice. We had only three groups in which TPA was painted on noninitiated mouse skin and all of them received cumulative doses of 1.6 µg in 4 days at different frequencies. Tumor development was again clearly dependent on the frequency of TPA application. Although the total applied dose was the same, there were less tumors when the interval between applications was longer. These results suggest that the frequency of TPA application plays an important role in complete tumorigenesis as well as in tumor promotion. Precise molecular mechanisms of complete carcinogenic activity of TPA are not known. Recently, we have shown that papillomas induced by TPA alone had no point mutation at the 61st codon of H-ras, as was seen in carcinomas and papillomas produced by a DMBA initiation-TPA promotion protocol (8). Although the identification of definite tumor-promoting agents in human carcinogenesis is difficult, there is good evi-
dence that most human cancers develop by a multistage process and that various factors are involved in each stage (12, 31, 32).

It is likely that carcinogens also act through both genotoxic and nongenotoxic mechanisms in the multistage process of human carcinogenesis, as demonstrated in animal experiments (3-5). If we extend our results, therefore, the effect of "exposure interval" to carcinogens might play an important role in certain causes of human cancer. To our knowledge, there have been no epidemiological studies in which the "exposure frequency" to carcinogens, as demonstrated in animal experiments (3-5), application (or exposure) frequency has not been adequately considered. If animal experimental results such as those presented here can be extrapolated to the human situation, then there is scope for modifying human risk by changing the interval between exposures to carcinogens with promoting activity.

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Dose and Frequency Effect in Mouse Skin Tumor Promotion


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