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Approach to Risk Assessment for Genotoxic Carcinogens Based on Data from the Mouse Skin Initiation-Promotion Model

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Tumor induction data in the mouse skin initiation-promotion system were found to be consistent with a quadratic function where the coefficient of the linear term depended on the dose of the promoter. The model implies that the existence of promoters may be more important at low doses of the carcinogen than at high doses where most testing is performed. Experiments are described showing that the initiating effect of carcinogenic chemicals, such as benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene, nitroquinoline oxide and β-propriolactone, accumulates in a linear, irreversible manner at low doses. Even when 7,12-dimethylbenz(a)anthracene was applied intragastrically to pregnant females, initiating activity was found in the skins of exposed offspring about in proportion to dose applied and number of cells at risk. The initiated cells essentially represent a potential for cancer that has a high probability for expression in the presence of a promoter.

Risk then can be interpreted in terms of the accumulated dose of initiator which alone presents a small risk of cancer. However, a promoter may substantially expand the overall risk, possibly by clonally expanding the initiated cells. Promotion needs to be sustained since there is a reduction of cancer risk if promotion is ended early. Some tissues, such as mouse bladder, may be intrinsically promoted more than others so that comparisons between tissues and between species are best made when the combination of intrinsic promotion and response to extrinsic promotion are comparable.

Introduction

In an attempt to explain mouse skin tumor data in terms of multistage progression, we have formulated a cell generation hypothesis (*1-3*). In this formulation, the initial event in carcinogenesis is assumed to be an interaction between the carcinogen and the cell (probably the DNA of the cell) that changes the cell in such a way that it is identifiable as initiated, i.e., it forms a benign clonal growth, known as a papilloma, when exposed to a promoter, such as, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (*4,5*). Furthermore, it is assumed that the original initiated cell and all of its progeny are unstable in the sense that they are subject at each cell division to the risk of additional changes leading to progressive acquisition of cancerous properties (*6*).

Papillomas that are dependent on continued promotion, i.e., those that would regress if the promotion were stopped, may acquire autonomy (*4*). The cells in autonomous papillomas continue to be at risk of progression to cancer whether or not promotion is continued. Probably many changes not produced directly by the carcinogen are needed to convert an initiated cell into a cancer cell. These additional changes or transitions are an indirect result of initiation and are assumed to be distributed in a stochastic manner in time subsequent to initiation (Fig. 1).

Multistage theory holds that the value of the exponent of the function of tumor rate versus time equals the number of stages in carcinogenesis. Accordingly, that exponent ought to be constant, but there are many examples where it varies. This can be handled by invoking clonal expansion as an important determinant of the temporal function whenever promotion, intrinsic or extrinsic, is present (*6*). Thus, there are two routes a cell may take as it progresses to cancer. The first route involves one carcinogen-induced alteration (initiation) followed by additional alterations that are observable only be-

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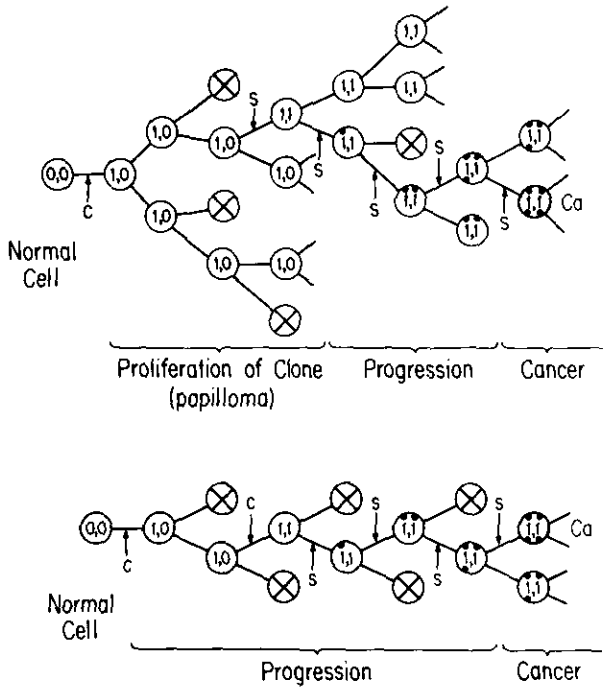

FIGURE 1. Schematic diagram of the multistage-multigenerational hypothesis. The initial event is depicted as being produced by a carcinogen (arrow with C). In the upper panel the cell (1,0) with one event multiplies into a clone, and at a subsequent cell division suffers a spontaneous event (arrow with S) to become a cell that is equivalent to one having suffered two carcinogen induced (without clonal multiplication) events as depicted in the lower panel. The further progression of 1,1 cells is depicted as requiring four additional events. As discussed in the text, this number is somewhat uncertain, because it depends on the amount of clonal multiplication assumed for intermediate stages.

cause of the multiplication of probability implicit in clonal growth. The second route involves two or three carcinogen-induced alterations that presumably damage the cell so severely that additional alterations are probable even without substantial clonal growth.

To make these ideas more concrete, we can formulate them into a mathematical framework. First let it be assumed that cancer requires n stages. These stages correspond to transitions that can be described by transition probabilities (for the i-th transition) of the form:

$$K_i = a_i + b_i d \qquad (1)$$

where a_i is the spontaneous transition rate, b_i is the dose coefficient of the i-th transition and d is the dose of the carcinogen (*2*).

If a_i and b_i are constants and the dose rate is constant, the cumulative probability of the i-th transition is given by $K_i\ t$ where t is elapsed time. Accordingly, the cumulative probability per unit time of cancer occurrence, often referred to as the cumulative hazard function $H(t,d)$ can be written:

$$H(t,d) = \prod_{i=1}^{n} K_i\, t^n \qquad (2)$$

For any transition where the spontaneous rate is negligible in comparison to the carcinogen-induced rate, i.e., $a_i << b_i d$, the transition probability can be written as $b_i d$. If there are m such transitions, the cumulative hazard becomes:

$$H(t,d) = \prod_{i=1}^{m} b_i \prod_{i=m+1}^{n} K_j\, d^m t^n \qquad (3)$$

Since the tumors are assumed to be randomly distributed among the animals, the proportion with tumors, $P(t,d)$, is related to the cumulative hazard, $H(t,d)$, by the equation:

$$P(t,d) = 1 - e^{-H(t,d)} \qquad (4)$$

It follows that 50% of any given group of animals will have at least one tumor when $H = 0.693$ (*8*). If the time when 50% have developed at least one tumor is designated t_{50}, it follows from Eq. (3) and (4) that:

$$d\ t_{50} n/m = \text{constant} \qquad (5)$$

Equation (5) can be considered to be a dose-response function where m is the number of dose-dependent transitions and n is the total number of transition in carcinogenesis.

Equation (5) was originally found empirically by Druckrey to apply to liver carcinogenesis when either AAF or DEN were given in the diet, and the value of n/m was estimated to be about 2.3 (*9*). Values of n/m were derived from the epidemiological data on lung cancer in cigarette smokers and found to be 2.6 (*10*). The multistage theory seems generally to fit the experimental and epidemiological data quite well for situations where the carcinogen exposure is prolonged, and the dose rate is constant.

The principle defect of the multistage theory is that the stages are not identifiable and their order or sequence is unknown. Peto concluded that the carcinogen-dependent stages were probably early in the sequence because older mice, that presumably had accumulated many of the non-carcinogen related stages, showed no greater response to topical application of a carcinogen, benzo(a)pyrene, than younger mice (*11*). The stages themselves are purely speculative entities but certainly can encompass the concept of carcinogenic progression as it is currently understood. Cancer cells may need to acquire a number of specific properties, such as the ability to stimulate host blood vessels by means of angiogenesis factor, the ability to dissolve connective tis-

sue proteins by secreting proteases, the ability to block the immune defenses of the host by producing blocking factor. The acquisition of each of these properties occurring sequentially in a given period of time could be considered to represent transitions between stages in the multistage model. Of course, there are many other properties of cancer cells that might represent stages and certainly more biological studies are needed to refine our understanding of stages and the transitions between them.

The mouse skin is an ideal model system to test these ideas and to study dose and time related aspects of benign and malignant tumor formation. A series of experiments were performed in an attempt to answer certain specific questions related to these ideas. The first and most important question was what shape to assume for the dose-response function of benign and malignant tumors.

Animals were observed every other week and the progress of individual tumors was charted. Regression of tumors and progression of benign lesions to malignancy was noted. Animals were sacrificed when moribund or when tumors exceeded 1.0 cm^3 in size. Representative benign skin tumors and all carcinomas diagnosed grossly were excised, fixed in 10% formalin and blocked in paraffin. Slides were prepared and stained with hematoxylin and eosin for histopathological diagnosis.

For each observation interval, the number of new tumors was divided by the average number of mice alive to obtain the rate of tumor occurrence. The rates were added cumulatively to obtain the yield of tumors in tumors per mouse (cumulative hazard) as a function of time.

Figure 2 shows the yield of papillomas and carcinomas in mice receiving only TPA thrice weekly. These data are based on 63 mice. Median survival was 550 days and there were 7 mice left at 650 days. Papilloma formation began at 150 days after the start of promotion and increased continuously thereafter. All carcinomas appeared from pre-existing papillomas. The conversion of papillomas to carcinomas was initially evident at 350 days and continued thereafter with a ratio of papillomas to carcinomas at about 7:1. No tumors were observed in 40 acetone treated mice during the same period of time.

Figure 3 shows the papilloma and carcinoma yield after a single initiating dose of 128 μg B(a)P followed by TPA thrice weekly. The temporal pattern is typical of that for other initiating doses of B(a)P. Papilloma formation began at about 50 days and continued fairly steadily thereafter. A few carcinomas developed from pre-existing papillomas beginning at about 350 days.

The temporal pattern of tumor formation after single and multiple applications of B(a)P as an initiating agent are similar. These data are shown in Figure 4.

The lack of fractionation effect permits the display shown in Figure 5 of the tumor response per fractional dose. For example, if 128 μg were given in eight fractions with a tumor incidence of 5.3 tumors/mouse at 200 days, the net tumor yield per fraction, $(5.3 - 0.06)/8 = 0.66$, would be plotted against the dose per fraction. $128/8 = 16$. Figure 5 shows these

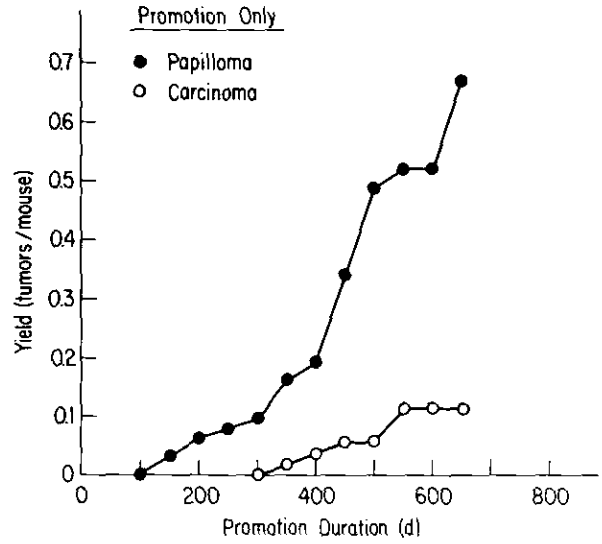

FIGURE 2. Yield of (•) papillomas and (○) carcinomas in mouse (Ha/ICR) skin treated only with 5 μg TPA in 0.2 mL acetone three times weekly.

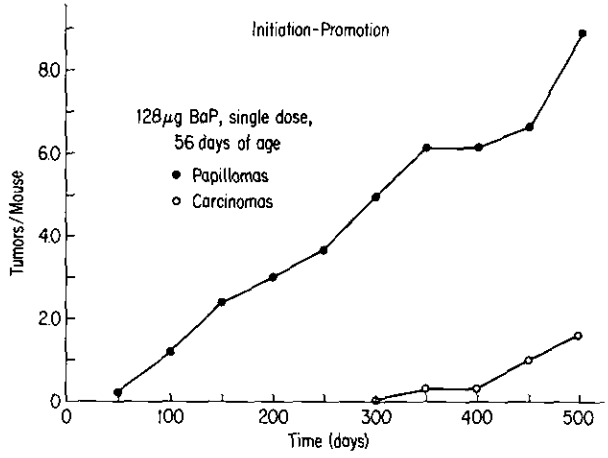

FIGURE 3. Cumulative yield of (•) skin papillomas and of (○) skin carcinomas after topical treatments. A single dose of 128 μg B(a)P administered at 56 days of age was followed by treatment three times weekly with 5 μg TPA in 0.2 mL acetone.

data for all single and fractionated exposures. The regression line drawn through the data points has a slope of 0.90. The data support the linear dose-response relationship of papilloma formation for B(a)P initiation throughout a dose range extending three orders of magnitude. The slope is such that one initiated tumor site is produced by each 30 μg dose of B(a)P.

The data support the linear nonthreshold character of the dose-response relationships for the initiation of carcinogenesis by B(a)P in the mouse skin. A linear nonthreshold dose-response was also obtained

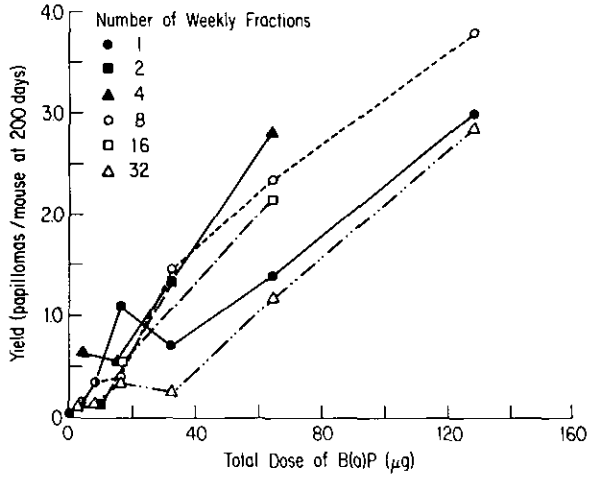

FIGURE 4. Yield of papillomas at 200 days of promotion as a function of the initiating dose of benzo(a)pyrene. Each set of data represents a different number of fractions as indicated. The fractions were administered weekly.

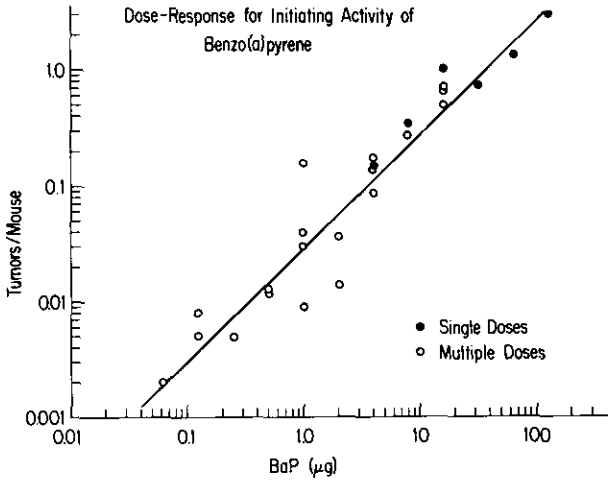

FIGURE 5. Double logarithmic plot of the yield of papillomas per fraction as a function of the dose per fraction. The solid line was fitted by regression analysis and indicates a very good linear fit (exponent 0.9).

from B(a)P in the skin of Sencar mice at a dose of 2 μg TPA twice a week (*12*). The lack of a fractionation effect is consistent with the linear response to single carcinogen exposures and implies a mechanism based on a single irreversible event.

If each papilloma is considered to be a clonal expansion of an initiated cell, and an initiated cell is the first stage in carcinogenesis, the above data permit an evaluation of $K_1 = a_1 + b_1D$. For example, from the data in Figure 2, $a_1 = (0.5/575)$ transitions/mouse/day and from the data in Figure 5, $b_1 = (2/16000)$ transitions/mouse-μg-day. The pattern of papilloma induction and conversion of papillomas to carcinomas in the uninitiated mice treated thrice weekly with TPA suggests that the skin has a background of initiation equivalent to about (4000/575) μg of B(a)P, i.e., a_1/b_1. Since the area of mouse skin treated contains about 3×10^6 epidermal cells, the corresponding transition constants in units of transitions per cell per day are $a_1 = 290 \times 10^{-12}$ and $b_1 = 42 \times 10^{-12}$ in transitions/cell μg day.

DNA Binding

The dose dependency of the binding of benzo(a)pyrene [B(a)P] with DNA of mouse epidermis was investigated. B(a)P-conjugated epidermal DNA was isolated and enzymatically degraded to deoxyribonucleosides. The B(a)P-DNA adducts were separated by Sephadex LH-20 column or high-performance liquid chromatography. Two major B(a)P-DNA adducts were found. One was in the region of the elution profile that contained polycyclic aromatic hydrocarbons adducted to deoxyribonucleosides. The other adduct was eluted from Sephadex LH-20 and high-performance liquid chromatography columns before the deoxyribonucleosides and after deoxyribonucleotides. Both adducts of B(a)P in epidermal DNA reached a maximum 7 hr after a single skin application, and subsequently little, if any, loss of adducts was observed for 49 hr. Both adducts varied as a linear function for topical doses in the range from 0.01 to 300 μg/mouse. The formation of DNA adducts by B(a)P occurred in proportion to dose at doses several orders of magnitude below those that are feasible to test for carcinogenicity.

When B(a)P is applied to mouse skin (*13*), added to cell culture (*14*) or incubated in the presence of microsomes and DNA (*15*), electrophilic metabolites are formed that covalently bind to DNA. The initiation of carcinogenesis by polycyclic aromatic hydrocarbons is believed to involve covalent binding to cellular macromolecules, and there appears to be an especially good correlation between their carcinogenic potency and DNA binding (*13*). Thus, the bind-

ing to DNA might serve as a sensitive indicator of carcinogenicity since analytical techniques are available to detect such binding at extremely low doses. Actually, the formation of a number of different DNA adducts as well as tritium exchange onto the bases is possible. Thus, specific B(a)P:DNA adducts could serve as a better marker for biological potency than the total amount of binding in B(a)P-conjugated DNA. The major hydrophobic B(a)P adduct to deoxyribonucleosides formed *in vivo* has been identified and studied extensively (*16,17*). Hydrophilic B(a)P:DNA adducts present in the elution profiles obtained from the chromatography systems used in the isolation of B(a)P:deoxyribonucleoside adducts remain to be characterized (*18*).

Promotion in Combination with a Carcinogen

Promoters have important consequences in determining the temporal pattern of cancer induction by accelerating the development of neoplasia. Promoters may act by stimulating clonal expansion of potentially cancerous cells, or they may stimulate the expression of a neoplastic event or they may actually produce neoplastic events. In order to determine the temporal effect of promoters on carcinogenesis, mouse skin was exposed to weekly doses of benzo(a)pyrene either alone or in combination with various twice weekly doses of TPA. The cancer yield as a function of time when B(a)P was given alone is shown in Figure 6. As the B(a)P dose increased from 16 μg weekly to 128 μg weekly, the tumor curves were displaced progressively to earlier times in such a manner that n/m in the expression, $dt_{50}^{n/m}$ = constant, was about 2.1, where d was weekly dose and t_{50} was time to 50% prevalence.

The results when various weekly doses of TPA were added to a given weekly dose of B(a)P are shown in Figure 7. As the TPA dose was increased, the cancer yields were progressively displaced to earlier times, although there seemed to be a plateau effect in the sense that the increase from 0.5 to 5.0 μg per week produced much less displacement than the increase from 0.05 to 0.5 μg per week. There is little doubt from these data that TPA, a promoter of papillomas, accelerated the development of carcinomas. The maximum degree of temporal displacement (the highest TPA dose) was equivalent to a 4-fold increase in B(a)P dose. These data suggest that part of the temporal displacement associated with different doses of B(a)P could be derived from the promoting action of B(a)P.

A dose-effect relationship for carcinoma induction under conditions of repeated weekly B(a)P exposure can be generated by considering the tumor yield at a specific point in time, specifically 300 days after the B(a)P was started. These data are shown in Figure 8. In the absence of TPA, the cancer yield increased sharply with dose consistent with a squared or cubed function. However, when TPA was added weekly along with B(a)P, the dose effect function shifted markedly to the left, i.e., to lower B(a)P doses, and more importantly lost the squared or cubed dose dependence and became nearly linear.

One explanation of these data is that papillomas contain cells, probably a clonal expansion of cells, that have undergone one or more events, presumably involving damage to DNA, that are early

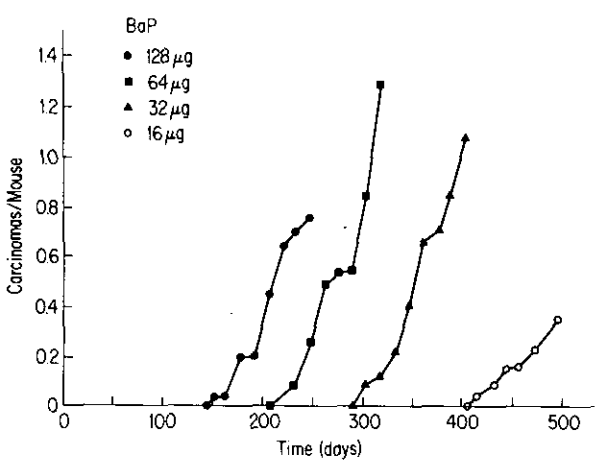

FIGURE 6. Cumulative yield of skin carcinomas per mouse. Numbers identifying each curve indicate the topically applied weekly dose of B(a)P treatment at 56 days of age.

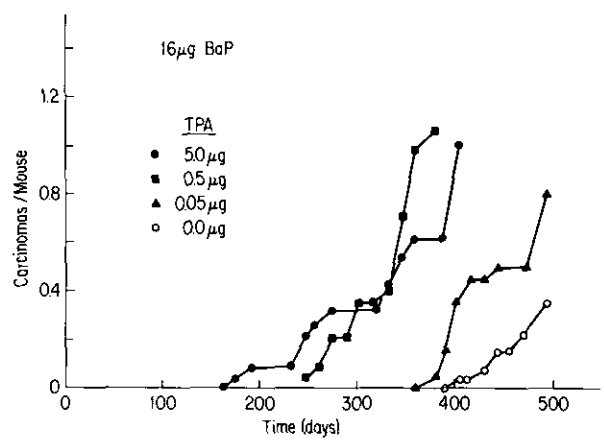

FIGURE 7. Cumulative yield of skin carcinomas per mouse. Each animal received weekly treatments of 16 μg B(a)P (Monday) plus twice weekly treatments of 0, 0.5, 0.5, or 5.0 μg TPA (Wednesday and Friday). Chemicals were topically applied in 0.2 mL acetone.

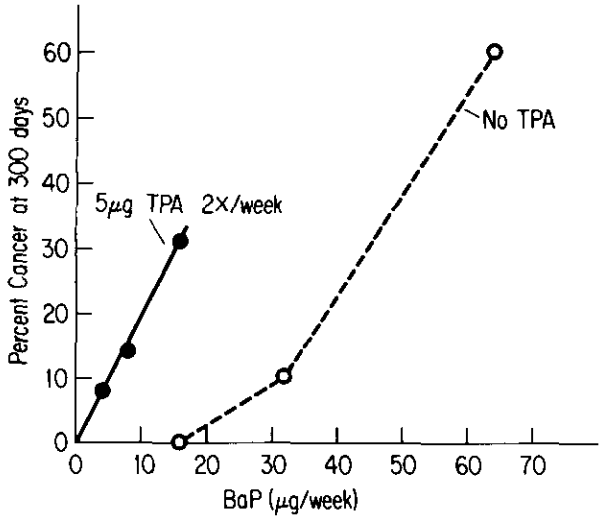

FIGURE 8. Dose-effect data at 350 days for carcinoma induction in mouse skin exposed to a weekly dose of B(a)P on Monday with or without 5.0 μg TPA on Wednesday and Friday. Doses refer to the amount of B(a)P given per week. The treatments were started at 56 days of age.

events in the production of a cancer cell. It is not suggested here that every event presumed to be involved in carcinogenesis necessarily produces cells that are expandable into clones by the action of phorbol ester. Neither can we eliminate the possibility at the present time that the promoter may have effects in addition to stimulation of clonal expansion, although such events must differ from initiator-induced events.

One hypothesis consistent with these data is that one or two events, e.g., chromosome breaks, produced either directly or indirectly by the action of a carcinogen, cause an instability leading to the accumulation of additional changes, possibly as many as four to six (*19*). Presumably any or all of these events may occur spontaneously, which is necessary to explain the occurrence of "spontaneous" cancer in untreated animals and the conversion of persisting papillomas to cancers without further treatment. The clonal expansion implicit in the growth of a papilloma greatly increases the chance that cancer will occur, because each papilloma cell has a spontaneous risk of occurrence of later events, including some that could be produced by action of a carcinogen. The promoter may also fix the initiation since short exposure to a promoter followed by a mitotic stimulation will produce tumors, whereas mitotic stimulation alone will not.

The shapes of the dose-effect functions for carcinoma induction with and without promotion can be explained in terms of the above ideas as follows. In the absence of promotion the carcinogen must act directly and repeatedly on target cells to produce whatever number of events are necessary for a malignant cell to occur (possibly two or three); hence the yield of cancers is proportional to (dose rate)$^{2-3}$, and a relatively high total dose is needed to produce a given yield. If the tissue is promoted, clonal expansion of some intermediate state leads to a greatly increased overall risk of malignancy because each initiated cell in a papillomatous clone is assumed to have acquired some risk, albeit small, of the occurrence of additional events that would complete the transition to malignancy. Cancers derived from such papillomas would be expected to follow the dose-response characteristic of the papillomas, since second and subsequent events would occur spontaneously without the necessity for action by the carcinogen. The cancer yield for a given dose of carcinogen would be much higher with promotion than without because of the risk multiplication inherent in clonal expansion.

The data in Figure 2 show that the rate of conversion of spontaneous papillomas to carcinomas was about 1/300 per day, while the data in Figure 3 show the comparable rate for induced papillomas was about the same (1/350 per day). If all papilloma cells are at risk, these values reduce to transition rates of about (1/300-350) $\times$ 10^{-6} per cell per day, since there are about 10^6 cells per papilloma. In our model the latter quantity is an estimate of a_2, the spontaneous rate of transition of papillomas to a cell type that has a high risk of further events in the progression to cancer.

Interactions between Initiators

Given that the neoplastically related cellular damage is not precisely known and that carcinogenic chemicals are chemically diverse, it is important to establish whether cells initiated neoplastically by one carcinogen exhibit differences in their interaction with a second carcinogen. Since single initiated cells cannot yet be isolated and studied, it is necessary to study interactions by applying carcinogens to whole tissues. The initiation-promotion system in mouse skin is one of the most useful for studies of this type (*7*). The objective of the study described here was to determine how the several chemically diverse initiators interacted when applied at different times to the same region of dorsal mouse skin.

The purpose of these experiments was to determine whether different classes of chemical carcinogens produce additive yields of papillomas when applied sequentially to mouse skin. The carcinogens were benzo(a)pyrene, nitroquinoline oxide (NQO) and β-propriolactone (BPL) applied topically to the shaved dorsal skin of mice (Ha/ICR) in 0.2 mL ace-

tone. The papilloma yield as a function of promotion time is shown by the open triangles in Figure 9 for mice that received 6 mg BPL and 16 μg B(a)P. The summation of the yields for separate groups of mice that received 6 mg BPL or 16 μg B(a)P as single doses is indicated by the closed triangles. The combined exposure produced about twice as many papillomas as expected from the summation of the individual single doses. No carcinomas were observed.

The papilloma and carcinoma yields for μg NQO and 16 μg B(a)P are shown in Figure 10. Here the sequence of administration markedly affected the yield of tumors. NQO prior to B(a)P produced about the same yield of papillomas as the summation of the yields for the individual exposures. However, the yield of carcinomas was much greater than expected from the summation of yields from the individual exposures. When the sequence was reversed (B(a)P first), the yield of papillomas was about the same as that produced by either a single dose of NQO or B(a)P alone. Only about 50% of the yield expected from the summation of the single-dose yields was realized by administering B(a)P before NQO. In marked contrast to the NQO-first sequence, no carcinomas were observed.

The papilloma yield as a function of promotion time is shown in Figure 11 for single doses of BPL as indicated. The yield reached a peak at about 100 days and declined slowly thereafter until a fairly stable level was reached beyond 250 days. The 48 mg yield was about twice as great as the 24 mg yield throughout the experiment. Carcinomas began to appear after 350 days.

The dose response at 200 days of promotion for single and split doses of BPL is shown in Figure 12. The yield of papillomas versus dose is reasonably

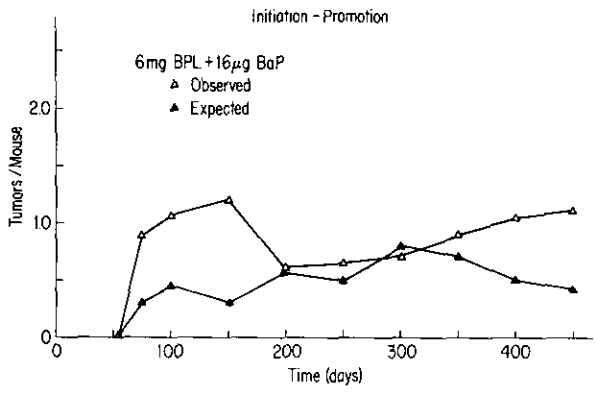

FIGURE 9. Plots of (Δ) the incidence of skin papillomas per mouse following initiation with 6 mg BPL plus 16 μg B(a)P and (▲) the numerical summation of the yields in groups of mice given single doses of 12 mg BPL or 16 μg B(a)P. All animals were promoted with 5 μg TPA three times per week.

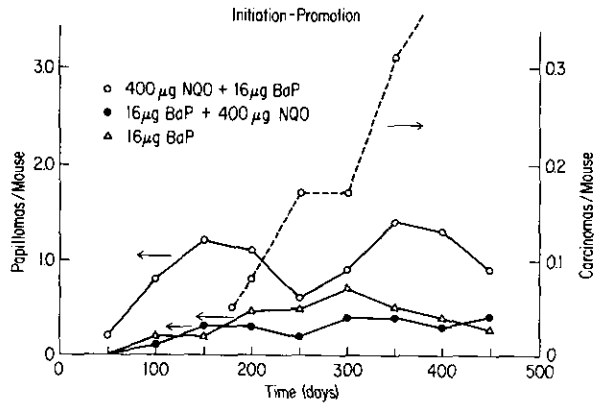

FIGURE 10. Incidence of skin papillomas (left) and carcinomas (right) per mouse observed following skin painting. Topical carcinogen treatments involved either a single dose of B(a)P or a single dose of either NQO or B(a)P followed two weeks later by another single dose of either NQO or B(a)P (whichever had not been applied the first time). Thrice weekly topical treatments with 5 μg TPA began one week after initiation.

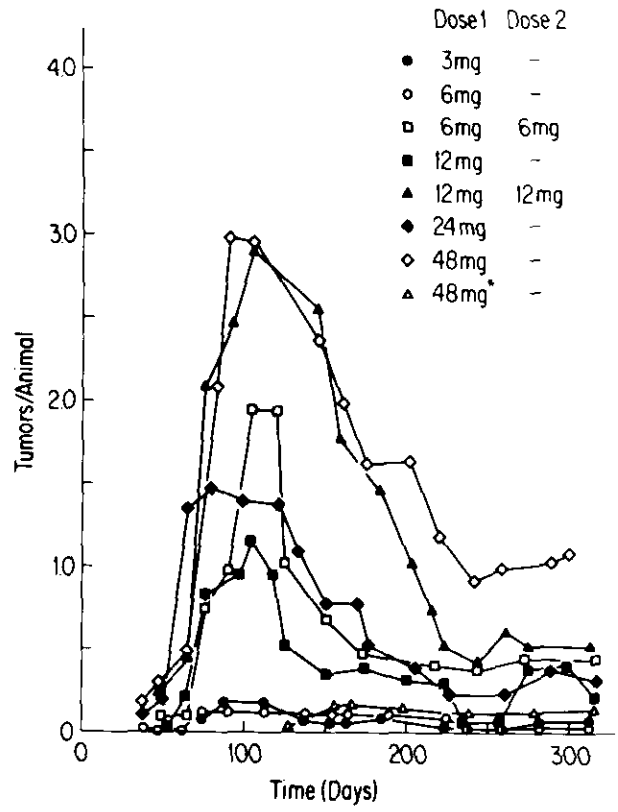

FIGURE 11. Yield of papillomas (average number of tumors per animal) as a function of promotion time. Single doses of β-propiolacetone (BPL) were given at 56 days of age followed by promotion with 5 μg TPA three times weekly.

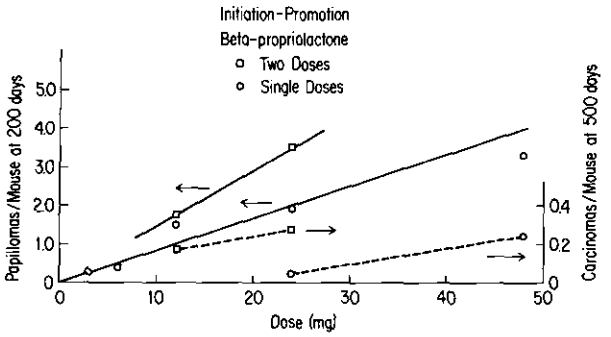

FIGURE 12. Yield of (left) papillomas and (right) carcinomas (average number of tumors per animal) as a function of applied dose of BPL. The papilloma yield at 200 days is plotted on the left scale and the carcinoma yield at 500 days is plotted on the right scale.

well fitted by a linear function, and the split doses produced up to 70% more papillomas than the corresponding single dose. Split doses produced as many as 10-fold more carcinomas than corresponding single doses.

The results reported here suggest that different carcinogens given far enough apart in time that chemical interaction is unlikely may interact in complex ways depending on the carcinogens. Even the same carcinogen may interact with earlier doses of itself to either enhance, as for BPL, or inhibit, as for NQO, the induction of papillomas by a promoter. Extensive evidence reported above indicates that polycyclic aromatic hydrocarbon, benzo(a)pyrene, produces papillomas in proportion to dose even when the dose is split into as many as 32 different fractions. From these results, B(a)P appears not to interact with earlier doses of itself.

It is difficult to explain results as seemingly diverse as these without considering the component processes of the carcinogenic mechanism (*5*). If initiation were the only effect being produced, the order of administration of two carcinogens ought not to matter. However, if one compound, e.g., NQO, were to have promoting activity relevant to B(a)P, then NQO prior to B(a)P ought to be more effective than vice versa. This effect was observed, especially with regard to carcinoma incidence. On the other hand, B(a)P or BPL could have approximately equal promoting activity relative to one another, since they were more effective in producing papillomas when given together in comparison to the summation of their individual effects. Since the enhancement observed here is inducible by a single exposure to the second initiator, the events involved could be analogous to what has been called the second stage of promotion (*20*).

It is not likely that B(a)P, NQO or BPL applied to skin persist in significant quantities after a few days, so that very little is expected to be present after one or two weeks. However, since inducible enzyme systems may persist after the inducing chemical is gone, the subsequent application of another chemical requiring activation after a dose could be influenced by a residual inducible enzymes still present at the time of the second application. Direct acting carcinogens, e.g., BPL and NQO, do not depend for their carcinogenic activity on enzyme activation, so the enhanced yield seen with the combination of these chemicals and B(a)P is probably not related to enzyme induction.

Progression of Papillomas to Carcinomas

Papillomas are focal, benign lesions consisting of folded layers of rapidly dividing cells that differentiate into squamous keratinizing cells almost as rapidly as they are produced (*21*). Such lesions may persist for many months growing slowly, other may regress, and still others may develop into invasive carcinoma (*22*). The latter papillomas are especially interesting in carcinogenesis. Generally about 5 to 7% of the papillomas underwent malignant conversion within the observation period (300 days).

Papillomas induced by initiation-promotion of mouse skin exhibit a spectrum of neoplastic properties in their ability to grow independently of the promoting chemical and their tendency to undergo conversion to carcinomas. Not unexpectedly, the greatest tendency for conversion to carcinomas was found among the papillomas with the greatest degree of autonomy, i.e., those having the least tendency to undergo regression when the promoting chemical is stopped.

Papillomas may be conceived of as clonal expansions of initiated cells, and the results here indicate that such cells, especially in autonomous papillomas, have a fairly high probability of undergoing malignant transition. It is not unreasonable to postulate that the precursor cells of the papillomas, i.e., the original initiated cells, retain the same probability of malignant transition as the cells in the papillomas. Since papillomas contain at least 10^5 cells, their overall probability of malignant transition would be at least that much greater than that of single initiated cells, and corresponding cancers would be expected earlier and with greater frequency in the papillomatous tissue. Obviously, more work is necessary to test such ideas, but the skin papilloma clearly provides an excellent model for studying the benign-to-malignant transition.

Prenatal Initiation of Skin Cells

It is known that exposure of a pregnant animal to carcinogenic chemicals is capable of producing a neoplastic response in her progeny, even without additional carcinogen treatment (*23*, *24*). However, comparatively little quantitative information is available concerning the effect of carcinogens on fetal cells at various periods of fetal development or to what extent growth and development affect the probability of a cell initiated in the fetus persisting and expressing its neoplastic potential in the adult organism.

In essence, this experiment was designed to determine if exposure to a carcinogen during fetal development results in initiated cells that can be promoted to form tumors in the same way and extent that has been found in adult skin. The goal of these experiments was to determine if fetal initiation could survive the dilution effect of the multiple cell divisions involved in development without alteration. These experiments were also aimed at obtaining information on the relative susceptibility of the fetal mouse skin at various periods of development and of adult skin to the tumorigenic effects of DMBA.

For each cell in the differentiating epidermal basal layer of the 9-day mouse fetus, there are at least 10 cells in 12-day fetus, 27 cells in the 15-day fetus, 63 cells in the 18-day fetus and 428 cells in the adult epidermal basal layer. In arriving at these relative numbers of cells, it was assumed that the relevant cells are contained in a monolayer covering the outer surface of the animal, that shape was not significantly changed by the growth, and that the number of cells was proportional to surface area.

Pregnant Ha/ICR Swiss mice were treated by gavage with DMBA on various days of gestation. Ten male and ten female offspring were obtained from mothers in each dose group. For the intragastric treatments, the powdered DMBA was freshly mixed with sesame oil (Erewhon, Cambridge, Massachusetts) on each treatment day. An 0.2 mL aliquot of the DMBA-sesame oil preparation was delivered via gastric intubation with a syringe fitted with a feeding needle. For the topical treatments, the DMBA was dissolved in acetone (Fisher Scientific Company, Fair Lawn, NJ) and applied with a Biopette (Schwarz/Mann, Orangeburg, NY). All carcinogen treatment occurred between 8:00 A.M. and 10:00 A.M.

Caesarean sections were performed on the pregnant mice on the afternoon of the nineteenth day of gestation. The offspring of the treated mothers were nursed by untreated foster mothers, who had given birth several days previously. The offspring were weaned at 4 weeks of age and were randomly assigned to one of the two secondary treatment groups.

The secondary treatments consisted of thrice weekly topical application of either 0.2 mL acetone (Fisher) of 5 μg TPA in 0.2 mL acetone beginning at 9 weeks of age. Treatment of pregnant mice with DMBA on day 15 of gestation produced skin tumors in their promoted offspring at all of the doses tested, 1, 2, 4 and 9 mg. No papillomas were observed in the acetone-treated or TPA-treated control groups. The time response of papilloma development in the offspring of mothers exposed to DMBA is shown in Figures 13, 14 and 15. These figures show that the first tumors appeared between weeks 6 and 8 of promotion in all groups. Figure 13 also shows that mice whose mothers received 8 mg DMBA developed fewer tumors than the offspring

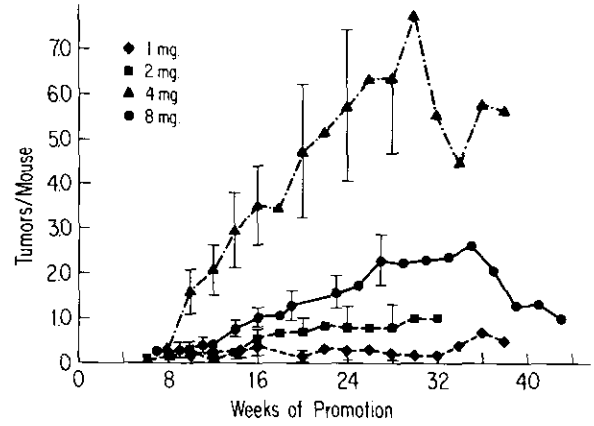

FIGURE 13. Time response of papilloma development following initiation with DMBA on day 15 of gestation.

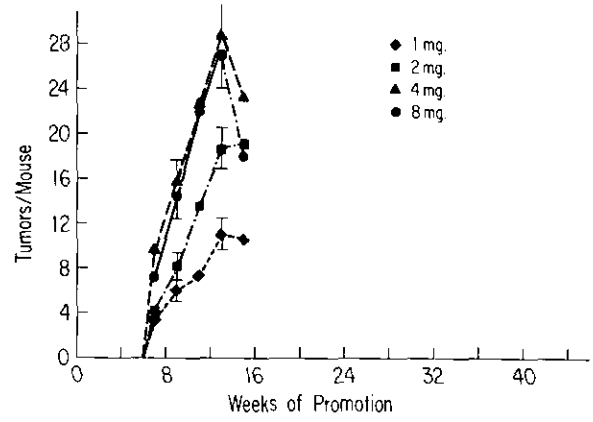

FIGURE 14. Time response of papilloma development following intragastric treatment of adults with DMBA. Experiment ended at 16 weeks because of high incidence of lymphoma.

of mice who received 4 mg DMBA. With the exception of the highest dose group, Figure 13 illustrates that reduction of DMBA dose results in a decrease in the multiplicity of tumors and total tumor yield.

The low tumor yield obtained from the highest dose group may be attributed to a generalized toxic effect. This dose produced some uterine bleeding, premature delivery, stillbirths and neonatal deaths.

When the yield of papillomas was plotted against dose (Fig. 16) or of epidermal cells (Fig. 17) in a given region of skin (the region receiving promotion in adults) for a given amount of intragastric carcinogen, a proportionality was obtained. Autoradiographic studies showed that the amount of carcinogen in the epidermal or presumptive epidermal cells varied very little between various stages of embryonic development and adults. These data are consistent with the concept that the risk of initiation per cell per unit dose is constant and that the tumor yield is proportional to the number of cells in the skin at time of exposure because a constant fraction of the cells are exposed to the risk. Furthermore, these data show that the initiated cell can survive and presumably transmit its initiated state to progeny in spite of a considerable amount of proliferation, growth and development from a 9 day embryo to adult.

This concept is similar to the idea proposed to explain an association between an increased tumor incidence and carcinogen exposure at later times of fetal development when more cells would be present (24). These workers suggested that the greater number of cells present at later times of fetal development was the basis for the enhanced tumorigenic response.

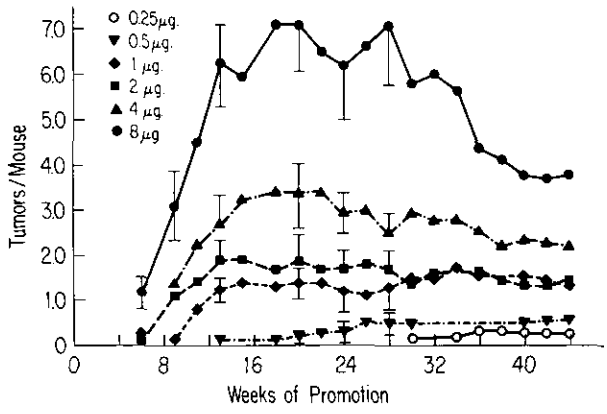

FIGURE 15. Time response of papilloma development following topical initiation of adults with DMBA.

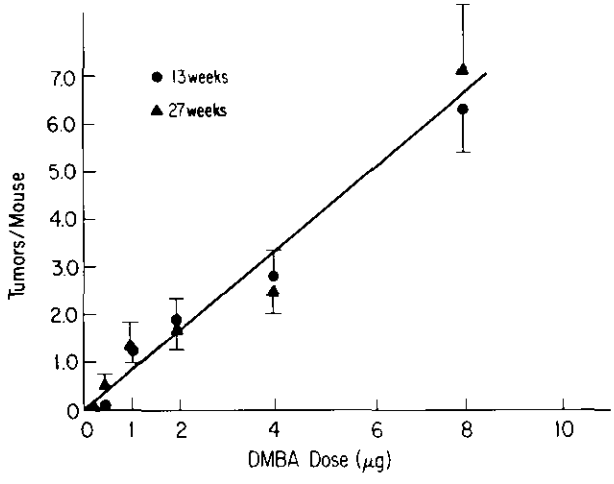

FIGURE 16. Dose response of papillomas at 13 and 27 weeks of promotion following topical application of DMBA to adults.

Conclusions

The model outlined here is tentative in the sense that additional confirmatory studies are necessary to define more pecisely the distinction between carcinogen-induced and spontaneous events and the role of clonal growth in whole carcinogenesis, and its applicability to organs other than skin is uncertain. Nevertheless there are several important and testable implications of the model at low carcinogen doses where considerable regulatory concern cur-

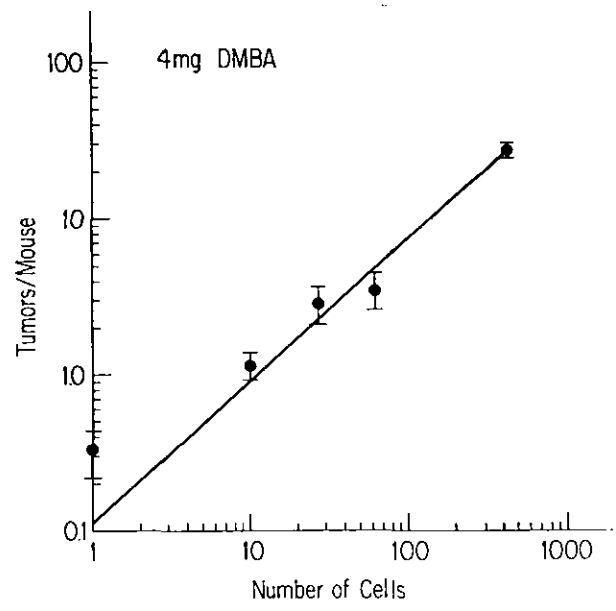

FIGURE 17. Tumor yield expressed as a function of the relative number of cells present at the time of exposure to 4 mg DMBA. In ascending order, the data points are for 9, 12, 15 and 18 day embryos and adults.

rently exists. One inevitable implication of the model is that as long as a_1 and a_2 are not zero, there must exist a region of dose where the dose-response function is linear. In the absence of promotion, the linear dose region is defined by the magnitude of the various transition constants, since the yield function has the form

$$y = \text{constant}\,(a_1 + b_1 d)(a_2 + b_2 d)$$

$$\sim a_1 a_2 + (a_2 b_1 + a_1 b_2) d$$

However, the clonal growth of cells containing the first transition effectively amplifies the value of a_1 and b_1 so that in the extreme as observed in our experiments the dose-response function becomes linear even at high doses. Clearly there must exist an intermediate situation where linear and dose-squared (or higher) terms coexist. The region of coexistence will be defined largely by the amount or intensity of the promotion. The model implies that the general dose-response function has the form $Y = (\text{constant})\ (A + Bd + Cd^2)$ where the magnitude of the constants A, B and C depends on promotion and/or clonal growth of one-event cells. Furthermore, the model implies that the constant may also be promoter dependent, because all additional non-independent events necessary to complete the transition to a cancer cell occur at a certain rate or probability in each cell generation, i.e., at each mitosis. Hence, an increased mitotic rate is expected to increase the rate of occurrence of such transitions.

In addition, clonal growth of two-event cells could affect the time function so that extreme care must be exercised when interpreting the exponent on the time function in terms of the number of events in carcinogenesis. At best, the exponent represents the upper limit of the number of events that might be involved but to the extent that clonal growth of intermediate stages is involved the exponent could reflect such growth. Since clonal growth is presumably stimulated by the action of promoters, these ideas emphasize the overriding importance of promotion and promoters in the temporal functions of cancer incidence.

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