Effect of the Promoter 12-O-Tetradecanoylphorbol-13-acetate on the Evolution of Carcinogen-altered Cell Populations in Tracheas Initiated with 7,12-Dimethylbenz(a)anthracene

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ABSTRACT

The aim of these studies was to investigate the effect(s) of the promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) on the evolution of different types of 7,12-dimethylbenz(a)anthracene (DMBA)-initiated rat tracheal epithelial cells in vivo. As described previously, upon exposure to carcinogens, cells appear in the tracheal epithelium which are distinguishable from the majority of the epithelial cells by a markedly enhanced in vitro growth capacity.

In the present study, tracheal transplants were exposed in vivo to 35 μg DMBA for 2 weeks and subsequently to 100 μg TPA. Controls were exposed to DMBA alone, TPA alone, or blank pellets alone. Tracheal cells were harvested by enzymatic procedures at 0, 3, 6, 12, or 18 months after the end of exposure to DMBA and at the same time points after the beginning of exposure to TPA or control pellets and were assayed in vitro with the epithelial focus (EF) assay for the frequency of different types of EF-forming units. Control tracheas yielded <1 EF/10^4 viable cells harvested. Of these EFs, 10% to 28% were subculturable (EFs), and no EFs derived from control tracheas exhibited growth in soft agarose (EFs_0). Exposure to TPA alone did not increase the yield of EF, EFs, or EFs_0 above control levels. Carcinogen exposure resulted in a 6- to 20-fold increase in yield of EF, a 2- to 3-fold increase in EFs, and a >15-fold increase in yield of EFs_0 above control levels. Neither the yield of EF nor the yield of EFs was affected by subsequent exposure to TPA. In contrast, there was a marked effect of subsequent TPA exposure on the maintenance and size of the cell pool giving rise to anchorage-independent offspring (EFs_0). By 3 months after DMBA exposure, 15% of EFs were EFs_0 in both the DMBA and DMBA-TPA exposure groups. However, in cultures established 12 months following exposure to DMBA alone, only 2% of EFs were EFs_0 following exposure to DMBA alone. In contrast, at this same time following exposure to DMBA and TPA, 18% of EFs were EFs_0. In a parallel two-stage carcinogenesis study with tracheal transplants, a significant enhancement of the DMBA-induced tumor response by TPA was observed. At 22 months after exposure, 4 and 37%, respectively, of DMBA- and DMBA-TPA-exposed tracheas developed invasive carcinomas.

In summary, it appears that initiation can be viewed as a series of complex cellular changes. With time, some of these changes are reversible. Exposure to TPA of cell populations initiated with low doses of DMBA results in the persistence of altered cell populations in the intact tissue. Without TPA treatment, some phenotypically altered cells appear to revert to a more normal state and/or fail to replicate.

INTRODUCTION

The phenomenon of multi- or 2-stage carcinogenesis has been most extensively studied in the mouse skin (1, 3, 13, 15, 17). More recently, it has been shown to also occur in other tissues such as lung (25), trachea (16, 19), urinary bladder (5), and liver (11, 12, 18). The studies herein reported were carried out to analyze 2-stage carcinogenesis at the cellular level in rat trachea. The experimental system used involves exposure of heterotopic tracheal transplants to DMBA and subsequently TPA released from intraluminal pellets (4, 6, 10). At various times after exposure, epithelial cells are harvested from tracheal transplants, cell cultures are established, and the EF assay is carried out. By means of this assay, we have been able to identify and isolate "carcinogen-altered" cells from tracheas, lungs, and esophagi exposed in vivo to DMBA or N-nitrosoheptamethyleneimine (19-22). These altered cells appear soon after exposure and can be found throughout the latency period. We have provided evidence suggesting that these cells are neoplastic since, compared with unexposed cells, they have an increased capacity to become neoplastic. These early neoplastic cells can be identified because of the acquisition of a markedly increased in vitro growth capacity. Under culture conditions which are "nonpermissive" for most normal tracheal epithelial cells, they not only survive but also proliferate rapidly, forming expanding EFs at a time when the cultures of normal tracheal cells have ceased to proliferate and senesce. Some of the epithelial colonies escape permanently from senescence (or terminal differentiation): they become "immortal" and can be propagated in vitro indefinitely (EFs). After repeated subculturing, some of the cell line cultures show anchorage-independent growth (EFs_0) and become neoplastically transformed, producing invasive carcinomas upon inoculation into compatible hosts. This sequence of (a) increased in vitro growth capacity (EF); (b) escape from senescence and immortalization (EFs); and (c) anchorage independence (EFs_0) commonly associated with neoplastic transformation is by no means unique for tracheal epithelial cells. It has been observed in a
number of epithelial and nonepithelial cell types after carcinogen exposure in vitro as well as in vivo (2, 7, 9, 16, 26), suggesting that it may represent an important series of events in neoplastic transformation. In normal tracheal epithelium, most of the clonogenic cells have only a limited replication capacity. After a limited number of population doublings, tracheal epithelial cells cease to proliferate and senesce. In contrast, cultures established from carcinogen-exposed epithelium contain clonogenic cells which proliferate extensively forming expanding EFs in late primary cultures. These altered clonogenic units were designated EFFUs. They often escape senescence permanently, thus giving rise to epithelial cell lines. Some of these become neoplastic after repeated subculturing. Those clonogenic units giving rise to subculturable progeny are termed EFFUs, and those giving rise to progeny which became anchorage independent are termed EFFUs\textsubscript{esp}. In the tracheal cell culture system, anchorage-independent growth is highly correlated with oncogenicity in vivo. The development and application of the EF assay for the analysis of cellular events of neoplastic development were described in several earlier publications (19–22).

The purpose of the present study is to investigate the effect of the promoter TPA on the induction of different types of EF-forming cells in tracheas initiated with DMBA.

**MATERIALS AND METHODS**

A previous tumor induction study (23) indicated that TPA causes a promotion-like enhancement of tumor formation in tracheas. We therefore decided to evaluate the effects in vivo of TPA on the development of “carcinogen-altered” tracheal cells as measured by the EF assay. Tracheal transplants were exposed in vivo to 35 ng DMBA for 2 weeks and subsequently to 100 ng TPA. Controls were exposed either to DMBA alone, TPA alone, or blank pellets alone. Tracheal transplants were harvested from host animals immediately; 3, 6, 12, or 18 months after the 2-week exposure to DMBA and the beginning of exposure to TPA or the control pellets.

**In Vivo Exposure of Tracheal Epithelium to DMBA and TPA.** Specific-pathogen-free female Fischer 344 rats were used. The methods for transplanting tracheas and exposing them to carcinogen and TPA have been described previously (4, 6, 10). Tracheal transplants were exposed over a 2-week period to a dose of 35 ng DMBA. The amount of DMBA actually released during the 2-week exposure interval was determined by using a spectrophotometric assay to measure the amount of DMBA remaining after 2 weeks in vivo (10). Control tracheas were exposed to beeswax-cholesterol (90%) without DMBA. After 2 weeks, pellets were removed and replaced by beeswax-cholesterol (20%) pellets with or without 100 ng TPA. These pellets were left in place for the duration of the experiment. In a previous experiment, the release rate of TPA was determined by measuring the change in amount of \( ^{3} \text{H} \) TPA in the pellet with time in vivo (10). Approximately 50% of the TPA was released within 4 months (10, 23). Ten tracheal grafts were harvested for in vitro studies at the following time points: (a) immediately after or 3, 6, 12, and 18 months after termination of DMBA exposure; or (b) at 3, 6, 12, or 18 months after the start of TPA exposure (TPA exposure was initiated at the end of DMBA exposure). For long-term tumor studies, approximately 40 tracheal grafts/exposure group were left in situ for up to 24 months after exposure.

**Preparation and Characterization of Tracheal Cell Suspensions.** Tracheas (10/group) were removed from host rats at different time intervals after the end of DMBA exposure or the start of TPA exposure (see above). Tracheal lumina were rinsed with HBSS and filled with 1% Pronase (protease type VI; Sigma Chemical Co., St. Louis, Mo.) in 0.9% NaCl solution and incubated for 20 min at 37° (19–22). Pronase was flushed out with 10 ml HBSS containing 0.5% bovine serum albumin (Sigma). Tracheal lumina were then gently scraped and again flushed out as described above. Cells collected from each trachea were pooled in separate centrifuge tubes and kept at 4°. Each cell sample was centrifuged at 150 × g for 10 min in a refrigerated centrifuge, the supernatant was removed, and 10-mi calcium and magnesium-free HBSS was added to each pellet. Cells were agitated for 1 min and again centrifuged. Following removal of the supernatant, 1 ml of 0.05% EDTA containing 1% crude trypsin (pH 8.2) was added. Cells suspended in trypsin were continuously agitated for 4 min at 37°. To each tube, 10 ml cold (4°) HBSS containing 20% FBS were added to stop enzyme action. Following centrifugation, supernatants were removed, and 2 ml Ham’s F-12 containing 10% FBS were added to each tube. The total number of viable (erythrosine B-excluding) cells stripped from each trachea was determined by counting each sample in a hemacytometer following a 1:1 dilution with erythrosine B. If any cell clumps were observed in the hemacytometer, the cell suspension was gently pumped through a 19-gauge needle 10 times, and the suspension was again checked for the presence of cell clumps.

The tracheal cell population obtained from each trachea was cultured separately. From each cell pool, \( 10^{2} \), \( 5 \times 10^{2} \), and \( 5 \times 10^{3} \) viable cells were seeded into two to four 60-mm tissue culture dishes per cell dose. The experimental protocol has been described in detail previously (see Ref. 21, Chart 1). Plating efficiency was estimated at 48 hr by counting the number of attached epithelial cells per 100 viable cells plated. All dishes were maintained in Ham’s F-12 plus 10% FBS, penicillin (100 IU/ml), streptomycin (100 Ìg/ml), insulin (0.1 Ìg/ml), and hydrocortisone (0.1 Ìg/ml). The culture medium was changed weekly.

**Determination of EFPU in Tracheal Grafts.** The cells recovered from tracheas (control and exposed) were seeded into culture dishes (see above) at various times after termination of exposure. One month after plating, the number of proliferating EFs per dish (Ref. 22, Fig. 1, A and B) was determined. If a focus of epithelial cells, persisting at least 1 month after seeding in culture, appeared to be proliferating, it was considered to be an EF. The EF data are expressed as EF/10\(^{3}\) viable cells.

**Isolation and Passage of EF-derived Cell Populations.** Dishes containing <4 EFs with at least 1 well-circumscribed EF were used for the isolation of EF cell populations. Except for 1 EF chosen at random, all other cells (epithelial cells and/or fibroblasts) were scraped from the dish. The remaining EF was maintained in culture until it attained a diameter of 2 to 3 cm (2 to 5 \( 10^{6} \) cells). The time required in primary culture for a focus to reach this size is referred to as the “growth rate” for that particular focus. The entire EF was then removed from the dish by brief trypsinization (3 to 4 min at 37°) and scraping with a rubber policeman and was transferred to a new 60-mm tissue culture dish (second passage). The number of cells transferred was estimated from counts of cells within a fixed area of an ocular grid performed on the primary cultures prior to trypsinization. When the secondary culture became confluent, the entire cell population was passaged into a 100-mm dish (third passage). All isolated EFs successfully completing the third passage were considered EF\(_{\text{esp}}\). When confluent, all cells were suspended in 0.5 ml of 10% dimethyl sulfoxide, frozen, and stored at liquid nitrogen temperatures. All EF\(_{\text{esp}}\) to be tested for growth in soft agarose were rapidly thawed 2 to 8 months later and seeded in two 100-mm dishes (fourth passage). In order to determine the effects of freezing and thawing on EF populations, EFs from 3 experimental groups were tested for growth in soft agarose before and after freezing. Freezing and thawing cells did not affect the frequency of EF\(_{\text{esp}}\).

**Testing for Anchorage-independent Growth.** We have reported previously (19) that anchorage-independent growth of carcinogen-exposed tracheal epithelial cells correlates well with oncogenicity in vivo when the minimum in vivo tumor latency is short (i.e., <42 days). In this study, we are, therefore, using a test for anchorage-independent growth to assess the oncogenicity of fourth-passage, EF-derived cell populations. The method used for preparation of the agarose plates was similar to that described by MacPherson (B). Cells (5 \( \times 10^{4} \)) were seeded...
in the top layer containing 0.3% agarose (Type II, electrophoretic grade; Sigma). The entire dish was stained with a tetrazolium salt (14) and was scored 3 weeks after plating. Any cell population yielding at least 1 colony of 20 or more cells on each of 2 duplicate dishes was considered positive (i.e., >0.004% plating efficiency).

**RESULTS**

The purpose of these studies was to investigate the effect of the promoter TPA on the induction of tumors and on the development of different types of EFFUs cells in tracheas initiated with low doses of DMBA. TPA exposure of DMBA-initiated tracheas was associated with a 7-fold increase in frequency of carcinomas above that observed following exposure to DMBA alone. While subsequent exposure of DMBA-initiated tracheas did not markedly affect the yield of EF or EF, there was a marked effect of TPA on the maintenance, in vivo, of that cell population giving rise to EF.

Effect of TPA Exposure on the Tumor Incidence in Tracheas Initiated with DMBA. Previous data indicated that tracheal tumor induction can be increased 3.5-fold by 100-μg TPA exposure given subsequent to 100 μg DMBA using a 2-stage experimental design (23). In the present experiment (Chart 1), the “initiation” dose was smaller (35 μg DMBA), and the duration of DMBA exposure was shorter (2 weeks instead of 4 weeks). DMBA alone induced a carcinoma incidence of 4.7% (3 carcinomas in 64 tracheal transplants). TPA exposure of tracheas initiated with DMBA resulted in a tracheal carcinoma incidence of 36%. It is noteworthy that this marked promotion effect becomes noticeable only about 14 months after the beginning of the TPA exposure. All of the tumors induced in this study were invasive squamous cell carcinomas.

Effect of Carcinogen and TPA Exposure on the Incidence of EF. The principal end point measured during the first phase of this study was the formation of EF (defined as a proliferating and expanding focus of epithelial cells) after 1 month in culture. The relevant data are summarized in Table 1 (Column 2) and expressed as EF/10^6 viable cells. Between 2 and 9 × 10^5 viable cells were harvested from each trachea regardless of exposure history.

Between 3 and 12 months after exposure to DMBA or to DMBA followed by TPA, the frequency of EF ranged from 6 to 13/10^5 viable cells, i.e., 6 to 20 times higher than in controls not receiving DMBA. It is important to note that, during this time, TPA treatment had no measurable effect on EF frequency.

At 2 important time points is the frequency of EF per 10^5 cells not or only marginally increased above controls in DMBA-exposed tracheas: (a) Immediately at the end of the 2-week DMBA exposure, only 2 EF/10^6 cells were detected. This may be due to the fact that the time required for optimal increase in enhanced *in vitro* growth capacity is longer than 2 weeks; (b) At 18 months after exposure to DMBA (without subsequent TPA treatment), the number of EFs has returned from its peak at 11 per 10^6 cells to 2 per 10^6 cells, which is close to or only slightly above control levels. In contrast, in the tracheas first exposed to DMBA and subsequently to TPA, the EF frequency remains at 7/10^6 cells at 18 months. The important point is that TPA treatment apparently prevents the reduction (p < 0.01) in EF frequency which occurs in tracheas exposed to low doses of DMBA.

In the present experiments, the frequency of EFFUs in vehicle-exposed and TPA-exposed tracheas is roughly 10-fold higher than that previously reported for beeswax-exposed control tracheas (19, 20). For reasons which are not clear at this time, the increase in control EFFUs may be associated with the addition of cholesterol to the vehicle matrix. Despite this, all carcinogen-exposed tracheas contained significantly more (p < 0.05) EFFUs than did vehicle-exposed control tracheas.

Effect of DMBA and TPA Exposure on the Growth and Subculturability of Isolated EF. After 4 to 12 weeks in primary culture, one well-isolated EF/dish was selected at random, and all other epithelial cells and fibroblasts were scraped from the dish with a rubber policeman. When the isolated focus expanded to 2 to 5 × 10^5 cells, the entire focus was transferred to a new 100-mm plastic tissue culture dish (second passage). The average time to attain this size for EF isolated from vehicle-exposed tracheas or from tracheas exposed to TPA ranged only from 15 to 16 weeks (Table 1, Column 4). In contrast, those EFs isolated immediately and up to 3 months after exposure to DMBA (or DMBA followed by TPA) grew more rapidly; less time (only 8 to 9 weeks) was required for the EFs to expand to 2 to 5 × 10^5 cells. By 12 to 18 months after exposure to DMBA (with or without subsequent TPA), the “growth rate” of EFs in primary culture had slowed down and was not markedly different from that observed for EFs isolated from vehicle-exposed controls.

During the second passage, the time required for EFs derived from DMBA- or DMBA-plus-TPA-exposed tracheas to yield confluent 100-mm dishes was less than that required by EF derived from vehicle control-exposed tracheas (namely, 3 to 4.5 weeks to reach confluence instead of 6 to 8 weeks). The relevant data are summarized in Table 1, Column 5. The only notable exceptions were the cultures established from tracheas at 18 months after exposure to DMBA only, which required a longer time (p < 0.01).

The frequency of EFs isolated from DMBA-exposed tracheas from which EFs could be successfully established after enzymatic dissociation was not affected by TPA (Table 1, Column 6). From both groups of tracheas, 82 to 95% of isolated EFs could be subcultured. In contrast, the EFs isolated from vehicle or TPA-exposed tracheas, only 6 to 39% (p < 0.001) were subculturable.

**Effect of Carcinogen and TPA Exposure on the Occurrence**

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*Unpublished data.*

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of Anchorage-independent Growth in EF-derived Cell Populations (EFs.ag*). A marked difference existed between EF-derived cell populations obtained from tracheas exposed to DMBA and DMBA plus TPA compared to those from the 2 groups of control tracheas (exposure to TPA or blank pellets). Between 2 and 25% of the former had acquired anchorage-independent growth characteristics, while none of the latter had developed the capacity for anchorage-independent growth. This clearly shows that only DMBA, but not TPA or blank beeswax-cholesterol, pellets, can induce anchorage-independent growth. TPA exposure did not appear to affect the maximum frequency of anchorage-independent growth in cultures derived from DMBA-exposed tracheas, which was reached at 3 months after exposure. However, it did prevent the subsequent reduction (p < 0.01) in EFs.ag frequency which was observed in cultures derived from DMBA-exposed tracheas not treated with TPA. In these tracheas, the EFs.ag frequency dropped from 16% at 3 months to 2 and 5% at 12 and 18 months, respectively. In contrast, in the tracheas exposed to DMBA as well as TPA, the frequency of EFs.ag of 15% at 3 months remained high at 12 and 18 months; namely, at 20 and 25%, respectively.

**DISCUSSION**

In the present study, we wanted to evaluate the effect(s) of TPA exposure on the sequential development of carcinogen-altered cell populations in initiated tracheal epithelium. The main observations can be summarized as follows. With one possible exception, exposure to TPA did not markedly affect the induction of "early" carcinogen-altered cell variants. Rather, the principal effect of TPA appears to be that it prevents (or reduces the rate of) the decrease in the size of the carcinogen-altered cell compartment(s) which occurs with time after exposure to low doses of DMBA, particularly the cellular phenotype which shows anchorage-independent growth in vitro and is probably a close precursor of fully transformed malignant phenotypes.

DMBA exposure (2 weeks) alone increased the frequency of EFFU/trachea roughly 10-fold above that observed in control tracheas. This increase was detected in tracheas harvested 3 to 12 months after exposure. Tracheas harvested immediately and 18 months after exposure to 35 μg DMBA for 2 weeks did not have an EFFU frequency above that observed in controls. Previous experiments suggest that 3 to 4 weeks are required for full development of EFFU in vivo. This is the most likely explanation for the low EFFU frequency observed immediately following exposure. By 18 months after exposure, it appears that EFFU present at earlier times have either reverted to a more "normal" state or have died.

Following carcinogen exposure, many of those cells which survive and proliferate in primary culture appear to have permanently escaped from senescence or terminal differentiation. Several parameters relevant to this phenomenon were examined: (a) the time required in primary culture for a focus to attain a size of 2 to 5 × 10^5 cells, referred to as "growth rate"; (b) the capacity for one or more cells within a focus containing 2 to 5 × 10^5 cells to survive and proliferate following enzymatic disruption and transfer into secondary culture; and (c) the time required for EFs to yield confluent secondary cultures. EFs isolated from both DMBA-exposed tracheas grew more rapidly in culture if isolated immediately and up to 3 months after exposure, compared to EFs harvested later (Table 1). The "growth rate" returned to control levels in EFs harvested 12 to 18 months following exposure. This may be a reflection of the population-doubling time and/or the fraction of cycling cells within each focus. In all carcinogen-exposed tracheas, the subculturable fraction of EF was larger than those in vehicle and TPA-exposed tracheas (p < 0.001). In addition, the time required for passage of EF to yield confluent secondary cultures was shorter for EFs from DMBA-exposed tracheas than the time required for EFs from control tracheas. The predominant factor affecting this time interval is the number of clonogenic cells transferred to the secondary
culture. Obviously, a culture inoculated with 1 clonogenic unit will take longer to become confluent than will a culture seeded with 100 clonogenic units. Secondary cultures seeded with 2 to $5 \times 10^6$ EF cells from control cultures often contained fewer than 5 proliferating epithelial colonies. In contrast, those established with the same number of EF cells from cultures inoculated with DMBA-exposed tracheal cells often contained 50 or more proliferating epithelial colonies. Thus, for reasons which are not entirely clear, EF from DMBA-exposed tracheae have a marked increase in clonogenic units over that seen in EF established from control tracheae.

It has been reported previously that TPA enhances neoplastic development in vivo (23) and in vitro (16) in carcinogen-exposed rat tracheal epithelium. In support of these observations, we found that exposure of DMBA-initiated tracheae to TPA was associated with a 10-fold increase, from 4 to 40%, in the frequency of carcinomas. This enhancement is more marked than that previously reported when a higher initiating dose of carcinogen was used (19, 21). In the present experiment, the most significant effect of TPA on the development of tumors was not observed until 12 to 24 months after the beginning of TPA exposure.

The most marked effect at the cellular level of TPA on DMBA-initiated tracheal epithelium was the long-term persistence of carcinogen-altered cells induced by low doses of DMBA in vivo. This effect was most marked with respect to the persistence of those cells giving rise to EFsagr in culture. In tracheae harvested up to 3 months after exposure, no difference in the frequency of EFFUSagr was noted between DMBA- and DMBA/TPA-exposed tracheae. By 12 months following exposure to DMBA alone, there was a significant decrease in the frequency of agarose-positive EF. This reduction in the number of anchorage-independent cells in the intact tissue was not observed in tracheae which were treated with TPA subsequent to DMBA exposure. It is not clear from these experiments whether exposure to TPA inhibits reversal of the potentially neoplastic state or whether it enhances those processes (progression and/or expansion) which tend to increase the conversion rate of EFFUagr to EFFUSagr or the growth rate of EFFUSagr in vivo. The net effect in either case would be persistence of the qq population in vivo.

How can the persistence of potentially neoplastic (EFFUSagr) cell populations in vivo be related to the development of carcinomas? Assuming that EFFUSagr are indeed precursors of neoplastic cells in the intact tissue, an increase in tumor incidence could be explained by either an increase in the frequency of EFFUSagr, and thereby an increased chance that one of these abnormal phenotypes will become malignant, or an increase in the frequency of conversion of EFFUSagr to neoplastic cells. The data presented herein tend to support the former hypothesis. The effect of TPA on the incidence of EFFUagr was not observed until 12 to 18 months after exposure, i.e., the time when marked TPA effects on the development of tumors in vivo were noted. Previous experiments seemed to suggest that roughly 50 EFFUagr/trachea (detected 16 weeks postexposure) had to be present for a tumor to develop (20, 21). In our present study, 2% of tracheae contained approximately 50 EFFUagr, following exposure to 35 $\mu$g DMBA. On the other hand, 50% of tracheae exposed to 35 $\mu$g DMBA followed by TPA when sampled at the same time contained roughly 50 EFFUagr. In order to definitively correlate changes in EFFUagr frequency with TPA-induced enhancement in tumor frequency, it will be necessary to carry out further experiments in which more EFs (at least 10 EF/trachea) are tested for anchorage-independent growth.

Whether or not initiation involves an irreversible process is clearly an important question and one which has received a great deal of attention since the early experiments of Berenblum and Shubik (1). The observation reported by these authors, namely, that extending the time interval between initiation and the start of promotion does not result in a decreased tumor yield, suggested that, at least in the skin, initiation may be irreversible. Since these early experiments, a number of others have been reported with similar results with skin (3, 17) as well as liver (11, 12, 24) as the target tissues. However, such tumor data do not necessarily accurately reflect the size of the initiated cell pool, as long as some initiated cells persist which are able to respond to TPA treatment with tumor formation. It is also notable that Roe et al. (13) have published results, using a mouse skin tumor promotion model similar to that described previously, which suggest a decrease in skin tumor yield with increasing time elapsing between initiation and the start of promotion. Roe et al. (13) suggested that the discrepancy between these and the earlier findings might be related to the fact that they applied a lower dose of initiator. Slaga et al. (15), in a series of initiator-dose-response studies, report data which suggest that DMBA may have reversible and irreversible effects and that these effects might be experimentally separable by a judicious selection of doses. It is possible that, in our studies, we were also able to detect reversibility because of the low dose of initiator selected. In previous experiments using the EF assay to study the dynamics of neoplastic development in DMBA-initiated tracheal epithelium and to explore carcinogen dose effects, no reversion of the initiated state was detected with passage of time up to 1 year after exposure (19, 21). In all cases, higher carcinogen doses or longer exposure times were used.

In summary, it appears that under the conditions used in the present experiments, initiation can be viewed as a series of complex cellular changes. With passage of time in vivo, some of these changes appear to be reversible. Exposure to TPA of cell populations initiated with low doses of DMBA results in the persistence of carcinogen-altered cell populations in the tissue. Without TPA treatment, some of the phenotypically altered cells may revert to a more normal state or may simply fail to replicate. Additional experiments are needed to further elucidate the effect of tumor promoters on carcinogen-altered cell populations.

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