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I am submitting herewith a dissertation written by Don Ray Miller entitled "Development of Improved Culture Conditions that Allow Optimal Proliferation and Differentiation of Mouse Epidermal Cells: Effects of Polycyclic Aromatic Hydrocarbons and 12-O-tetradecanoylphorbol-13-acetate (TPA) on Epidermal Cells." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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# DEVELOPMENT OF IMPROVED CULTURE CONDITIONS THAT ALLOW OPTIMAL PROLIFERATION AND DIFFERENTIATION OF MOUSE EPIDERMAL CELLS: EFFECTS OF POLYCYCLIC AROMATIC HYDROCARBONS AND 12-O-TETRADECANOYLPHORBOL-13-ACETATE (TPA) ON EPIDERMAL CELLS

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# ABSTRACT

Previous attempts to transform newborn mouse epidermal cells into neoplastic cells resulted in continuous cell lines that had lost most of the epidermal characteristics of the primary cultures. Normal epidermal cells had a limited life span and could not be subcultured (severely limiting the usefulness of these cultures), and the transformed (neoplastic) lines produced undifferentiated tumors in suitable hosts, not the squamous cell carcinomas characteristic of epidermal carcinogenesis *in vivo*. In order to overcome these drawbacks, I sought to improve the culture conditions used in past *in vitro* studies of epidermal cells by combining the best features of several different systems. Using an enriched Waymouth's medium, I found that normal mouse epidermal cells could be subcultured if grown at 31° on an irradiated BALB/c 3T3 Clone A31 feeder layer. Primary cultures also regularly gave rise to highly differentiated, continuous lines that would, if transformed, form squamous cell carcinomas in nude mice. Primary epidermal cells, targets for polycyclic aromatic hydrocarbon (PAH) carcinogenesis *in vivo*, were also shown to metabolize the PAH *in vitro*, forming from benzo[a]pyrene primarily the 7,8-dihydrodiol (immediate precursor to the proposed ultimate carcinogenic form of benzo[a]pyrene) with lesser amounts of other metabolites, some of which were conjugated to glucuronic acid. The 7,8-dihydrodiol has been shown to be formed at lower levels by most other cell types examined in previous studies.

In order to examine the biological effects of chemicals associated with epidermal carcinogenesis on normal epidermal cells,

I developed a quantitative assay for proliferation (secondary cloning efficiency) using newborn mouse epidermal cells. With this assay, I found that epidermal cells from SENCAR mice (a strain bred for sensitivity to skin carcinogenesis) were more susceptible than BALB/c epidermal cells (from a strain less sensitive to skin carcinogenesis) to PAH toxicity *in vitro*, implying that the SENCAR sensitivity may reside at the cellular level. SENCAR cells also proliferated much better *in vitro*, indicating a greater autonomy perhaps important in epidermal carcinogenesis. Since subsequent colony development did not necessarily parallel initial toxicity, factors other than toxicity were apparently involved in long-term epidermal proliferation. Toxicity of several different PAH roughly paralleled their carcinogenic potency, similar to results obtained in the past with fibroblast systems.

The epidermal-A31 interaction, which may reflect phenomena associated with carcinogenesis or differentiation *in vivo*, was found to be specific for the feeder type, probably requiring cell-cell contact since secondary epidermal colony production was not supported by A31-conditioned medium or culture surfaces. The tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA), previously shown to interfere with metabolic cooperation *in vitro*, also blocked epidermal colony formation, probably not through simple toxicity, These data imply that the A31-epidermal effect is apparently not a typical mesenchymal-epithelial interaction (as has been suggested for the similar Swiss 3T3-human epidermal interaction), since the basement membrane would prevent this contact in intact skin, and may involve

direct transfer of some critical molecule. TPA may function as a promoter by interfering with normal cellular communication, a phenomenon that appears to be very important for normal epidermal growth *in vitro*.

The biological properties of epidermal cells have long been neglected because of the lack of a suitable *in vitro* system. Epidermal cells grown under the conditions I have described should be more suitable for studies of cellular carcinogenesis and differentiation *in vitro* and *in vivo*.

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# CHAPTER I

# INTRODUCTION

## Background

Most of this material has been reviewed by many authors, and rather than give an exhaustive list of all the individual publications, I have wherever possible referenced review articles that cite those publications.

Percival Pott in 1775 was the first to notice that an organic combustion product (soot) was probably responsible for the high incidence of scrotal cancer in English chimney sweeps (Kipling, 1976). Later work showed that tar, pitch, and mineral oil were also associated with occupational skin cancer, although the actual causative agents were unknown. The era of experimental carcinogenesis began in 1915 when Yamagiwa and Ichikawa caused papillomas and skin carcinomas by the repeated painting of tar onto rabbit ears (Suss et al., 1973). Mice soon became the preferred experimental animal because they were easier to house and handle. The latency period of tumors induced on the dorsal skin of mice was also much reduced.

The ubiquitous carcinogen benzo[a]pyrene (BP) was finally purified from pitch in 1933 (Dipple, 1976). BP was only one of a large number of polycyclic aromatic hydrocarbons (PAH) that were later shown to be carcinogenic. The most important mechanistic concept to arise from the early animal studies was that skin carcinogenesis could be separated into two sequential stages: initiation and promotion (Boutwell, 1974; Scribner and Suss, 1978). Complete

carcinogens, such as the PAH, were thought to have both initiating and promoting activity, although the promoting activity diluted out at low concentrations. In 1941, Berenblum discovered the cocarcinogenic (potentiating) effect of croton oil (from the seeds of the Euphorb, Croton tiglium L.) applied simultaneously with BP to mouse skin. Three years later, Mottram showed that only a single subcarcinogenic dose of the carcinogen (acting as the initiator) was required, followed by repeated croton oil treatment. Further research demonstrated that initiation was necessarily the first stage and that it was additive and irreversible. On the other hand, promotion was found to be reversible, but not additive. The structures of the active phorbol esters from croton oil were determined in 1967 (Van Duuren, 1976). The most active phorbol ester (and still the most potent of all promoters) was later shown to be 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Other phorbol esters with different substituents at the 12- and 13-positions were much less active. Initiation-promotion has since been demonstrated in other rodent tissues in vivo (liver, mammary gland, colon, bladder, and lung) and in vitro (fibroblasts) with various initiators (chemicals and radiation) and chemical promoters (Scribner and Suss, 1978).

Recent investigations have been designed to reveal the mechanisms behind initiation and promotion. Crucial to the understanding of these mechanisms is a knowledge of the cellular changes that occur during neoplastic transformation. The many reports of chemically induced neoplastic transformation of cultured cells (Casto and DiPaolo, 1973; Heidelberger, 1973a, 1975) showed that cancer was a cellular

(not a systemic) defect. Most of these studies were done with cultured fibroblasts, since these cells were easier to grow and had a distinctive neoplastic (transformed) morphology in vitro (multilayered, crisscrossed cells, DiPaolo et al., 1969b and Reznikoff et al., 1973) that allowed early quantitation of transformed foci. Transformed fibroblasts also developed other characteristics (immortality, chromosomal aberrations, lectin agglutinability and other membrane modifications, loss of contact inhibition, loss of anchorage dependence, increased cloning efficiency, increased fibrinolytic activity, and decreased serum dependence--Freeman and Huebner, 1973; Tooze, 1973; Pitot, 1976; DiPaolo and Casto, 1977; Barrett et al., 1979), but these properties were not suitable for quantitating transformation because they were either not sufficient or not early markers. Neoplastic transformation was found to occur progressively over a period of time (Hayflick, 1967). Barrett and his colleagues (Barrett et al., 1977; Barrett and Ts'o, 1978a,b) investigated this in some detail, showing that growth in soft agar (a generally reliable marker) and tumorigenicity in vivo arose only after many population doublings. Several investigators developed quantitative transformation systems based on fibroblast morphology. Syrian hamster embryo cells (Berwald and Sachs, 1963, 1965; DiPaolo et al., 1969a) or cells of a cloned line from C3H mouse prostate (Chen and Heidelberger, 1969) were plated at low densities, treated with a chemical carcinogen, and the transformed colonies scored after sufficient incubation (usually one and one-half or six weeks, depending upon the system). Our basic concepts of cellular carcinogenesis came from these studies. In

*vitro* transformation quantitatively paralleled *in vivo* carcinogenicity for a series of PAH (DiPaolo and Casto, 1977). A direct role for toxicity in selecting for pre-existing transformed cells was ruled out when toxicity and transformation were shown to follow different curves. Heidelberger and co-workers later showed that single cells could be transformed at a very high frequency, implying that transformation involved a direct conversion of cells to a neoplastic state. Nevertheless, toxicity was recognized to be at least a secondary characteristic of chemical carcinogens (Suss et al., 1973), perhaps playing an indirect role in carcinogenesis (Griffin et al., 1979) or resulting from a similar biochemical mechanism.

The initial attempts to relate carcinogenicity to PAH structure were concerned with relative reactivities of the different bonds of various PAH (Dipple, 1976). Pullman suggested that an active "K-region" (e.g., the 4- and 5-carbons of BP; see diagram in Chapter III, page 34) and an unreactive (substituted) "L-region" (the 6- and its *para*-carbon) were required for carcinogenicity, but these criteria did not hold for all PAH, primarily because this as well as other similar theories did not consider metabolic activation of the carcinogens (Brookes et al., 1979). The Millers subsequently found that the ultimate carcinogenic forms of most, if not all, chemical carcinogens were very electrophilic (Miller and Miller, 1976, 1979). For the PAH, the ultimate carcinogenic forms are currently thought to be diolepoxides (Lehr et al., 1978; Yang et al., 1978; Brookes et al., 1979). *In vitro* carcinogenesis with PAH showed that they could be activated by microsomal mixed function oxidases of the target

cells. Although the actual molecular target for the reactive metabolites remains unclear, DNA has probably received the most attention. Ames's bacterial mutagenesis system revealed that 85% of the carcinogens tested were also mutagenic (Scribner and Suss, 1978); this correlation did not hold up quantitatively, however. Using Chinese hamster V79 cells, Huberman showed that mutagenesis was quantitatively correlated to initiating ability for a series of PAH. This finding fit in with the concept of an initiator acting to induce a somatic mutation that permanently altered a cell to a latent neoplastic state, and a promoter then acting to express the mutation. This is a very reasonable hypothesis, except that there are at least several instances where DNA damage does not seem to be involved. Teratocarcinomas of mice and kidney carcinomas of frogs were shown to have all the genetic information necessary to proceed along normal ontogenetic pathways if this information were put into the appropriate embryonic environment (Rubin, 1980). Nonporous sheets of chemically inert material caused tumors in rats, supposedly by disrupting tissue communication. Large amounts of certain body chemicals (e.g., steroid hormones) or tissues transplanted into unusual places also led to tumors. Finally, transformation was shown to occur at a much higher frequency than what would be expected for a simple mutation. Because of these findings, it is just as plausible that permanent epigenetic changes (such as those involved in normal tissue differentiation) are responsible for tumor growth. Ahuja and Anders (1977) developed the concept of gene regulation as the fundamental defect of cancer, although they recognized that aberrant regulation might also be induced by a somatic mutation.

The mechanism of promoter action also remains unclear. In addition to causing inflammation and hyperplasia in vivo, properties not by themselves sufficient for promoting activity (Scribner and Suss, 1978), TPA also brings about an altered state of differentiation. Raick (1973a,b) showed that adult mouse epidermis assumed a more embryonic morphology with TPA treatment. Superficial, flattened cells swelled, resembling hyperplastic basal cells. The rough endoplasmic reticulum and Golgi complexes of basal cells increased, resulting in an increased secretory activity not seen in normal adult epidermis. Scattered, electron-dense cells ("dark cells") filled with ribosomes also appeared, and the upper cell layers keratinized in a manner more characteristic of embryonic skin. In vitro, TPA prevented terminal differentiation of most cell types tested, including murine Friend erythroleukemia cells, preadipocytic Swiss and BALB/c 3T3 cells, C1300 neuroblastoma cells; newborn mouse epidermal cells (see Yuspa et al., 1976; Fusenig and Samsel, 1978); and chick embryo fibroblasts (with respect to collagen production only), myoblasts, and chondroblasts (Diamond et al., 1980). On the other hand, TPA caused further differentiation of Rauscher virus-transformed murine erythroid cells, HL-60 human promyelocytic leukemia cells, and HO human melanoma cells. Cells grown in TPA-containing medium also adopted (reversibly) certain phenotypic characteristics of transformed cells: plasminogen activator activity, transformed morphology, enhanced growth, altered cell surface, and novel enzymes (Weinstein et al., 1979). These data argue that TPA can affect gene expression, and that prior initiation may fix in some way the TPA-stimulated

effects. The molecular target(s) for TPA is unknown. Because TPA is active at such low concentrations ($10^{-8}$ to $10^{-6}$ M *in vitro* and $10^{-8}$ mol/application *in vivo*, Boutwell, 1974) and because of its many established cell membrane effects, it has often been suggested that a membrane component may be involved, perhaps a receptor that would normally bind to some endogenous growth factor or hormone. A physical perturbation of membrane structure that could interfere with endogenous growth regulators is also a possibility (Diamond et al., 1980).

Whether altered differentiation (gene expression) is the mechanism of promotion is unknown. TPA's mode of action on epidermal differentiation is unclear because the factors that control epidermal differentiation *in vivo* are unclear. Mesenchymal-epithelial interactions, of course, have been known for some time (Wessells, 1967; Flaxman and Maderson, 1976). One mesenchymal function is merely support, but there also appear to be others, as the two types of tissue must cooperate to form various skin structures. Early ectoderm, at least of amphibians, is apparently totipotent, but with subsequent growth the developmental pathways available to ectoderm become more and more restricted. Initially, these pathways are influenced (programmed) by the subjacent mesenchyme, but the local ectoderm eventually becomes totally committed to a given pathway and cannot be reprogrammed. It can be modified slightly, however, since epidermal thickness appears to be determined by the underlying connective tissue. Keratinization is thought to be the default pattern of epidermal differentiation, as its appendages (e.g., sebaceous gland cells during wounding) generally revert to keratinization if

mesenchymal influences are removed. The exact nature of mesenchymal-epithelial interactions, aside from the fact that they exist, are essentially unknown.

## My Research

More than 85% of all human tumors are epithelial in origin and a significant number are thought to be caused by environmental factors. The PAH are ubiquitous environmental chemicals that have been proven carcinogenic in experimental animals, primarily in studies using mouse skin. The natural *in vitro* extension of these studies would be to mouse epidermal cells, but this was prevented in the past because of the difficulty in growing epidermal cells. Epithelial cells that had previously been used also did not have early, reliable markers of neoplastic transformation. Most investigators therefore continued to use fibroblast systems for *in vitro* transformation studies in spite of the fact that epithelial cells were responsible for most human tumors.

In addition to these, there were other reasons that warranted the development of an improved epidermal cell culture system. The epidermis is a tissue that is in immediate contact with the environment. The toxic effects of pollutants (e.g., PAH) on these cells and the response of these cells to them or to TPA would also be of critical importance in helping us interpret responses to such chemicals *in vivo*. In addition, because the epidermis is a very highly differentiated epithelial tissue, differentiation itself could also be examined with a good *in vitro* system.

For all these reasons, I felt it was necessary to try to improve the published epidermal cell culture procedures. Ultimately, I sought to develop a quantitative assay for epidermal growth so that I could examine the biological effects of the PAH and TPA at the cellular level. Yuspa and Harris (1974) had developed a technique for preparing pure epidermal cultures from newborn mice. As was the case with previous epidermal systems, epidermal cells with or without contaminating fibroblasts could not be subcultured, and started to senesce after a very short period of time (about two weeks) in vitro. Somewhat earlier, Elias et al. (1974) had found that whole skin cultures could be treated with 7,12-dimethylbenz[a]anthracene and would eventually give rise to cell lines that formed undifferentiated tumors in suitable hosts. Colburn et al. (1978a,b) and Slaga et al. (1978b) then showed that carcinogen-treated cultures of pure epidermal cells would also produce the same types of cell lines and tumors. Since epidermal differentiation could not be maintained for long periods of time in culture, the culture conditions used in these studies (medium 199 with 10% fetal bovine serum, 37°) were obviously deficient. Rheinwald and Green (1975a,b) had had success using Swiss 3T3 cells as a feeder layer for human epidermal cells; human cells could be subcultured and cloned on irradiated 3T3 cells but would not give rise to permanent cell lines. Since human epidermal cells normally grow much better than mouse epidermal cells, it was not clear whether this technique would also work with newborn mouse cells. In other published work, Hennings et al. (1976) had noted improved growth of mouse epidermal cells at 31°, and Marchok et al.

(1976) had developed an enriched Waymouth's medium for growing rat tracheal epithelial cells. There were no short-term quantitative assays of epidermal cell growth with which to evaluate these conditions. The best assay for long-term growth was the production of continuous cell lines. I therefore examined the effects of these culture conditions (except that I used BALB/c 3T3 Clone A31 cells instead of the similar Swiss 3T3 line) on cell line production in BALB/c and SENCAR (a mouse strain bred for sensitivity to chemically induced skin carcinogenesis) epidermal cells. In Chapter II, I show that these improved culture conditions (enriched Waymouth's medium, 31°, irradiated A31 feeder layer) allow the production of highly differentiated continuous cell lines and the subculture of normal mouse epidermal cells. If transformed by chemical carcinogens, these lines form squamous cell carcinomas in nude mice, although the absence of early neoplastic markers so far precludes any quantitative assays for transformation.

With the improved culture conditions, I could then look at the in vitro effects of chemicals associated with epidermal carcinogenesis. Cultured cells are known to metabolize the PAH to more easily excretable, water-soluble products. Since the PAH are also activated during this metabolism, these biochemical pathways are important for our understanding of tissue susceptibility to PAH carcinogenesis. BP metabolism has been studied in many other systems. In Chapter III, I report its metabolism in SENCAR epidermal cells and show that these cells (as representative epidermal cells) produce comparatively high

levels of the 7,8-dihydrodiol, the immediate precursor to the ultimate carcinogenic form of BP.

Many of the PAH metabolites produced by the microsomal mixed function oxidases are cytotoxic. Toxicity is a biological effect generally associated with carcinogenic chemicals. In Chapter IV, using as an assay secondary epidermal cloning efficiency, I show that SENCAR cells are more susceptible than BALB/c cells to PAH toxicity. SENCAR cells are thus less able to deal with both the carcinogenicity and toxicity of the PAH, although they proliferate much better than BALB/c cells in culture. I also show that long-term colony development does not necessarily follow initial toxicity, implying that factors other than toxicity also have an effect on long-term epidermal growth.

In view of the finding that A31 cells stimulate epidermal proliferation, I decided to further examine this effect, since it may reflect in vivo processes. In Chapter V, I establish that the epidermal-A31 interaction (specific for the feeder type) is not transmitted by the culture medium and probably not by the culture surface. Because of my work and the results of past work, I conclude that the interaction is probably mediated by epidermal-A31 contact. Since TPA, previously shown to inhibit direct transfer of label (metabolic cooperation) between cultured cells (Murray and Fitzgerald, 1979; Yotti et al., 1979), also inhibits the epidermal-A31 interaction, the A31 effect may be mediated by direct transfer of some critical factor.

# CHAPTER II

## THE MAINTENANCE OF A DIFFERENTIATED STATE IN CULTURED MOUSE EPIDERMAL CELLS


### Abstract

Previous attempts to transform normal mouse epidermal cells into neoplastic cells yielded continuous lines that had lost most of the morphological characteristics of the primary cultures. These lines were classified as epidermal in origin because they arose from pure epidermal cultures, had intermediate junctions (but not true desmosomes), and if tumorigenic formed undifferentiated carcinomas (not fibrosarcomas). A major drawback to these studies was that keratinizing squamous cell carcinomas were not routinely produced. In our ongoing efforts to produce tumorigenic epidermal lines that retain their differentiation, we examined several culture modifications for their effects on mouse epidermal cells. Using an enriched Waymouth's medium, we found a set of conditions that (1) allows normal mouse epidermal cells to be subcultured and (2) yields continuous lines that retain many differentiated epidermal traits. Critical conditions are a BALB/c 3T3 Clone A31 feeder layer and incubation at 31°.


### Introduction

We have been examining the growth of mouse epidermal cells in long-term culture to develop a reliable epithelial system for neoplastic transformation. Pure epidermal cultures can be obtained easily with the technique developed by Yuspa and Harris (1974). Primary

cells are harder to maintain and are less defined than established lines, but are initially normal. Worst et al. (1974) have shown that these cells are able to regenerate a normal epidermis in vivo after several days in culture.

To be applicable to the past in vivo work, any in vitro epidermal transformation system must yield lines that produce differentiated epidermal tumors in suitable hosts. Initial attempts to transform mouse epidermal cells have usually yielded "dedifferentiated" lines that produce undifferentiated tumors (Fusenig et al., 1973; Colburn et al., 1978a,b; Slaga et al., 1978b). Dedifferentiated is perhaps the best descriptive term for these lines since they arose from cultures that had desmosomes (Colburn et al., 1978a) and were initially producing keratin (Steinert and Yuspa, 1978), although the resultant lines had neither of these characteristics. These lines did possess other types of junctional complexes and were designated as epithelial in origin. The vast majority of cells in the epidermis are highly differentiated epidermal cells; however, the presence of a small undifferentiated population that could have given rise to these lines cannot be ruled out. Whether or not this occurred, the highly differentiated epidermal colonies themselves dedifferentiated with time in culture. We found that primary epidermal colonies grown under those conditions progressively ceased to pile up and produce squames and keratinized debris, although viable cells were still present and were not overtaken by undifferentiated cell types.

Since epidermal cells spontaneously dedifferentiated, the culture conditions in these early studies were obviously deficient.

Normal epidermal cells could not be subcultured and most cultures died after several months *in vitro*. The subculturable dedifferentiated colonies arose only in some flasks after months in culture. In the process these cultures lost most of their epidermal characteristics. It was necessary to find alternative culture conditions that would allow epidermal cells to remain differentiated *in vitro*. These epidermal cells could then be expected to behave more like epidermal cells *in vivo*. With an enriched Waymouth's medium, a 3T3 feeder layer, and a lower incubation temperature, normal mouse epidermal cells can be subcultured and will yield continuous lines that retain many differentiated epidermal characteristics. These lines eventually become independent of the 3T3 feeder layer.

## Materials and Methods

### Cell Culture

Epidermal cells were isolated from the skins of newborn (1-3 day) SENCAR mice (a population specially bred by R. K. Boutwell, University of Wisconsin, to be sensitive to chemically induced skin carcinogenesis) by the trypsin flotation method of Yuspa and Harris (1974). Briefly, the skins were floated dermis-side-down on a trypsin solution overnight at 4°. The epidermis was then peeled away from the dermis, minced, and the epidermal cells plated at either 31° or 37°, either on plastic at $3 \times 10^6/25\ cm^2$ flask or onto nearly confluent feeder layers at $3 \times 10^5$/flask. The culture medium was a modification (Fischer et al., 1980) of an enriched Waymouth's MB 752/1 medium developed by Marchok et al. (1976), supplemented with 10% fetal bovine

serum (FBS) (Gibco or Microbiological Associates). Horse serum (HS) or heat-inactivated horse serum (IHS) was used in some of the long-term 3T3 feeder studies. Because these sera only produced minor differences in line morphology, the FBS, HS, and IHS data were pooled for subsequent analysis. BALB/c epidermal cells and/or medium 199 with 10% FBS were used for some of the other long-term studies.

Epidermal cells were removed for replating (for the experiments below) with 1 ml of 0.25% trypsin--0.04% EDTA (Gibco), applied at 37° for up to 90 minutes. Passage of the differentiated epidermal lines also required this treatment, followed by replating onto irradiated 3T3 feeder layers for the first few population doublings; the 3T3 cells gradually died off and were displaced by the proliferating epidermal cells. Other lines were passaged with 0.05% trypsin--0.02% EDTA. The lines were initially split 1:4, but could eventually be split 1:16 or 1:64 with increasing time in culture. Staining with Rhodanile blue (for keratin) was done according to Rheinwald and Green (1975a).

Feeder Layers

Dermal fibroblasts from SENCAR mice were prepared on a Ficoll gradient, after the procedure of Yuspa et al. (1976). Primary fibroblast cultures were either used as is or replated for use in the epidermal cloning efficiency assays. BALB/c 3T3 Clone A31 cells were donated by R. W. Tennant, Oak Ridge National Laboratory. These cells were maintained subconfluent at 37° in enriched Waymouth's medium with 10% FBS. An additional 0.2 mM L-glutamine was included

for 3T3 cells. Cells to be used for feeder layers were lethally irradiated with 5000 R X-rays.

Cloning Efficiency

Primary epidermal cells were plated on plastic ($3 \times 10^6/25$ $cm^2$ flask) at 31°. For secondary cloning efficiencies, $2\text{-}5 \times 10^5$ epidermal cells were replated onto $2 \times 10^6$ irradiated feeder cells in 25 $cm^2$ flasks at 31° or 37°. Epidermal colonies were counted two weeks later. These colonies were easily identifiable under phase contrast and required no special staining.

Electron Microscopy

Cultured cells were prepared for electron microscopy by the method of Brinkley et al. (1967). Prior to fixation, the cultures were rinsed twice with 0.1 M sodium cacodylate buffer (pH 7.2) containing 0.001 M calcium chloride. The cell layer was fixed with buffered 2.5% glutaraldehyde for 30 minutes, rinsed twice with buffer, and post-fixed 30 minutes in buffered 1% osmium tetroxide. Following dehydration and epon embedding in situ, the polymerized epon sheet containing the cells was removed from the culture flask. Some pieces of the epon sheet were glued cell side out to old epon blocks for cutting sections parallel to the cell layer. Other pieces were cut into 2-mm strips and placed in capsules filled with liquid epon, which was polymerized so that sections could be cut perpendicular to the cell layer. Thin sections were stained with uranyl acetate and lead citrate.

Statistical Analyses

All statistical analyses were done according to Sokal and Rohlf (1969). Cloning efficiencies without a 3T3 feeder layer at 31° were so low that we were not sure the assumptions of analysis of variance could be met; we selected non-parametric tests for these data. Appropriate data transformations were used for calculating means and confidence intervals to correct for data that were probably not normally distributed.

## Results

Short-Term Studies: Effects of Feeder Layer and Temperature on Secondary Cloning Efficiency of Normal Mouse Epidermal Cells

Table 1 shows that the subculture of mouse epidermal cells (assayed by cloning efficiency) requires a 3T3 feeder layer. Cloning efficiency with a 3T3 feeder layer was 20X higher than with a dermal fibroblast feeder layer. A small number of clones on the 3T3 feeder layer (approximately one per $10^5$ cells plated) grew to be grossly visible at six weeks after cloning, but no clones on the dermal fibroblast feeder layer ever reached such sizes.

Table 2 shows that the subculture of mouse epidermal cells requires incubation at 31°. These data are from a study to investigate the effect of primary culture age on cloning efficiency. Colony formation at 37° was significantly less than at 31°. The efficiency at 31° decreased with age of the cultures. Only a small fraction of the clones grew to be grossly visible at six weeks, and none occurred with cells that had been in primary culture more than five days. Thus

Table 1. Effects of 3T3 Cells and Dermal Fibroblasts on the Secondary Cloning Efficiency of Epidermal Cells from Two-Day Cultures (SENCAR Cells, Enriched Waymouth's Medium, 31°)[a]

| Feeder Layer | Cloning Efficiency: Mean (Sample Size) (%) | 95% Confidence Interval (%) | Test Statistic |
|---|---|---|---|
| None | < 0.01 (4) | 0.0 | |
| Dermal Fibroblasts | 0.02[b] (4) | 0.0 - 0.06[b] | $U_s = 20^{*c}$ |
| 3T3 Cells | 0.43[d] (5) | 0.37 - 0.49[d] | |

[a]Plating conditions as described in Materials and Methods.

[b]Values calculated using the square-root transformation.

[c]The cloning efficiency on 3T3 cells is significantly greater than the cloning efficiency on dermal fibroblasts at the 5 percent level (*) according to the non-parametric Wilcoxon two-sample test.

[d]Values calculated using the arcsine transformation.

Table 2. Effect of Temperature on the Secondary Cloning Efficiency of Epidermal Cells on a 3T3 Feeder Layer, After Various Periods of Time in Primary Culture at 31° Without a Feeder (SENCAR Cells, Enriched Waymouth's Medium)[a]

| Days in Primary Culture | Flask Number | Cloning Efficiency (%) | |
|---|---|---|---|
| | | At 31° | At 37° |
| 1 | 1a | 0.40 | < 0.004 |
| | 1b | 0.32 | < 0.004 |
| 2 | 2a | 0.14 | 0.009 |
| | 2b | 0.13 | 0.009 |
| 3 | 3a | 0.14 | < 0.009 |
| | 3b | 0.03 | < 0.009 |
| 4 | 4a | < 0.009 | < 0.009 |
| | 4b | 0.04 | < 0.009 |
| 5 | 5a | 0.16 | < 0.009 |
| | 5b | 0.14 | 0.009 |
| 6 | 6a | 0.090 | < 0.009 |
| | 6b | 0.13 | < 0.009 |
| 7 | 7a | 0.13 | 0.01 |
| | 7b | 0.12 | < 0.02 |
| 8 | 8a | 0.08 | 0.01 |
| | 8b | 0.01 | < 0.01 |
| 9 | 9a | 0.04 | < 0.01 |
| | 9b | 0.10 | 0.01 |
| Test Statistic | | $T_s = 0^{**}$[b] | |

[a]Plating conditions as described in Materials and Methods.

[b]Wilcoxon's signed ranks test for paired comparisons shows that cloning efficiency at 31° is significantly greater at the one percent level (**).

the loss of epidermal differentiation in cultures plated without a feeder layer is apparently irreversible and occurs after about one week *in vitro*. We have also found that 3T3 cells could not restore a healthy epithelial morphology (1) to 17-day, deteriorating, primary epidermal cultures when 3T3 cells were plated onto such cultures, or (2) to two of our dedifferentiated epidermal lines that had been passaged onto 3T3 feeder layers for up to 18 population doublings. 3T3 feeder layers probably allow the production of continuous differentiated epidermal lines (discussed later) by preventing early loss of epidermal differentiation.

Long-Term Studies: Effects of Improved Culture Conditions on the Production of Continuous Lines

Figure 1 shows that enriched Waymouth's medium is much better than 199 for the production of continuous lines. Some of these lines were carried to the fortieth population doubling level and did not senesce. These early studies were done without a feeder layer under conditions that favored the production of dedifferentiated lines similar to those described by Colburn et al. (1978 a,b) and Slaga et al. (1978b); differentiated lines are not considered in this figure. The data are a composite of transformation experiments with several different polycyclic aromatic hydrocarbons or *N*-methyl-N'-nitro-*N*-nitrosoguanidine. Neither these chemicals (0.01 - 1 µg/ml applied for two days beginning the day after plating) nor continuous treatment with 0.1 µg/ml of the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) affected line production, so these data were pooled in the figure. Neither the particular inbred mouse strain nor the

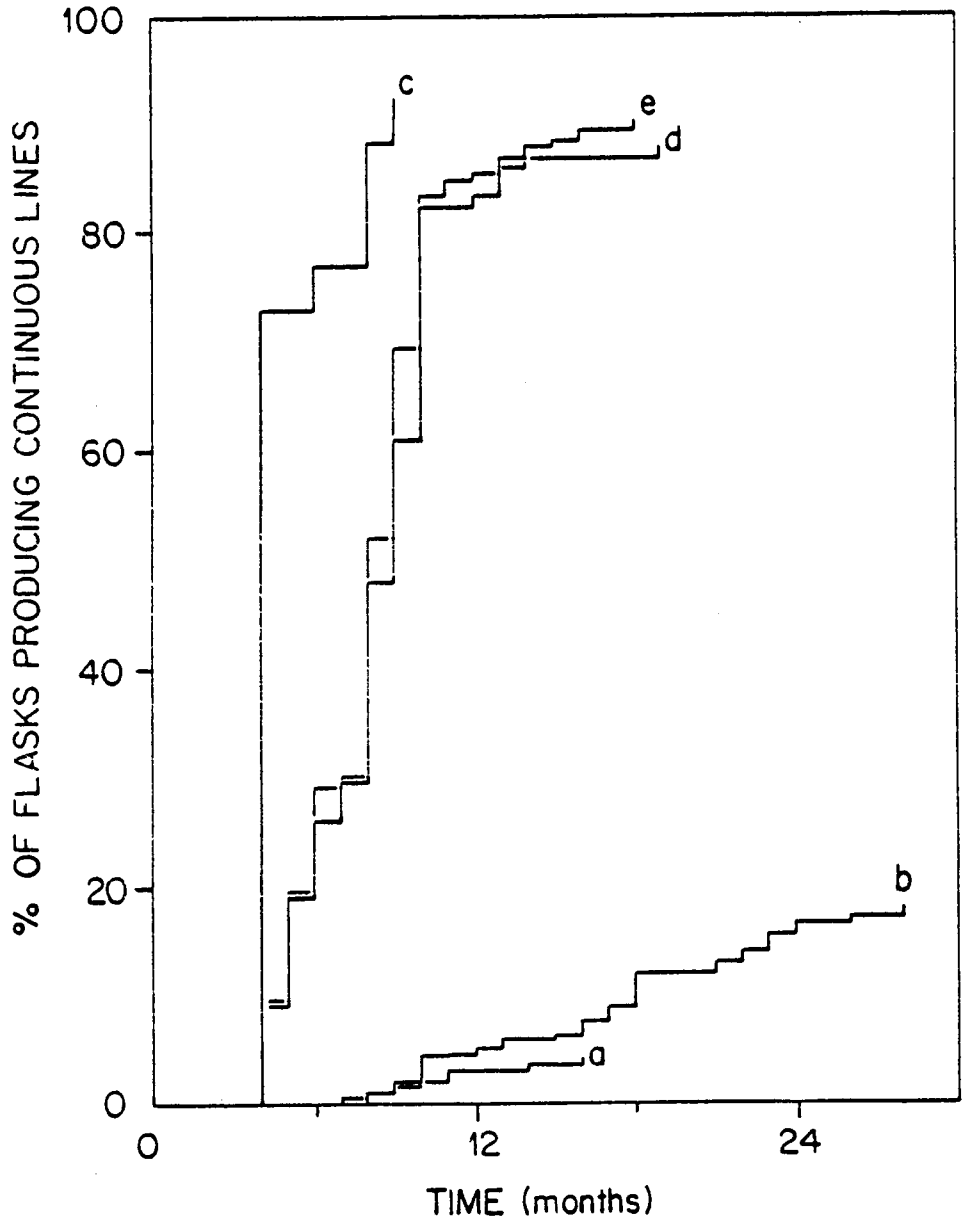



Figure 1. Production of continuous dedifferentiated epidermal lines vs. time in primary culture, as a function of mouse strain, medium, and incubation temperature (no feeder layer). Primary cultures of BALB/c epidermal cells grown at 37° in medium 199 (a) or enriched Waymouth's medium (c); or SENCAR epidermal cells in medium 199 at 37° (b) or in enriched Waymouth's medium at 37° (d) or at 31° (e).

incubation temperature had much of an effect on the production of lines. The reason for the slight significant difference between SENCAR and BALB/c cells in 199 at 37° (Table 3, a vs. b) is unclear. There was a difference between the two types of media (a,b vs. c,d,e); enriched Waymouth's medium yielded a much higher percentage of continuous lines. The period in primary culture (abscissa, Figure 1) was determined by the subjective decision to make the first subculture. We decided to subculture the cells in a particular flask if the cells in it were apparently proliferating and taking over the flask. We feel that enriched Waymouth's medium also decreased this period in primary culture, but a numerical value for the decrease would be hard to determine.

Certain culture modifications yielded classically epithelial, differentiated epidermal lines (Table 4). Some of these lines have retained their epithelial morphology past the fortieth population doubling level (PDL) and have not senesced. The data in Table 4 were pooled over all chemical treatments. Substitution of HS or IHS for FBS did not affect line production so these data were also pooled. Differentiated lines arose in approximately 10% of the flasks started at 31° without a feeder layer. A dermal fibroblast feeder layer had no effect, and incubation at 37° almost totally abolished the effect. If 3T3 cells were also used, this frequency rose to 95%. The temperature effect without feeders and its potentiation by 3T3 cells at 31° are both statistically significant.

Table 3. Production of Continuous Dedifferentiated Lines under Conditions that Favor Their Development (Data of Figure 1)

| Culture Conditions[a] | Line Production[b] | | Homogeneous Subset at the 5% Level of Significance[c] | Test Statistic[c] |
|---|---|---|---|---|
| | Number of Lines | Number of Flasks Discarded | | |
| a | 11 ( 1) | 269 (11) | | $G_H$ = 316.069** |
| b | 50 ( 6) | 232 (19) | | |
| c | 24 ( 3) | 2 ( 0) | | |
| d | 139 (22) | 19 ( 7) | | |
| e | 141 (40) | 15 (3) | | |

[a]Primary cultures of BALB/c epidermal cells grown at 37° in medium 199 (a) or enriched Waymouth's medium (c); or SENCAR epidermal cells in medium 199 at 37° (b), enriched Waymouth's medium at 37° (d), or at 31° (e).

[b]Numbers in parentheses refer to untreated controls. Various hydrocarbons or TPA appeared to have little effect on line production. Flasks were discarded if they remained apparently empty for at least six months.

[c]Replicated G-test for goodness of fit and a posteriori determination of homogeneous subsets. The significant $G_H$ (**) implies heterogeneity of groups at the one percent level.

Table 4. Production of Continuous Differentiated Lines as a Function of Incubation Temperature and Feeder Layer (SENCAR Cells, Enriched Waymouth's Medium)

| Temperature | Feeder Layer | Line Production[a] Number of Lines | Number of Flasks Discarded | Homogeneous Subset at the 5% Level of Significance[b] | Statistics[b] |
|---|---|---|---|---|---|
| 37° | None | 1 ( 0) | 157 (29) | | |
| | Dermal Fibroblasts | 0 ( 0) | 33 ( 6) | | G = 14.184** |
| | 3T3 Cells | 0 ( 0) | 31 ( 6) | | |
| 31° | None | 14 ( 2) | 142 (41) | | |
| | Dermal Fibroblasts | 0 ( 0) | 16 ( 6) | | G = 244.216** |
| | 3T3 Cells[c] | 102 (20) | 5 ( 2) | | |

[a]Numbers in parentheses refer to untreated controls.

[b]G-test for goodness of fit and a posteriori determination of homogeneous subsets. A significant G(**) implies heterogeneity of groups at the one percent level.

[c]Data pooled for media supplemented with FCS, HS, or IHS.

## Morphology of Normal Epidermal Cells and Continuous Lines under Conditions that Maintain the Differentiated State

The phase contrast morphology of a primary epidermal culture is shown in Figure 2. Secondary colonies of these cells replated onto a 3T3 feeder layer (Figure 3) were very similar. Both types had a classic epithelial cobblestone appearance. The continuous lines were also quite similar, producing apparent keratinized debris (Figure 4) (Yuspa and Harris, 1974) and overlying cornified cells (Figure 5) (Sun and Green, 1976), as did finite epidermal cells. Rhodanile blue staining of these lines was indicative of a keratinizing epithelium as suggested by Rheinwald and Green (1975a).

Electron microscopy revealed stratification both in the primary cultures (Figure 6) and in the continuous lines (Figure 7). Note the cuboidal nature of the basal layer cells and the flattened cells overlying them. Numerous intercellular spaces were present in both sections, although they were more widespread in the established lines. Abundant desmosomes and keratin bundles (Figure 8) were also characteristic of the continuous lines, as were terminally differentiating keratinocytes with little cytoplasmic ultrastructure (Figure 9). In these respects the continuous lines were very similar to primary cultures and to the epidermis *in vivo*.

## Discussion

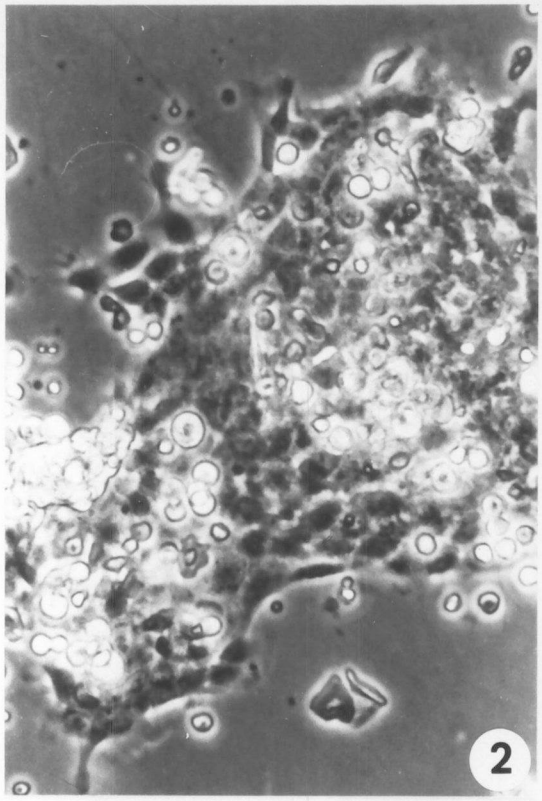
A 3T3 feeder layer and incubation at 31° are both required to subculture normal mouse epidermal cells. That only a small fraction of the secondary colonies reach large sizes is perhaps indicative of

Figure 2. Day 1 primary culture (SENCAR, enriched Waymouth's medium, 31°, no feeder). Phase contrast, X 260.

Figure 3. Secondary epidermal colony (bounded by arrows) on an irradiated 3T3 feeder layer (SENCAR, enriched Waymouth's medium, 31°). Phase contrast, X 160.

Figure 4. A differentiated epidermal line (untreated) at the twenty-second PDL (SENCAR, enriched Waymouth's medium, 31°). Overlying debris (D). Phase contrast, X 100.

Figure 5. Same culture as in Figure 4. Arrows indicate several overlying cornified cells; others are visible in the field of view. The basal layer is slightly out of focus. Phase contrast, X 160.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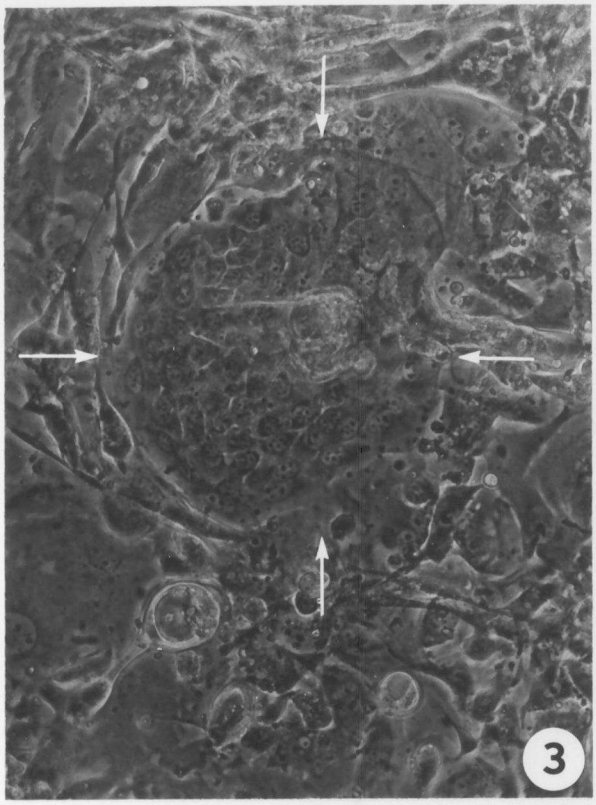
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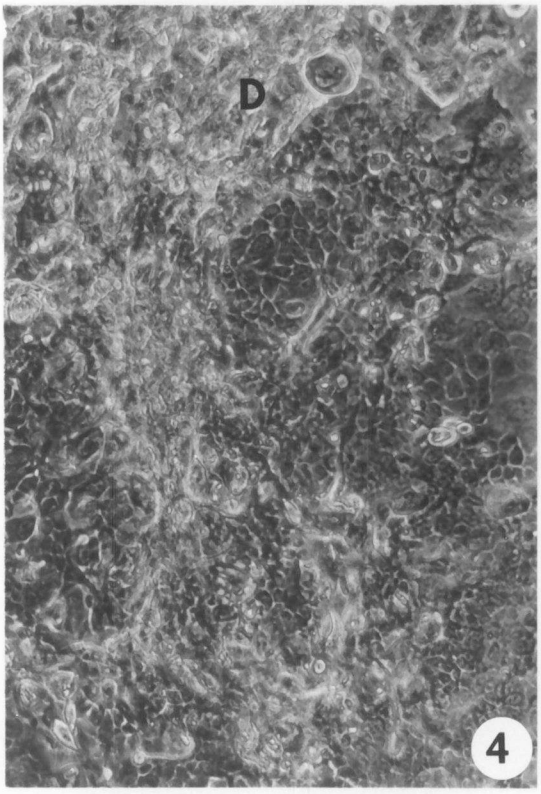
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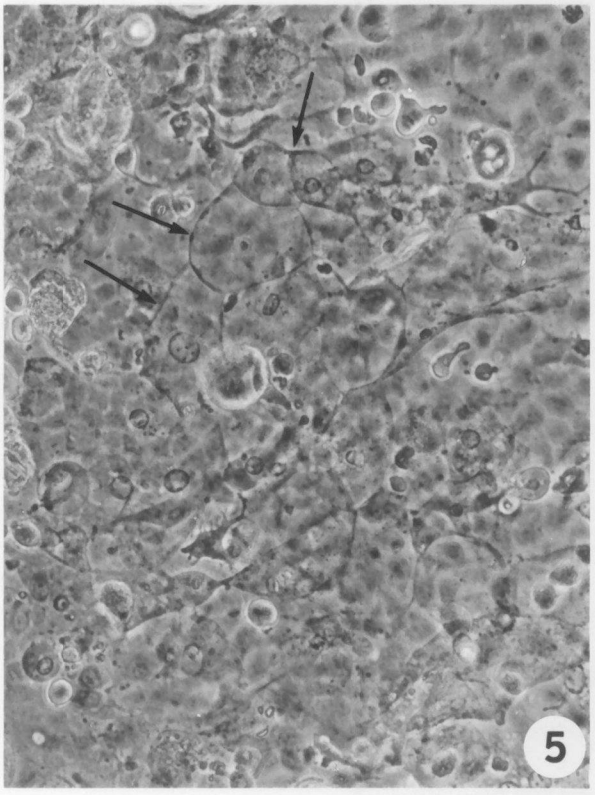
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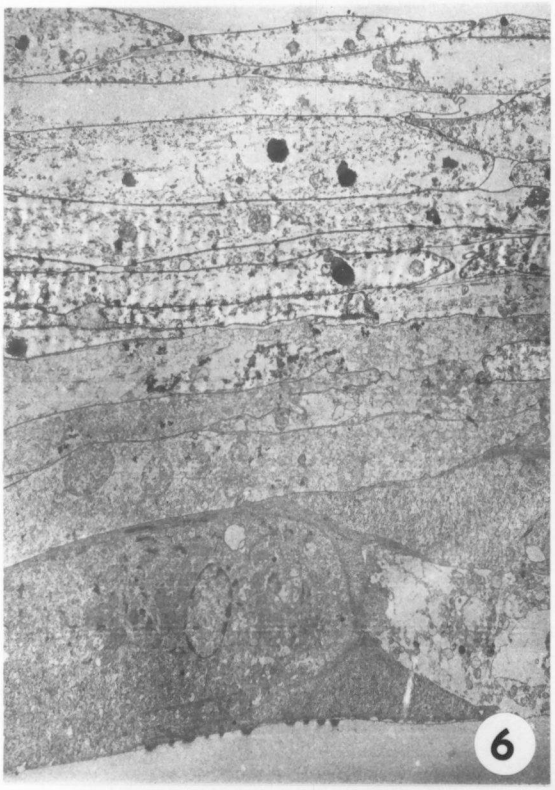
Figure 6. Day 7 primary culture (SENCAR, enriched Waymouth's medium, 31°, no feeder); vertical section through cell sheet. Electron micrograph, X 2,800.

Figure 7. A differentiated epidermal line (untreated) at the eighth PDL (SENCAR, enriched Waymouth's medium, 31°); vertical section. Electron micrograph, X 4,900.

Figure 8. Same culture as Figure 7; horizontal section. Desmosome, (d); keratin bundles (k). Electron micrograph, X 43,000.

Figure 9. Same culture as Figure 7; vertical section. Terminally differentiating keratinocytes. Electron micrograph, X 6,700.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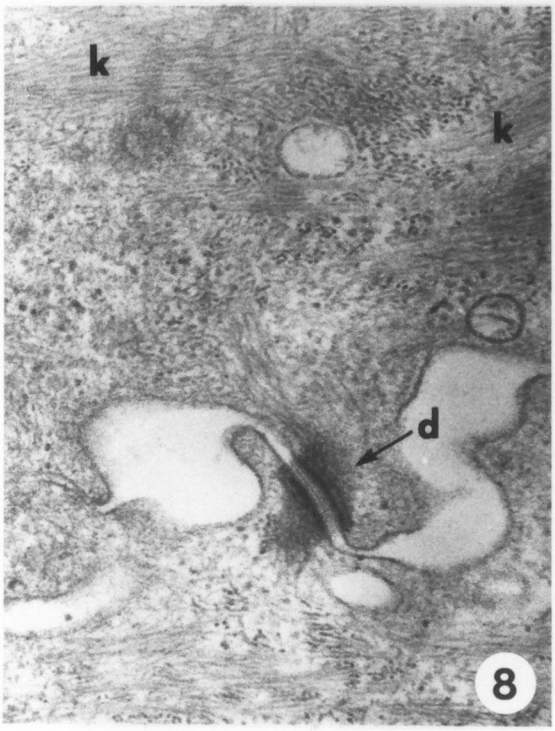
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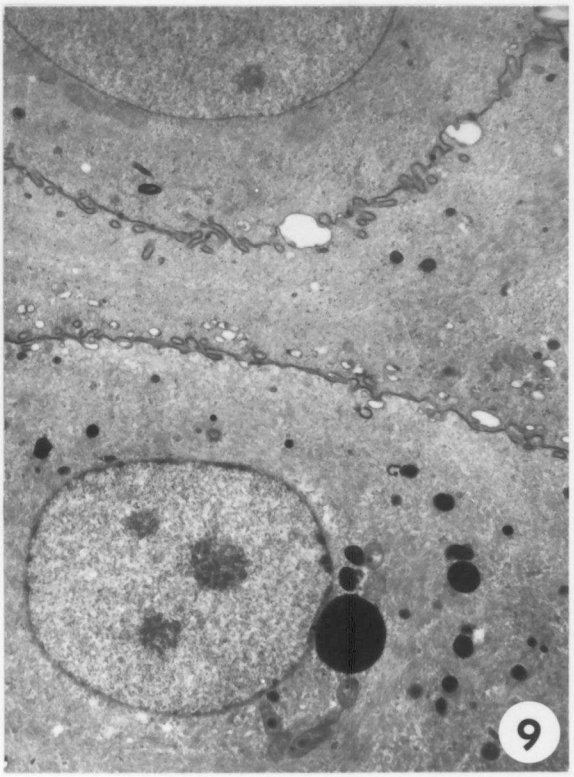
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two classes of basal cells, as suggested by Potten (1978). One class comprises most of the basal cells, those capable of a limited number of divisions. A much smaller class consists of the true epidermal stem cells, capable of a large number of divisions.

Enriched Waymouth's medium yields continuous lines from virtually every flask. The production of differentiated lines has an almost absolute requirement for 31°. BALB/c 3T3 Clone A31 feeder layers enhance this production at 31° but have no effect at 37°. How 3T3 cells potentiate this temperature effect is unclear. Hennings et al. (1976) noted improved growth of mouse epidermal cells at 31°. Other than the early studies of mesenchymal dependence of various epithelial cell types (for review, see Green et al., 1977), there has been little study of epidermal interactions with other specific cell types. Rheinwald and Green (1975b), using a Swiss 3T3 feeder layer with human epidermal cells, reported an effect similar to the one we have noted; however, they did not find a 31° requirement with those cells. They concluded that 3T3 cells directly affected epidermal differentiation, apart from any nonspecific feeding. There are two established cell lines that enhance primary epidermal growth: Swiss 3T3 and BALB/c 3T3 Clone A31. The 3T3 regimen used in developing those lines may be important for the effect since other established lines were less effective in promoting epidermal growth (Green et al., 1977). Diploid fibroblasts tended to enhance the growth of an established epidermal line derived from a mouse teratoma but were less effective with human epidermal cells (Rheinwald and Green, 1975b).

3T3 cells probably enhance the production of differentiated lines by preventing an early loss of differentiation in primary cultures. On the other hand, the resulting lines quickly lose any dependence on 3T3 cells with serial passage. It is apparent that epidermal cultures can be locked into either a permanent dedifferentiated state or a permanent differentiated state, depending upon the culture conditions. This is reminiscent of general ontological development, where cells are locked into some state of differentiation as a direct result of their local environment. Our experiments show that temperature can be an important factor in this environment and that some mesenchymal functions may be replaced by those present in established cell lines.

Since continuous differentiated epidermal lines can be regularly produced, it is more likely that tumors produced by cells transformed under these conditions will be differentiated epidermal carcinomas. This should overcome one of the major drawbacks to early studies of epidermal transformation. Our current studies have yielded lines from all treatments as early as three months after primary plating. These lines are currently being tested for growth in soft agar and tumorigenicity.

Cells from one of the nontumorigenic, highly differentiated SENCAR epidermal control lines that we have designated SECa were treated for two days at the sixteenth PDL with 1 µg/ml of the carcinogen benzo[a]pyrene. The treated cells, and not the cells of the parent line, were tumorigenic in nude mice at the fortieth PDL. The first tumor taken was a squamous cell carcinoma.

# CHAPTER III

## BENZO[A]PYRENE METABOLISM IN CULTURED MOUSE EPIDERMAL CELLS

### Abstract

The metabolism of benzo[a]pyrene (BP) and the subsequent conjugation and release of BP metabolites was studied in cultured mouse epidermal cells, specific target cells for BP carcinogenesis in vivo. Primary epidermal cultures initially plated with 20 X $10^6$ cells from newborn SENCAR mice metabolized [$^3$H]-BP to both organic- and water-soluble products that accumulated with time in the medium. At 24 hours, the cell fraction contained about 1.6% of the total counts added (12 µg BP at 1 µg/ml medium) per $10^6$ cells successfully plated. The metabolites retained by the cells were primarily the 7,8-dihydrodiol (precursor to the proposed ultimate carcinogen) and another metabolite, the 3-phenol, with much smaller amounts of the 9,10-dihydrodiol and the 9-phenol.

More than 75% of the total counts at 24 hours were found in the medium. Most of these were organic-extractable, the metabolites consisting primarily of the two dihydrodiols of BP at equal concentrations with much less of the phenols. About two-thirds of the remaining water-soluble metabolites were glucuronide conjugates of the 3-phenol and of several quinones. The other water-soluble metabolites were not sulfate conjugates, but their identities were not established.

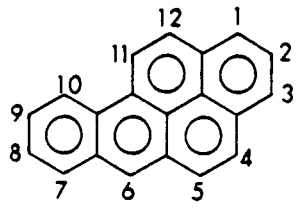
Thus the major BP dihydrodiol produced by primary epidermal cells was the 7,8-dihydrodiol. Many other cell types examined by other labs produced primarily the 9,10-dihydrodiol. The high level of 7,8-dihydrodiol production may have some bearing on epidermal carcinogenesis, since the 7,8-dihydrodiol is the immediate precursor to the 7,8-dihydrodiol-9,10-epoxide, thought to be the ultimate carcinogenic form of BP.

## Introduction

Many cancer-causing chemicals must first be activated by microsomal enzymes to reactive intermediate(s) (Selkirk et al., 1980). One class, the polycyclic aromatic hydrocarbons (PAH), are major environmental pollutants formed during combustion or distillation of organic products (Everall and Dowd, 1978). The PAH are metabolized by detoxifying enzymes to water-soluble (polar) compounds more easily excreted from the body (Gielen, 1978). The first enzyme involved, aryl hydrocarbon hydroxylase (AHH), adds oxygen to form various epoxides (DePierre and Ernster, 1978). These may spontaneously rearrange to phenols, or be further oxidized to trans-dihydrodiols by epoxide hydrase (Oesch, 1972). Other enzymes (Sims and Grover, 1974; Smith et al., 1977; DePierre and Ernster, 1978) may attach the various metabolites to sulfate (aryl sulfotransferase--Nemoto and Takayama, 1977; Nemoto et al., 1977), glutathione (glutathione S-transferase--reaction also spontaneous), or glucuronic acid (uridine diphosphoglucuronyl-transferase--Nemoto and Gelboin, 1976). The phenols and dihydrodiols can also be further metabolized and conjugated. The

conjugates are water-soluble and can ultimately be excreted.

Benzo[a]pyrene (BP) is the PAH most often studied (Selkirk et




Benzo[a]pyrene

al., 1980) and is metabolized by AHH to epoxides at the 4,5-; 7,8-; and 9,10-positions. Epoxide hydrase then oxidizes them to the respective trans-dihydrodiols. The presumed 1,2- and 2,3-epoxide intermediates are unstable and are thought to spontaneously rearrange to give the 1- and 3-phenols. The 7- and 9-phenols also arise from epoxide rearrangement. The origin of the 6-phenol is thought to be through a separate free-radical mechanism (Selkirk, 1980). It rapidly oxidizes to the 1,6-; 3,6-; and 6,12-quinones. Another metabolite enzymatically formed is the 6-hydroxymethyl derivative (6-hydroxymethylbenzo[a]pyrene synthetase--Sloane, 1975). These metabolites undergo further reactions as described in the previous paragraph.

Of the common BP metabolites, only the 7,8-dihydrodiol (BP-7,8-D) is as carcinogenic as BP in mouse skin (Slaga et al., 1978a). These data argue that BP-7,8-D, generated inadvertently by detoxification pathways, may be one of the metabolites involved in BP

carcinogenesis. Borgen et al. (1973) initially found that BP-7,8-D with further activation binds to DNA, thought to be a critical target for carcinogens (Lutz, 1979), to a ten-fold greater extent than does BP. Sims et al. (1974) demonstrated that BP-7,8-D epoxidated at the 9,10-position (BP-7,8-D-9,10-E) and reacted with DNA produces adducts that chromatograph in the same position as adducts formed by activated BP or BP-7,8-D, and distinct from 4,5-epoxide adducts. The fluorescence of DNA adducts of BP-7,8-D-9,10-E indicates an intact pyrene ring system (Daudel et al., 1975); binding can therefore only be through positions 7-10. BP-7,8-D-9,10-E has been shown to be an effective tumor initiator in mouse skin (Slaga et al., 1977, 1979a) and complete carcinogen in mouse lung (Buening et al., 1978), especially if the appropriate isomer is used. Diolepoxides are also thought to be involved in activation of other PAH (Smith et al., 1978): benz[a]anthracene (Wislocki et al., 1979; Cooper et al., 1980; Vigny et al., 1980) and 7,12-dimethylbenz[a]anthracene (Dipple et al., 1979; Slaga et al., 1979b), (Marquardt et al., 1979); 3-methylcholanthrene (Thakker et al., 1978); chrysene and phenanthrene (Wood et al., 1979).

We recently developed improved culture conditions for growing newborn mouse epidermal cells (Chapter II). These conditions allowed the subculture of, and the routine production of highly differentiated continuous lines from, primary cells. Since we were interested in using these culture conditions to study epidermal carcinogenesis *in vitro*, we wanted to know whether cultured epidermal cells were able to metabolize PAH as they do *in vivo* (Thompson and Slaga, 1976; Pyerin and Hecker, 1980). Differences in metabolism might explain

differential species (Conney and Levin, 1974) and tissue (Montesano and Magee, 1974; Bartsch et al., 1978) susceptibility to carcinogenic chemicals, including the PAH (Nebert et al., 1974; Wiebel and Gelboin, 1974; Dees et al., 1980). Since BP metabolism has been well characterized in fibroblasts and liver cells, we looked at the spectrum of BP metabolites produced by cultured epidermal cells, which are specific target cells for PAH. The nature of BP metabolism may be important in explaining epidermal susceptibility to PAH carcinogenesis.

## Materials and Methods

### BP Treatment of Epidermal Cells

Pure epidermal cultures ($10^7$ cells/100 mm culture dish) were prepared from newborn SENCAR mice (a population bred by R. K. Boutwell, University of Wisconsin, for sensitivity to chemically induced skin carcinogenesis) and grown at 31° in an enriched Waymouth's medium with 10% fetal bovine serum as previously described (Chapter II). The day after plating, the medium was changed to 6 ml/dish fresh medium containing 1 µg/ml [$^3$H]--BP (Amersham) diluted to 1 Ci/mmol with cold BP (Aldrich Chemical Company). Twenty-four hours later, the medium from two dishes was collected, the cells washed twice with phosphate-buffered saline (PBS), and the washes added to the medium samples. We then added 1 ml PBS to each dish and scraped the cells loose. We removed the cell suspension and rinsed the dishes two more times with PBS, combined the cell suspension and the last two rinses, and pelleted the cells. Pellets and medium samples were stored

at -70° until analyzed. All BP manipulations were done in subdued light.

Each pool analyzed consisted, as mentioned, of the cells or medium from two dishes. We examined several such pools for consistency and found that they gave very similar results. Since the epidermal preparations sometimes differed in their plating efficiencies, we also counted the nonterminally differentiating, attached cells released by 0.25% trypsin-0.04% EDTA (Chapter II) in representative dishes and expressed the BP metabolism results per $10^6$ cells. In other experiments, several time points of 12-96 hours were used. Except for overall quantitative differences, the metabolite profiles were very similar, so we did not report them. We did, however, report here the time course of [$^3$H]-BP metabolism into organic- and water-soluble metabolites.

High Pressure Liquid Chromatography (HPLC)

We prepared ethyl acetate/acetone extracts of cell pellets and medium pools as previously described (DiGiovanni et al., 1980). The pellets were homogenized and extracted twice. The two extracts were pooled and evaporated to dryness under nitrogen. We redissolved the residue in 1 ml methanol for the HPLC. The medium pools were also extracted twice, and then treated as described above. We analyzed the water-soluble glucuronide conjugates remaining in the medium by treating the aqueous phase with 2000 units/ml β-glucuronidase for two hours in the presence of ascorbic acid or butylated hydroxytoluene (BHT) (1 mg/ml, antioxidants), and then re-extracting as described. For any possible water-soluble sulfate conjugates, we instead used

30 units/ml aryl sulfatase with the β-glucuronidase inhibitor saccharo-1,4-lactone (20 mM).

The HPLC analysis of [$^3$H]-BP metabolites was done as described by Juchau et al. (1978) modified by DiGiovanni et al. (1979). Samples coming off the column were counted to generate the metabolite profiles. We interpreted the profiles in terms of known BP metabolite standards. The qualitative uniformity of BP metabolites from tissue to tissue (Selkirk et al., 1980) justified this procedure.

## Results

### Time Course of Overall BP Metabolism

By 96 hours, SENCAR primary epidermal cells (3.24 X $10^6$/pool successfully plated) had metabolized 72% of the BP initially added (12 μg, 47.6 nmol/pool). BP did not spontaneously break down over this time period, corroborating previous results (Jensen et al., 1977). Plotted over the entire time period (Figure 10), the amount of BP remaining decreased by about 4.7% (0.57 μg, 2.2 nmol)/$10^6$ cells per day. Further metabolism to water-soluble compounds (Figure 11) occurred at the rate of about 3.8% (1.8 nmol)/$10^6$ cells per day. Glucuronide conjugates formed at about 2.1% (1.0 nmol)/$10^6$ cells per day. The remaining water-soluble compounds after β-glucuronidase treatment might have been glutathione conjugates, as they were not released by aryl sulfatase.

In this particular experiment, because of a somewhat lower plating efficiency, BP did not become limiting at the later time points. This resulted in linear plots (after initial equilibration)

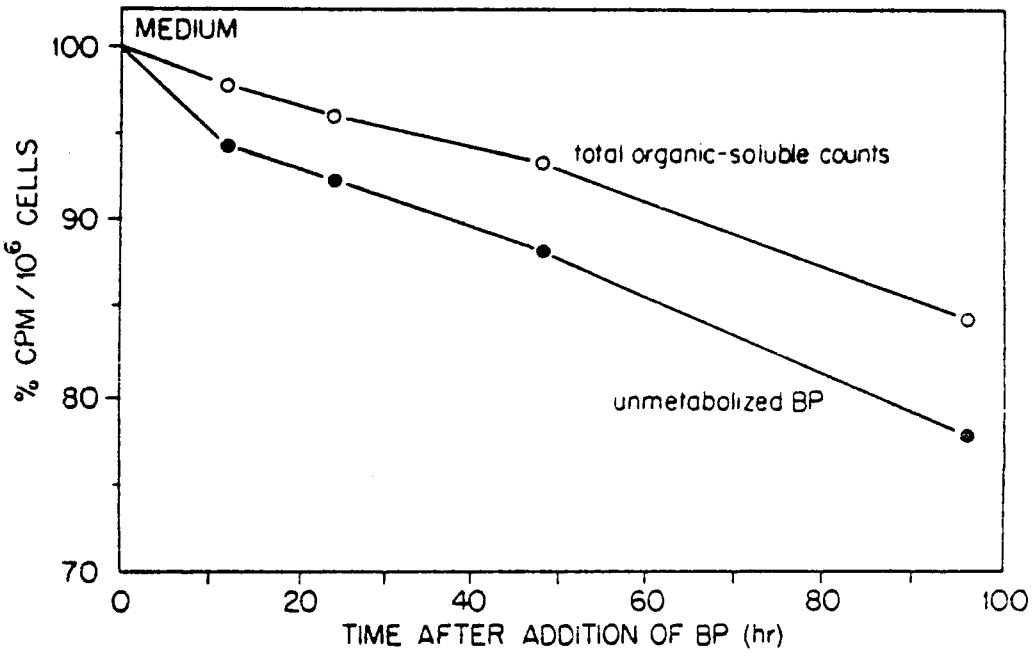



Figure 10. Depletion of benzo[a]pyrene (BP) and its organic-soluble metabolites from the medium of primary mouse epidermal cultures. Cells were treated the day after plating with 1 µg/ml [$^3$H]-BP (12 µg, 47.6 nmol/pool). At 12, 24, 48, and 96 hours, BP and its ethyl acetate/acetone-soluble metabolites in the medium were separated on a high pressure liquid chromatograph (HPLC) and counted. Total extractable counts and those due to unmetabolized BP were expressed as a fraction (percent cpm) of the total medium counts before extraction [91-94% (43.3-44.7 nmol) of the total counts initially added]. This value was further adjusted for cell number (3.24 X $10^6$/pool) determined at the 24 hour time point.

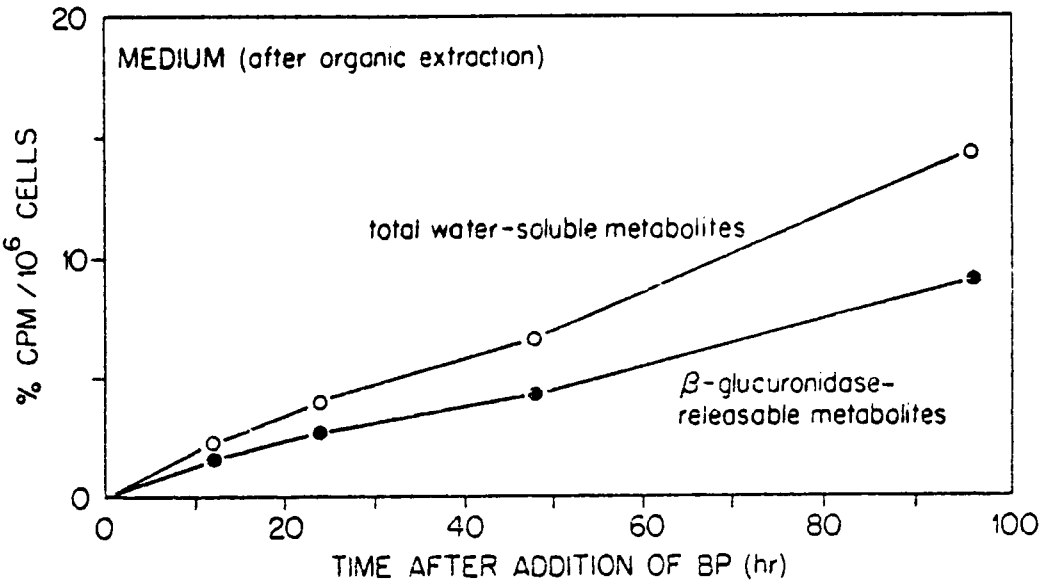



Figure 11. Accumulation of glucuronic acid-conjugated and other non-extractable, water-soluble BP metabolites in the medium of primary mouse epidermal cultures. The aqueous phase remaining after the ethyl acetate/acetone extraction described in Figure 10 was treated with β-glucuronidase and re-extracted. The β-glucuronidase-released counts and the total water-soluble counts remaining after the first extraction were expressed as a fraction of the total medium counts and adjusted for cell number, as described for Figure 10.

of overall metabolism with time, allowing the above rate calculations to be made. (The entire cell fraction retained only 6-9% of the total counts and were disregarded in these calculations.)

Profile of BP Metabolites at 24 Hours

The HPLC separation of the cell-associated BP metabolites produced by SENCAR primary epidermal cells is shown in Figure 12. The major metabolites, tentatively identified by their mobilities, were the 3-phenol (3-OH-BP) and the 7,8-dihydrodiol (BP-7,8-D), with lesser amounts of the 9-phenol (9-OH-BP) and the 9,10-dihydrodiol (BP-9,10-D), and few quinones. There may have been other metabolites that co-migrated with some of these peaks (Selkirk et al., 1976a; Harris et al., 1977; Autrup et al., 1978), but the other metabolites are present only at very low levels in other cell types.

The culture medium contained the large majority of counts in all experiments described. The organic-extractable metabolites released into the medium by primary epidermal cells were primarily the two dihydrodiols at approximately equal levels, with trace amounts of the two phenols and with some activity in the quinone region (Figure 13). The water-soluble glucuronide conjugates were primarily 3-OH-BP with large amounts of the quinones (Figure 14). The presence of ascorbic acid or BHT to reduce spontaneous oxidation during the β-glucuronidase treatment implies that the quinones were apparently bound as such to glucuronic acid (Selkirk et al., 1980).

We have not attempted to calculate absolute quantities of the various metabolites for reasons mentioned in the Discussion. The published literature on BP metabolism by intact cells primarily

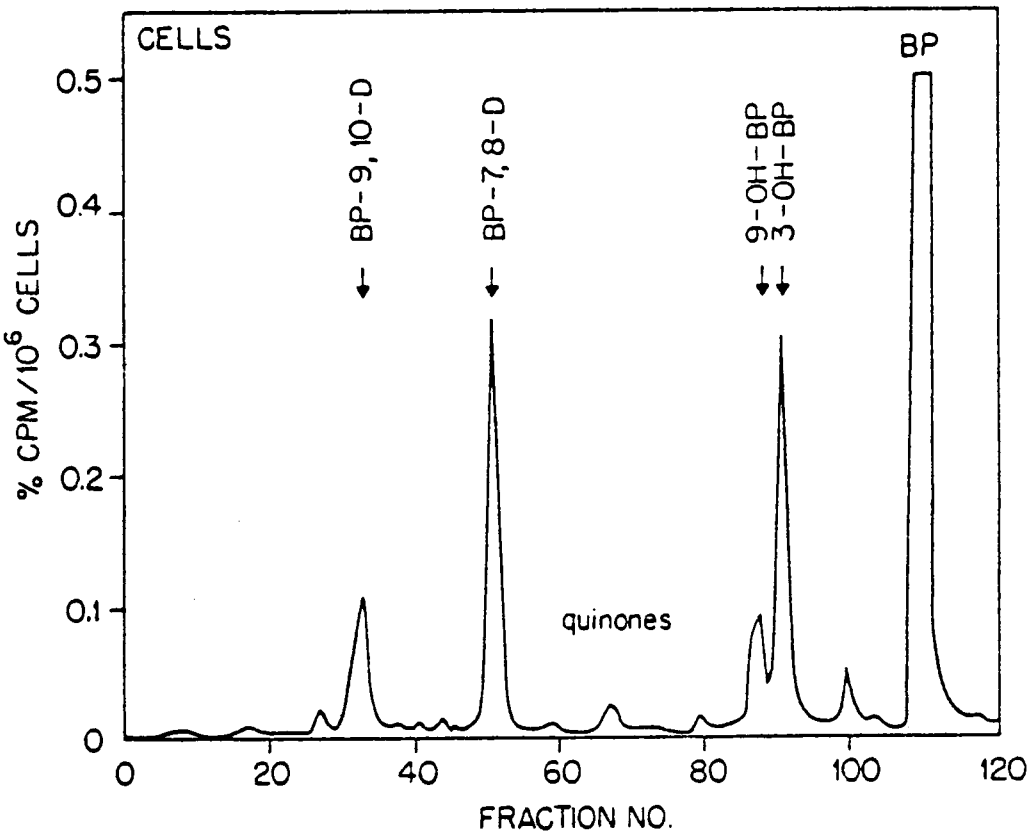



Figure 12. Cell-associated metabolites 24 hours after addition of BP. Cells were treated the day after plating with 1 µg/ml [$^3$H]-BP (12 µg, 47.6 nmol/pool). The next day, the cells were homogenized and extracted, and the extract [containing 22.5% (10.7 nmol) of the total counts initially added] run on an HPLC. The data were expressed as percent cpm of the total cell-associated counts [22.7% (10.8 nmol) of the total counts added] and adjusted for cell number (14.3 X $10^6$/pool).

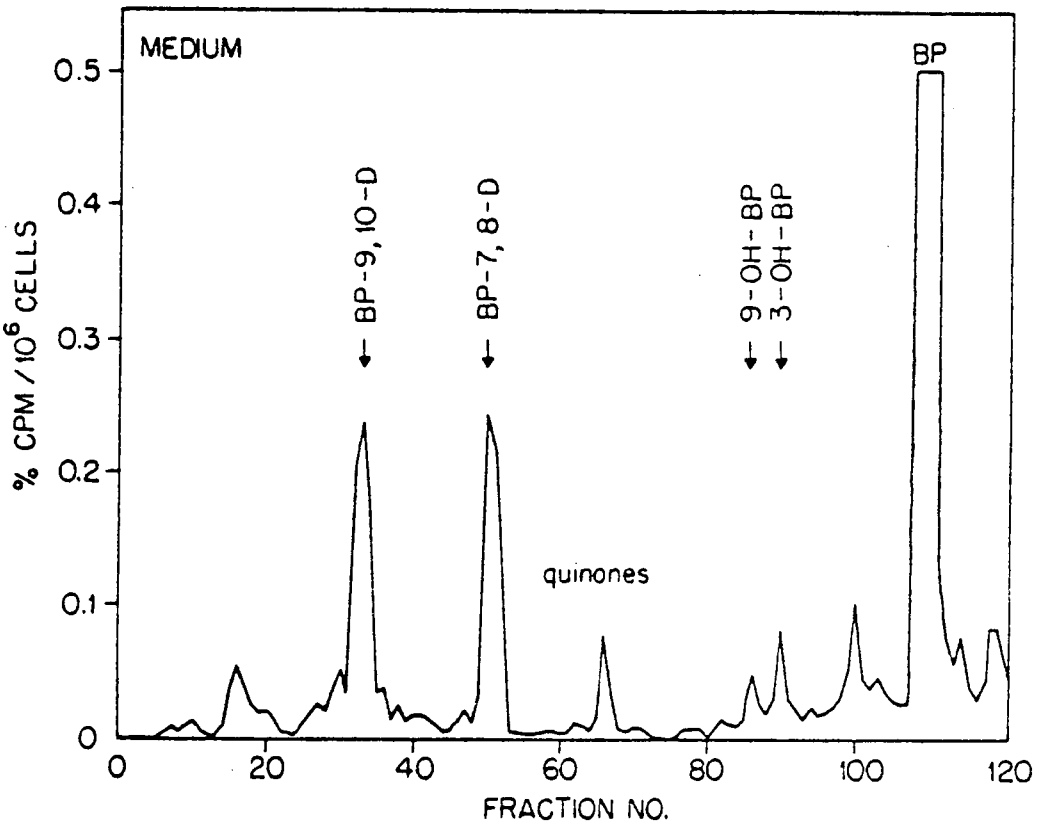



Figure 13. Organic-soluble metabolites in the medium 24 hours after addition of BP. BP and its metabolites were extracted [extract containing 47.7% (22.7 nmol) of the total counts initially added] from the medium of cultures treated as described for Figure 12 and run on an HPLC. The data were expressed as percent cpm of the total medium counts before extraction [87.2% (41.5 nmol) of the total counts added] and were adjusted for cell number (8.01 X $10^6$/pool).

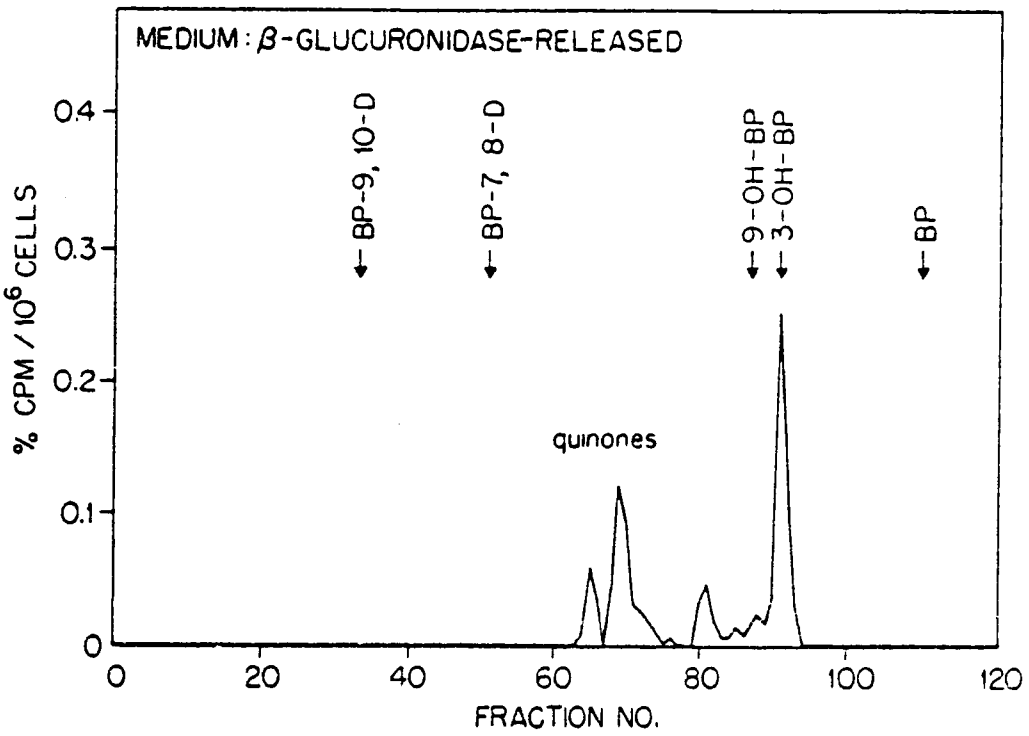



Figure 14. Glucuronide conjugates in the medium 24 hours after addition of BP. The aqueous phase remaining after the extraction described for Figure 13 was treated with β-glucuronidase and re-extracted. The extract [containing 25.3% (12.0 nmol) of the total counts initially added] was run on an HPLC and the data expressed as for Figure 13.

considers relative amounts of the various metabolites and how they are partitioned. We have adhered to that convention to best compare these data.

## Discussion

Microsomal preparations produce an altered profile when compared to BP metabolism by intact cells (Selkirk et al., 1980). For this reason, we used living epidermal cells in these experiments. The BP metabolite profiles for primary epidermal cells were different in several respects from BP metabolism in other types of intact cells. The major phenol of the cell-associated metabolites was 3-OH-BP, the major metabolite of rat liver cells (Burke et al., 1977). With hamster embryo fibroblasts, 9-OH-BP is the major cell-associated metabolite, with 3-OH-BP present in small amounts (Selkirk and MacLeod, 1979; Selkirk et al., 1980). Epidermal cells retained as much BP-7,8-D as 3-OH-BP, with less BP-9,10-D. The 9,10-dihydrodiol is also the lesser dihydrodiol in rat liver cells (also see Jones et al., 1979) and hamster embryo fibroblasts. Hamster hepatocytes retain BP-9,10-D and BP-7,8-D in equal amounts, while ferret hepatocytes tend to eliminate all dihydrodiols (Jones et al., 1979). The 4,5-dihydrodiol (BP-4,5-D) is a major metabolite in all liver cells. Epidermal homogenates form primarily the 3- and 9-phenols with almost no dihydrodiols (Slaga et al., 1976; Berry et al., 1977). The differences between BP profiles of intact epidermal cells and epidermal homogenates may be due to the disruption of normal cellular architecture by homogenization.

The profile of organic-soluble metabolites released into the medium by primary epidermal cells indicated that BP-7,8-D and BP-9,10-D were produced in approximately equal amounts. From this and the cell-associated dihydrodiol distribution discussed above, it is obvious that the overall production of BP-7,8-D was at least as great as that of BP-9,10-D. With most other cultured cell types, the extracellular BP-9,10-D peak is much larger than the BP-7,8-D peak (human lung and bronchial epithelium, Cohen et al., 1976; human fetal hepatocytes and fibroblasts, Pelkonen, 1976; human bronchus, Harris et al., 1977; hamster lung and trachea, Cohen and Moore, 1978 and Mass and Kaufman, 1978; rabbit lung, Warshawsky et al., 1979; hamster embryo cells, Baird et al., 1979 and Selkirk et al., 1980). Large amounts of BP-4,5-D are also produced by human fetal hepatocytes and rabbit lung cells. Short-term suspension cultures of rat, hamster, and ferret hepatocytes also produce more BP-9,10-D than BP-7,8-D, but with substantial amounts of BP-4,5-D (Burke et al., 1977; Jones et al., 1979); BP-4,5-D is in fact the major dihydrodiol of hamster liver cells. The other cell types that produce roughly equivalent amounts of BP-7,8-D and BP-9,10-D are all human (lymphocytes, Selkirk et al., 1976b; WI-38 fibroblasts, Baird and Diamond, 1978; colon, also with a large BP-4,5-D peak, Autrup et al., 1978). Of these experiments, the 9-OH-BP peak is higher than the 3-OH-BP peak in hamster trachea (Mass and Kaufman, 1978) and embryo fibroblasts, but is lower in human lymphocytes and bronchus, and rat hepatocytes (Burke et al., 1977). In all cases, there are more dihydrodiols than phenols.

The above findings demonstrate that there is a lot of variability in the basic spectrum of BP metabolites. This may be partly due to slight differences in experimental procedures among the various labs (e.g., serum, which binds BP and can reduce its carcinogenic effect, Stenback et al., 1979). AHH stimulation by PAH pretreatment as used in some of these experiments normally makes only quantitative differences overall. Also, while the concentration of labeled carcinogen may affect the route of its metabolism by isolated microsomes, this does not appear to be the case with intact cells (Bigger et al., 1980). The qualitative differences in BP metabolism noted are probably due to real differences in cellular activation. Whether this differential metabolism among the various cell types is due to the involvement of different cytochrome P-450's (the critical component of AHH--Wiebel et al., 1975; Tsibris, 1976) or other differences is unclear.

A large proportion of the water-soluble PAH metabolites formed by cultured cells are glucuronide conjugates, easily releasable with β-glucuronidase (BP treatment of hamster embryo cells, Baird et al., 1977; 7,12-dimethylbenz[a]anthracene treatment of mouse epidermal cells, DiGiovanni et al., 1980). The major glucuronide conjugate formed by primary epidermal cells in our studies was 3-OH-BP, with some quinones and no dihydrodiols. The remaining water-soluble metabolites were not sulfate conjugates and have not been identified. The absence of glucuronide-conjugated dihydrodiols was also noted in epidermal cells treated with DMBA (DiGiovanni et al., 1980). Hamster embryo cells (Baird et al., 1977; Selkirk et al., 1980) also form no

dihydrodiol-glucuronic acid conjugates, instead conjugating 9-OH-BP primarily, with less 3-OH-BP (probably due to phenol availability) and the quinones. Rat liver cells form glucuronide conjugates primarily with 3-OH-BP. They also conjugate, at high levels, BP-4,5-D (the free dihydrodiol formed in large amounts by liver AHH) and other unidentified BP metabolites migrating close to the position of the 9,10-dihydrodiol after β-glucuronidase treatment (Jones et al., 1979). Hamster trachea produces a similar spectrum of glucuronide conjugates, although with reduced formation of the BP-4,5-D conjugate (Cohen and Moore, 1978). Hamster lung, however, is different in that the major glucuronide-conjugated dihydrodiol is BP-7,8-D, although at very low levels compared to 3-OH-BP. WI-38 cells form no water-soluble glucuronic acid or sulfate conjugates (Baird and Diamond, 1978), while β-glucuronidase plus aryl sulfatase treatment of water-soluble metabolites produced by human colon explants release primarily BP-7,8-D, with lesser amounts of the quinones and BP-9,10-D, and almost no 3-OH-BP (Autrup et al., 1978).

Since the liver is particularly resistant to PAH carcinogenesis (Brookes, 1976), it must be able to effectively deal with those molecules. AHH activity is much greater (on the order of 100X, Pelkonen, 1976) in the liver than in other tissues; hence, the liver can very quickly inactivate the PAH. Liver-mediated activation has been shown to be immaterial to skin carcinogenesis, however (Pyerin and Hecker, 1980). The predominantly local tumors produced by the PAH may be due to inefficient detoxification in those tissues. Although the amount of metabolism may differ among tissues (also related to the particular

mouse strain, Wang et al., 1976 and Kouri, 1976), the metabolite profile is in most cases very similar (Selkirk et al., 1980). The absolute rate of BP metabolism in SENCAR epidermal cells is difficult to compare with rates in other cells either because the various procedures used are often not directly comparable or not enough data is given. An epidermal rate substantially less than that of liver is generally accepted.

In addition to the overall rate of metabolism, there are other qualitative differences that could also be important in terms of predicting particular tissues at risk during exposure to PAH. Because BP-7,8-D is the immediate precursor to the proposed ultimate carcinogen BP-7,8-D-9,10-E, it is a critical metabolite. Most liver cells form primarily BP-9,10-D (total production) with a substantial amount of BP-4,5-D. Most other cells, while not forming much of the latter metabolite, preferentially form BP-9,10-D. These cells would be expected to be less susceptible to PAH carcinogenesis than cells that form larger amounts of BP-7,8-D. In actuality, these susceptibilities would be hard to quantitate since cellular transformation assays only apply to cultured fibroblasts; it would be hard to interpret _in vivo_ because of the possible interference of other cell types. Also important to consider in assessing risk is the ability to conjugate and eliminate BP-7,8-D. In that respect, human colon is far more effective than any other cell type; hamster lung is a poor second.

Those cell types that tend to form more BP-7,8-D (human lymphocytes, colon, and WI-38 fibroblasts; mouse epidermal cells) would be expected to be more susceptible to BP carcinogenesis. (This would

tend to be offset in colon by subsequent BP-7,8-D conjugation.) These susceptibilities would be even more difficult to ascertain since human tissues are involved. Our previous studies (unpublished) demonstrated that BP metabolism in BALB/c epidermal cells was very similar to metabolism in SENCAR cells as discussed here, although SENCAR mice were about 30X more sensitive to chemically induced skin carcinogenesis. There are obviously many factors that affect susceptibility to carcinogenesis, of which the relative level of BP-7,8-D production is one. Elevated BP-7,8-D in mouse epidermis, however, may have contributed to the usefulness of mouse skin as a model tumor system.

# CHAPTER IV

## POLYCYCLIC AROMATIC HYDROCARBON TOXICITY IN CULTURED MOUSE EPIDERMAL CELLS: IMPLICATIONS FOR CARCINOGENESIS

### Abstract

SENCAR mice are much more sensitive than BALB/c mice to skin carcinogenesis induced by polycyclic aromatic hydrocarbons (PAH). Since epidermal carcinogenesis can only be quantitated *in vivo*, it is not clear whether the difference is extra-epidermal (systemic or mesenchymal) or within the epidermal cells. Our prior, unpublished *in vivo* studies with these two populations of mice revealed no major differences in PAH metabolism that could explain their different susceptibilities to skin carcinogenesis.

Cytotoxicity is also a characteristic effect of chemical carcinogens. Using a secondary cloning efficiency assay, we have shown that SENCAR epidermal cells are more sensitive than BALB/c cells to PAH toxicity *in vitro*. Cytotoxicity was linear with respect to the logarithm of the carcinogen concentration, a characteristic of many chemical toxicity curves. SENCAR epidermal cells also proliferated to a much greater extent in culture. This increased autonomous growth of SENCAR cells and their increased susceptibility to carcinogen toxicity are consistent with the concept of an intrinsic defect in SENCAR epidermal cells making them more prone to convert to a malignant state. Extra-epidermal effects *in vivo* cannot, however, be completely ruled out.

Initial toxicity and sustained colony growth often followed different curves, in line with previous data from fibroblast culture systems showing that cytotoxicity and subsequent proliferation resulting in neoplastic transformation are not related. In contrast, in vitro toxicity with several different PAH (benzo[a]pyrene, 3-methylcholanthrene, and 7,12-dimethylbenz[a]anthracene) in epidermal cells correlated reasonably well with their established in vivo carcinogenic activities. While not directly involved in carcinogenesis, toxicity may result through a similar mechanism. Toxicity should be easier to study than carcinogenesis in epidermal cells in vitro since quantitative epidermal transformation assays do not yet exist.

## Introduction

The polycyclic aromatic hydrocarbons (PAH) are an important class of environmental pollutants carcinogenic to the skin (Everall and Dowd, 1978) and other tissues (Suss et al., 1973). Their effects have been extensively studied both in vivo and in vitro (e.g., Heidelberger, 1973a, 1975; Suss et al., 1973). Most carcinogens are also toxic (Suss et al., 1973). Early in vitro studies comparing toxicity and carcinogenesis were done with fibroblasts (Heidelberger, 1973b; DiPaolo and Casto, 1978), which have a characteristic neoplastic morphology in vitro (Reznikoff et al., 1973). Cellular carcinogenesis was quantitated by the development of these altered colonies or foci. When plotted against carcinogen concentration and compared with toxicity, it was found to follow a different curve

(Huberman and Sachs, 1966; DiPaolo et al., 1971). Selection (via toxicity) is therefore thought to play no direct role in carcinogenesis in cultured fibroblasts (see Mondal and Heidelberger, 1970), although it is associated and may reflect a similar biochemical mechanism.

Most human tumors are epithelial in origin. In vitro carcinogenesis in these cells is not presently quantifiable, perhaps because of the poorer growth of epithelial cells in culture. These cells, and especially epidermal cells, tend to differentiate away from a proliferative state in vitro, and do not have a distinctive neoplastic morphology (Kuroki, 1975). We recently reported improved culture conditions that permitted the subculture of newborn mouse epidermal cells (Chapter II). Using these conditions, we have developed a quantitative assay (secondary cloning efficiency) for chemical toxicity in mouse epidermal cells. Although SENCAR mice are about thirty times more sensitive than BALB/c mice to chemically induced skin carcinogenesis, they do not differ in epidermal PAH metabolism (unpublished). In contrast, we have now found SENCAR cells in vitro to be more sensitive than BALB/c cells to PAH toxicity. Long-term survival of secondary colonies after chemical treatment often followed a curve different from initial toxicity, implying no direct relationship between the two. Initial toxicity generally corresponded to the carcinogenic activity of several PAH, however, perhaps reflecting an indirect relationship.

## Materials and Methods

### Preliminary Experiment: Growth and Viability of Pure Epidermal Cultures

Epidermal cells from newborn mice rapidly degenerate without an irradiated BALB/c 3T3 Clone A31 feeder layer (Chapter II). To rule out extraneous metabolic activation of the carcinogens, we did not want to use a feeder layer during the PAH treatment. We also wanted to treat the epidermal cells as early as experimentally feasible so that they were the least removed from the *in vivo* state. Therefore, we examined the temporal viability of pure primary cultures to establish a time frame for the manipulations used in our later experiments. Epidermal cells from newborn SENCAR mice ($4 \times 10^6$ cells/25 $cm^2$ flask) were plated in an enriched Waymouth's medium with 10% fetal bovine serum (FBS) at 31° (Chapter II). Daily for thirteen days we estimated the degree of confluence of the cultures. We also counted the non-terminally differentiating attached cells released (as single cells) by 0.25% trypsin--0.04% EDTA, and replated $10^6$ cells onto $2 \times 10^6$ irradiated 3T3 feeder cells in each of five 25 $cm^2$ flasks. We counted the microscopic secondary epidermal colonies (at 125X) two weeks after replating and determined the cloning efficiency.

### Toxicity Assay

BALB/c mice were donated by H. Witschi, Oak Ridge National Laboratory. From the results of the above experiment (discussed later), we formulated the following protocol for assaying toxicity in mouse epidermal cells. We plated 20-30 $\times 10^6$ newborn epidermal

cells/75 $cm^2$ flask in medium with 10% FBS at 31°. Two days later these cells were replated at 5 X $10^5$ cells/25 $cm^2$ flask. (Epidermal preparations taken directly from newborn mice have too many clumps for cloning assays.) After the cells were allowed to attach for five hours, we added the carcinogens (Aldrich Chemical Company or Sigma Chemical Company), dissolved in medium with dimethyl sulfoxide (DMSO), to a final concentration of 0.01-1 µg/ml in 0.1% DMSO in 3 ml medium/flask. For experiments including carcinogen concentration above 1 µg/ml, which may have presented solubility problems, 1% DMSO was used. We removed the medium 24 hours later and added 2 X $10^6$ irradiated 3T3 cells in 5 ml medium with 10% horse serum and 1% DMSO. The cultures were thereafter maintained in this medium. These conditions were optimal for the proliferation of secondary epidermal colonies (D. R. Miller, manuscript in preparation). Microscopic epidermal colonies were counted two weeks later, those grossly visible were counted at later times, and the colony-forming efficiency was determined.

We used the more conventional culture conditions (i.e., FBS, 0.1% DMSO) during carcinogen treatment to be consistent with some of our previous experiments. One of the 3-methylcholanthrene (MC) experiments was done entirely under more conventional, but suboptimal conditions (described later) to show that those conditions gave similar results. The day after primary plating, these cells were treated for 24 hours and then subcultured onto 3T3 feeders.

Statistical Analyses

Most statistical analyses were done according to Sokal and Rohlf (1969). For parametric analyses in which the number of colonies counted was so small that the mathematical assumptions underlying the analyses could not be met, we corrected the data with a square root transformation, but reported the results in the original scale. The nonparametric correlation analysis of some of the MC data was done as described by Burr (1960).

## Results

Figure 15 shows that primary epidermal cultures (without 3T3 feeders) deteriorated with time. Although the cells expanded to nearly cover the dish by seven days, the number of attached, non-terminally differentiating (apparently basal) cells declined by 65% over the same period. The subculturability (cloning efficiency) of those cells onto irradiated 3T3 feeder layers declined by 80%. The cloning efficiency stayed high for the first three days, however. We concluded from these data that carcinogen treatment and any cellular manipulations should be completed within three days of primary plating. The corroborating data of Worst et al. (1974) showed that mouse epidermal cells after 1-4 days in culture can re-form an intact epidermis after reimplantation *in vivo* (i.e., the cells are normal).

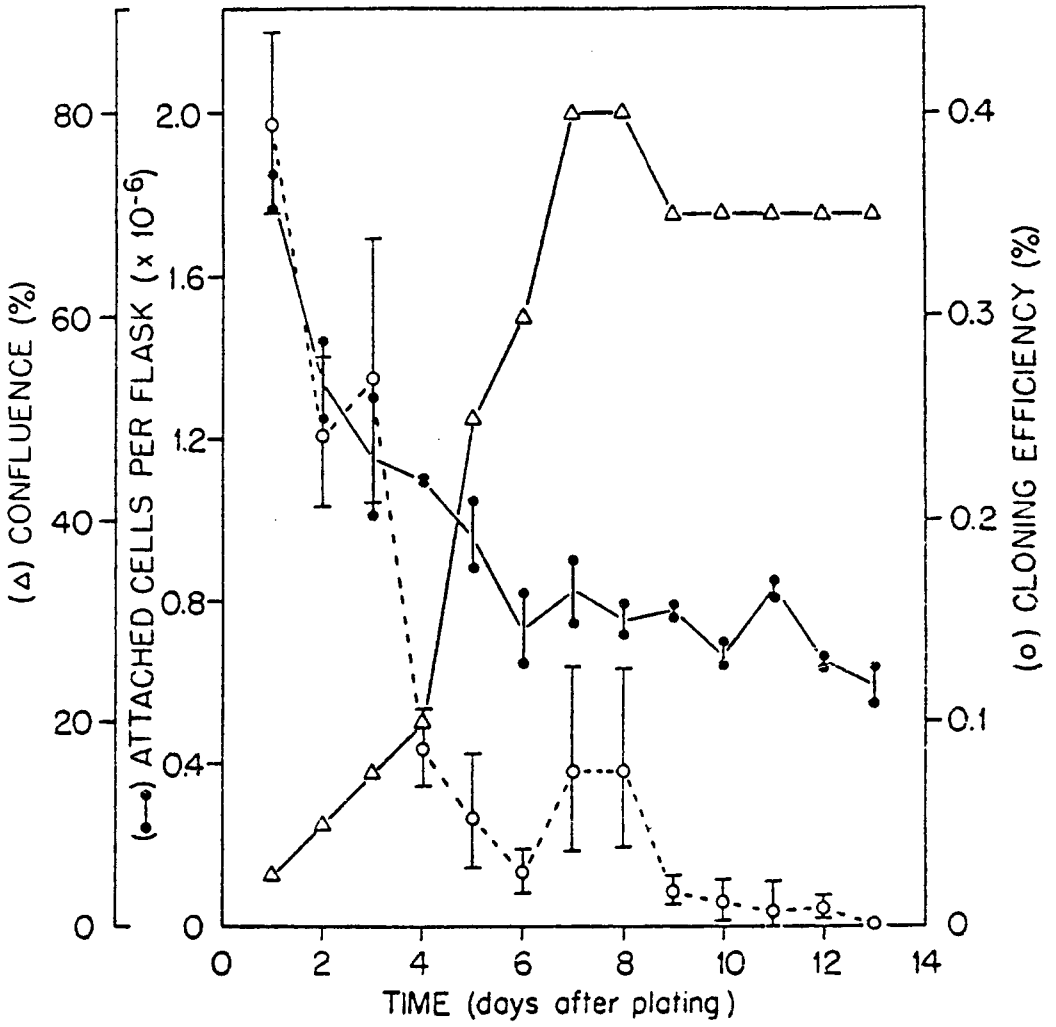



Figure 15. Growth characteristics of pure primary SENCAR epidermal cultures (4 X $10^6$ cells/25 $cm^2$ flask, plated day 0). Daily after plating, estimates were made of the fraction of the flask area covered by epidermal cells (percent confluence) in several flasks. Two counts (both shown) were also made of the enzymatically released, non-terminally differentiating attached cells. The released cells were replated onto 3T3 feeder layers, and the secondary epidermal cloning efficiency was determined two weeks after replating (5 secondary flasks/group; 95% confidence intervals shown).

Carcinogen Toxicity in BALB/c and SENCAR Epidermal Cells

7,12-Dimethylbenz[a]anthracene (DMBA) treatment of BALB/c and SENCAR epidermal cultures produced toxicity curves at two weeks as shown in Figure 16. Toxicity was significant and linear with respect to the logarithm of DMBA concentration for both cell types. This relationship is characteristic of many toxicity data (Sokal and Rohlf, 1969): proportional increases in chemical concentration reduce the number of colonies by a constant increment. This type of data is routinely expressed as percent of control, but is more directly analyzed as raw colony counts. We expressed the colony counts as cloning efficiencies to show that as DMBA concentration increased, SENCAR cloning efficiency dropped from twice to about one-fourth the corresponding BALB/c values.

The raw data are shown in Table 5. We counted more BALB/c than SENCAR microscopic fields to get an equivalent number of control colonies counted/flask (78.2 vs. 79.5). By counting the same respective numbers of fields at each treatment as for the controls, we could directly compare the BALB/c and SENCAR colony counts. Analyses of variance (ANOVA's) done for each treatment showed that the BALB/c and SENCAR results were not significantly different through 1 µg/ml DMBA. With higher concentrations of DMBA, there were significantly fewer SENCAR colonies. SENCAR epidermal cells were therefore more sensitive than BALB/c epidermal cells to DMBA toxicity as DMBA concentration increased.

The weaker carcinogen benzo[a]pyrene (BP) was less toxic (Figure 17). Toxicity was not detectable in BALB/c cells, but was

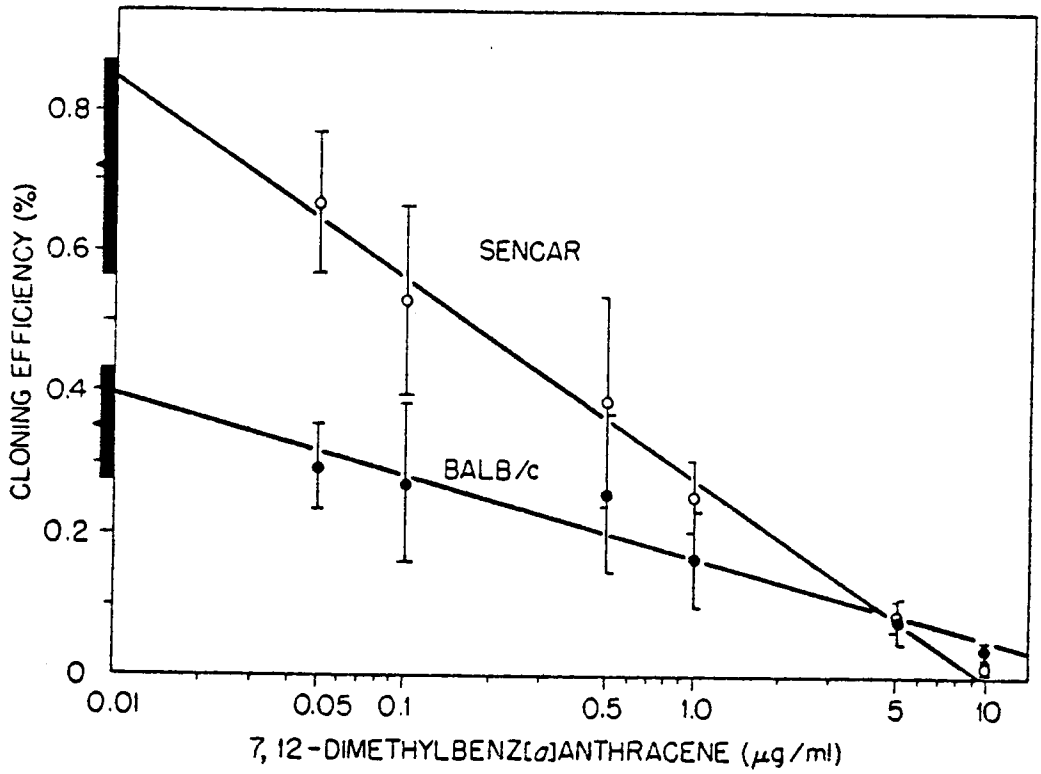



Figure 16. DMBA toxicity in BALB/c and SENCAR epidermal cells. Epidermal cells were subcultured (at 5 X $10^5$/flask) and treated for one day with DMBA. Irradiated 3T3 cells were then added, and the epidermal colonies were counted two weeks later (4 flasks/group, 95% confidence intervals shown; bars on ordinate indicate control values). Analyses of variance (ANOVA's) with regression showed that DMBA was toxic to both BALB/c ($F_S$ = 19.8***, 0.1% significance) and SENCAR ($F_S$ = 48.6***) epidermal cells. Regression explained a significant amount of the variation for each cell type ($F_S$ = 54.1**, 1% significance, and 233***, respectively); deviations from regression were not significant ($F_S$ = 1.70ns and 0.826ns). The SENCAR analysis did not include the 10 μg/ml group because its variance was significantly smaller (Bartlett's test), violating assumptions of both ANOVA and regression.

Table 5. Comparison of DMBA Toxicity in BALB/c and SENCAR Epidermal Cells[a]

| DMBA Concentration (μg/ml) | Colonies Counted/Flask[b] (95% Confidence Intervals) | | $F_s$[c] |
|---|---|---|---|
| | BALB/c | SENCAR | |
| 0 | 78.2 (61.05-95.45) | 79.5 (62.54-96.46) | 0.027ns |
| 0.05 | 65.0 (51.82-78.18) | 74.0 (62.83-85.17) | 2.75ns |
| 0.1 | 60.2 (35.15-85.35) | 58.8 (43.75-73.75) | 0.027ns |
| 0.5 | 57.0 (32.46-81.54) | 43.0 (26.26-59.74) | 2.25ns |
| 1 | 37.2 (21.75-52.75) | 28.0 (22.34-33.66) | 3.18ns |
| 5 | 17.7 (10.55-26.76) | 9.2 ( 6.62-12.17) | 12.4* |
| 10 | 8.7 ( 5.79-12.12) | 1.5 ( 0.68- 2.52) | 70.3*** |

[a]Raw data of Figure 2. Epidermal cells were subcultured and treated for one day with DMBA. Irradiated 3T3 cells were then added, and the epidermal colonies were counted two weeks later.

[b]Sample size, 4. Enough microscopic fields/flask were counted to give an equivalent number of control colonies/flask (78.2 BALB/c vs. 79.5 SENCAR). The same number of fields/flask were counted for each treatment as for the respective BALB/c or SENCAR controls. The numbers of colonies at each treatment could then be directly compared.

[c]ANOVA test statistic (ns, not significant; *, ***, significant at the 5%, 0.1% levels, respectively).

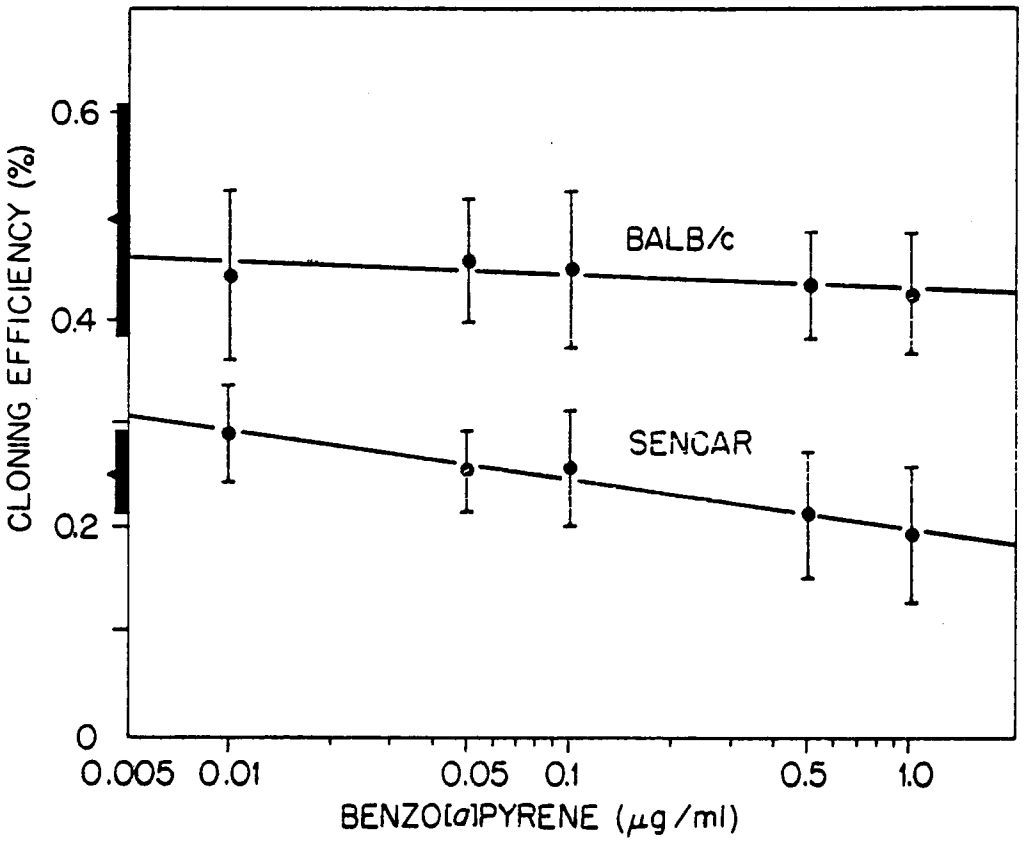



Figure 17. BP toxicity in BALB/c and SENCAR epidermal cells (as described for Figure 16, except that BALB/c control and 0.01 μg/ml groups each had three flasks). The BALB/c data were not significantly different over this concentration range ($F_S$ = 0.409ns). The SENCAR data were significant ($F_S$ = 4.89*, 5% significance). Regression explained a significant amount of the SENCAR variation ($F_S$ = 76.7**); deviations from regression were not significant ($F_S$ = 0.245ns).

significant in SENCAR cells. Overall cloning efficiency varied from experiment to experiment, which may have been due to the different epidermal cell preparations, serum batches, and 3T3 feeder populations. The higher cloning efficiency of BALB/c cells at two weeks shown in Figure 17 did not always occur (cf. Figure 16). In spite of their lower initial cloning efficiency, SENCAR cells formed colonies that subsequently proliferated to a much greater extent than BALB/c colonies (Figure 18, macroscopic colonies visible at seven weeks). The initial toxicity of BP in SENCAR cells at two weeks (Figure 17) was not reflected at seven weeks. Initial toxicity was therefore not the sole determining factor in subsequent colony growth.

MC Toxicity in SENCAR Epidermal Cells

We obtained similar toxicity results using more conventional conditions (10% FBS, no DMSO) following carcinogen treatment (Table 6). Early toxicity (up to 86% at nine days) was again apparent, but was not related to long-term colony development at six months. Any healthy colonies (having small cells with a well-defined, stratified epithelial morphology) that remained at six months almost always developed into continuous, highly differentiated epidermal lines. Therefore, the production of continuous lines was not related to initial toxicity.

Comparative Toxicity of BP, MC, and DMBA in SENCAR Epidermal Cells

These PAH were all toxic to SENCAR epidermal cells at 1 μg/ml (Table 7). DMBA was significantly more toxic than BP or MC. Toxicity can vary substantially among experiments (Umezawa et al.,

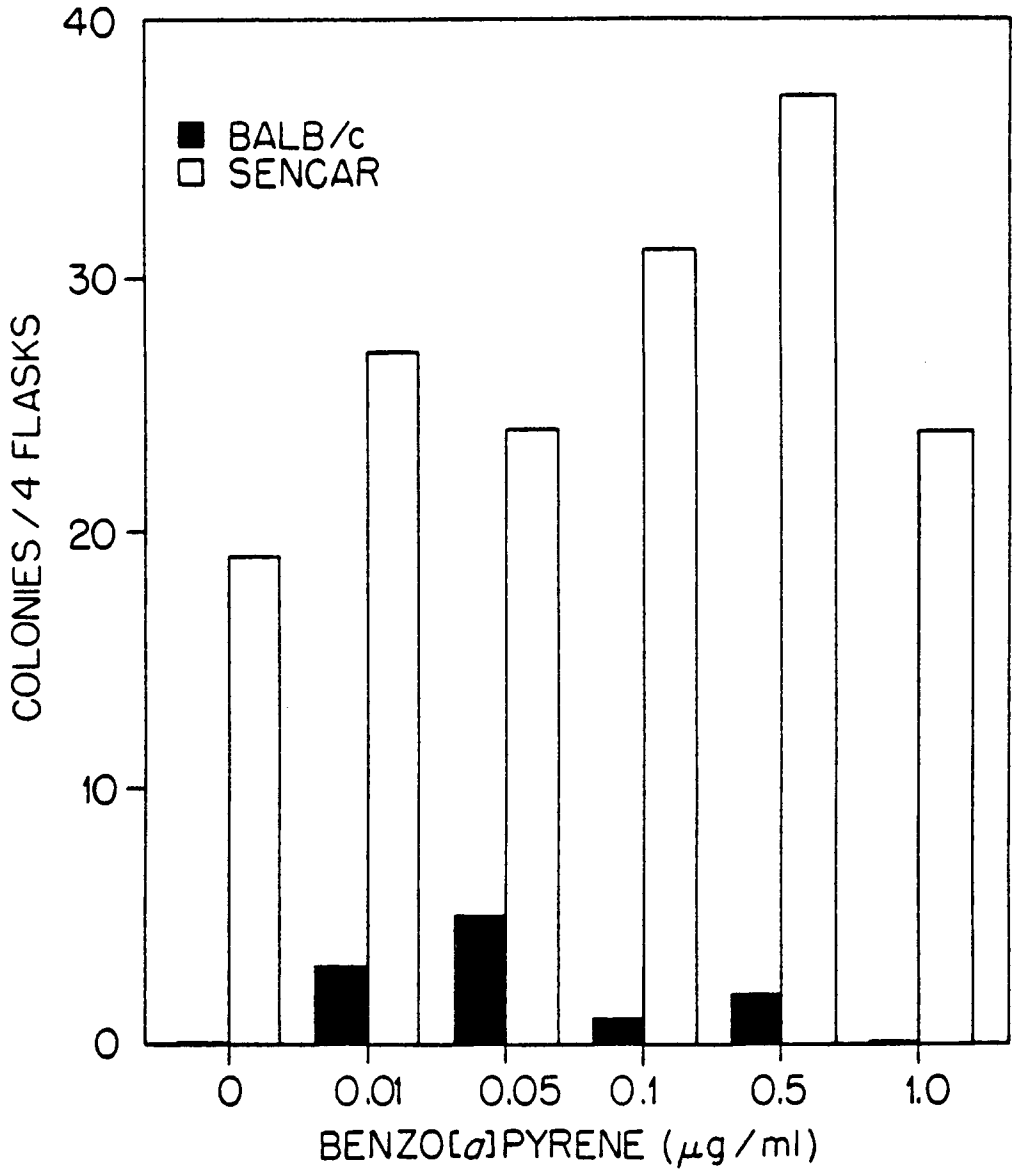



Figure 18. Long-term growth of BALB/c and SENCAR secondary epidermal colonies following BP treatment. Same experiment as in Figure 17. Macroscopic colonies were counted seven weeks after subculture (data from 3-flask groups were adjusted). Wilcoxon's signed ranks test for paired comparisons showed that there were significantly more SENCAR colonies than BALB/c colonies ($T_S$ = 0*). Correlation analysis of the two- and seven-week SENCAR data showed no association between initial toxicity and long-term colony growth ($r$ = -0.181ns).

Table 6. MC Toxicity and Long-Term Colony Growth in SENCAR Epidermal Cells[a]

| MC (μg/ml) | Cloning Efficiency [%][b] at Nine Days (95% Confidence Interval) | Healthy Colonies/ Flasks Remaining at Six Months |
|---|---|---|
| 0 | 1.06 (0.867-1.252) | 1/5 |
| 0.01 | 0.74 (0.526-0.963) | 3/5 |
| 0.05 | 0.34 (0.236-0.441) | 1/4 |
| 0.1 | 0.62 (0.470-0.760) | 1/4 |
| 0.5 | 0.15 (0.109-0.200) | 2/5 |
| 1 | 0.17 (0.113-0.228) | 2/5 |

[a]The cells were treated, subcultured (at $10^6$/flask) onto 3T3 feeder layers, and maintained in medium with 10% FBS without DMSO. Kendall's test of rank correlation showed that the nine-day and six-month data were not significantly correlated (S = 5ns).

[b]Sample size, 5. An ANOVA on these data showed significant toxicity ($F_s$ = 60.5***).

Table 7. Toxicity of Several PAH in SENCAR Epidermal Cells[a]

| Carcinogen | Cloning Efficiency [%][b] (95% Confidence Interval) | Homogeneous Subset[c] |
|---|---|---|
| Control | 0.71 (0.562-0.867) | |
| BP | 0.54 (0.493-0.591) | } |
| MC | 0.60 (0.455-0.737) | } |
| DMBA | 0.25 (0.201-0.303) | |

[a]Epidermal cells were subcultured and treated for one day with 1 µg/ml of each carcinogen. Irradiated 3T3 cells were then added, and the epidermal colonies were counted two weeks later.

[b]Sample size, 4. An ANOVA on these data showed significant variation ($F_s$ = 32.6***).

[c]At the 5% level of significance.

1978; Richter-Reichhelm et al., 1979); hence, comparison of results from our different experiments is somewhat tenuous. Based upon the results in Table 7, however, the toxic effects of these PAH on mouse epidermal cells could be ranked in the following order: DMBA > BP ≅ MC.

## Discussion

Our early attempts to detect biochemical differences between BALB/c and SENCAR epidermal cells that could explain their 30-fold difference in sensitivity to chemical carcinogenesis were unsuccessful. We found no differences in aryl hydrocarbon hydroxylase activity or in BP and DMBA metabolism as analyzed by high pressure liquid chromatography and DNA binding (unpublished observations). This led us to pursue other biological effects of carcinogens assayable in culture, where epidermal-specific differences could be detected. Any differences found might suggest fundamental cancer mechanisms.

Cloning efficiency is the best indicator of carcinogen cytotoxicity. In the past mouse epidermal cells could not be cloned. Subjective visual estimates of overt toxicity in epidermal cells are not very quantitative (Dietz and Flaxman, 1971) and are deceptive because this type of estimate does not necessarily parallel proliferative capability (Figure 15, p. 57). Epidermal cells enlarge and terminally differentiate with age in pure culture, confusing visual estimates. Secondary mouse epidermal cells will proliferate at 31° on a 3T3 feeder layer, permitting quantitation of toxicity.

Our results showed that SENCAR epidermal cells are more sensitive than BALB/c cells to carcinogen toxicity (proportional to the logarithm of the dose). This may be due to the better *in vitro* growth of SENCAR cells we noted. Previous results indicated that SENCAR epidermal cells also have a greater tendency to form continuous lines under limiting conditions (Colburn et al., 1978b; see also Chapter II). The heightened toxic sensitivity of rapidly proliferating cells is the primary rationale for cancer chemotherapy and may be involved here. The better growth of SENCAR epidermal cells when removed from their natural environment may be of fundamental importance. They may have greater autonomy or be sensitive to fewer feedback controls than BALB/c cells. This may explain their sensitivity to *in vivo* carcinogenesis.

Apart from effects due to proliferative differences, increased SENCAR sensitivity to toxicity may also reflect a greater susceptibility to the type of damage caused by chemical carcinogens (probably not due to differences in carcinogen metabolism, since we detected none earlier). Toxic mechanisms have largely been ignored since early work showed that *in vitro* toxicity and carcinogenesis are not directly related. They are associated, however, since the established ranking of the major PAH's based on their carcinogenic potency (DMBA > MC > BP -- see Chen and Heidelberger, 1969 and DiPaolo et al., 1969a) is very similar to their ranking based on toxicity (DMBA > MC $\cong$ BP -- see Chen and Heidelberger, 1969 and Table 7). Carcinogen metabolism is thought to play a major role in cytotoxicity (Iype et al., 1979), as it does in carcinogenesis (DePierre and

Ernster, 1978). With X-irradiation, two different classes of lesions seem to be involved (Little et al., 1979), although this has not been established with carcinogenic chemicals. Our results demonstrated that PAH have very similar toxic effects in both fibroblasts and epidermal cells.

That subsequent phenomena are often unrelated to initial carcinogen toxicity implies one of two things: either the cultures consist of two different populations of cells that respond differently, or the carcinogens have two different effects. Potten (1978) developed the concept of a small number of epidermal stem cells with a much larger proliferative life span than the remaining basal cells. This possibility was considered by us in a previous publication (Chapter II). Early and late effects of DMSO on secondary epidermal cultures also differ (D. R. Miller, manuscript in preparation), perhaps implying different sensitive populations. On the other hand, the carcinogens may kill some cells while converting others to preneoplastic states (Colburn et al., 1978b; Barrett et al., 1977; Barrett and Ts'o, 1978a,b) of sustained proliferation, a phenomenon characteristic of fibroblast experiments. Indo and Wilson (1977) noted improved growth of rat epidermal sheets after BP treatment of mass cultures, but Dietz and Flaxman (1971) found only toxicity in epidermal outgrowths from BP- and MC-treated human explants. Human cells rarely form continuous lines, perhaps accounting for the lack of stimulation in Dietz and Flaxman's data. These findings are not interpretable on a cellular basis and are, therefore, difficult to compare with our data. We sometimes saw late effects that paralleled

early toxicity. Since long-term growth of epidermal cells was more sensitive to suboptimal conditions (D. R. Miller, manuscript in preparation) minor inadequacies in serum, trypsin, etc. probably tended to suppress the late effect.

Although undifferentiated tumors of presumed epidermal origin have been produced several times before (Elias et al., 1974; Colburn et al., 1978a; Slaga et al., 1978b), epidermal cell lines that produced squamous cell carcinomas were formed less frequently from mouse cultures (Fusenig et al., 1973, 1978). Lines of the latter type have recently been established from rat epidermal cultures (Indo and Miyaji, 1978), and we have developed conditions for routinely producing highly differentiated continuous epidermal lines from mouse cells (Chapter II). Cells from one of these lines were recently treated for two days with 1 μg/ml BP at the sixteenth population doubling level. At the fortieth doubling, only cells from the treated subline caused squamous cell carcinomas in nude mice. Use of conditions for more favorable cell growth should make biochemical and biological studies on isolated epidermal cells more relevant to the fundamental mechanisms underlying epidermal carcinogenesis *in vivo*.

# CHAPTER V

## CONTACT-STIMULATED PROLIFERATION OF CULTURED MOUSE EPIDERMAL CELLS BY 3T3 FEEDER LAYERS: INHIBITION BY 12-O-TETRADECANOYLPHORBOL-13-ACETATE (TPA)

### Abstract

Mouse epidermal cells can be subcultured at 31° onto an irradiated BALB/c 3T3 Clone A31 feeder layer. A31 Cells (supposedly derived from embryonic fibroblasts) were found to be specifically required for the optimal production of secondary, keratinizing epidermal colonies. This effect was not transmitted through the medium nor by the culture surface, since A31 cells plated on one end of a flask did not stimulate epidermal proliferation at the other end, even if the other end had previously held A31 cells. Epidermal contact with metabolizing A31 cells was probably necessary for the effect. Fixed or freeze-thawed A31 cells were ineffective. The tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), recently shown to interfere with contact-mediated transfer of label (metabolic cooperation) between Swiss 3T3 cells and an epidermal cell line *in vitro*, also blocked epidermal colony formation, probably not through simple toxicity. The A31-epidermal effect is apparently not a typical mesenchymal-epithelial interaction, since the basement membrane would prevent this contact in intact skin.

## Introduction

Irradiated Swiss 3T3 cells, of supposed fibroblastic origin (Green, 1978), greatly improve the growth of normal human epidermal cells (Rheinwald and Green, 1975b). This is thought to be an *in vitro* manifestation of the established dependence of many epithelial cell types on adjacent mesenchymal tissues (Hata and Slavkin, 1978; Armstrong and Rosenau, 1978; DeBault and Cancilla, 1980; Kollar and Fisher, 1980; reviews by Wessells, 1967; Flaxman and Maderson, 1976; Green et al., 1977). Collagen-coated dishes improve the growth of rabbit and human epidermal cells (Liu and Karasek, 1978a,b; Liu et al., 1979), although this does not necessarily mean that Swiss 3T3 cells stimulate epidermal proliferation via a similar mechanism. We previously found that mouse epidermal cells form continuous, highly differentiated cell lines if cocultivated at 31° in an improved medium with an irradiated BALB/c 3T3 Clone A31 feeder layer (Chapter II). Normal epidermal cells can also be subcultured under these conditions upon the A31 feeder layer. The possible mediators of the epidermal-A31 interaction are the medium, the culture surface, or cell-cell contact.

We are very interested in the growth characteristics of normal epidermal cells *in vitro*. Differences at the cellular level are more easily detected in culture, and our findings should aid in the interpretation of *in vivo* epidermal phenomena where local and systemic influences are much more complex. We are particularly interested in epidermal carcinogenesis and the role in it played by cellular interactions. For this reason, we have pursued the A31 feeder effect. We

used secondary colony-forming efficiency of normal mouse epidermal cells as an assay. As Rheinwald and Green (1975a) found with the epidermal-Swiss 3T3 interaction, the A31 feeder effect was specific for the feeder type. Epidermal colony formation was probably mediated by active cell contact with A31 cells and was blocked by the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA), probably not through toxic effects.

## Materials and Methods

### Secondary Epidermal Cloning Efficiency Assay

Epidermal cultures from newborn SENCAR mice were prepared and grown as described previously (Chapter II). Routinely, 25 X $10^6$ cells/75 $cm^2$ flask were plated in an enriched Waymouth's medium with 10% serum and incubated two or three days at 31°. The cells were then removed with 0.25% trypsin--0.04% EDTA and replated at 0.5 or 1 X $10^6$ cells/25 $cm^2$ flask onto 2 X $10^6$ irradiated BALB/c 3T3 Clone A31 feeder cells. This medium was supplemented with 10% horse serum and 1% dimethyl sulfoxide (DMSO) to optimize epidermal proliferation (D. R. Miller et al., manuscript in preparation). An additional 0.2 mM L-glutamine was required for the A31 cells.

Secondary epidermal colonies were counted under phase contrast (125X) two or three weeks later. These colonies had a distinctive, stratified epithelial morphology and did not require special staining. In some cases macroscopic colonies were also counted at later times. The secondary cloning efficiencies for these cells usually ranged between 0.1 and 1% at two weeks.

Other Feeder Layers

Other irradiated feeder layers (Table 8) were tested against A31 feeders at equivalent cell numbers. The number of secondary epidermal colonies on each feeder was compared to that on the A31 feeder, and expressed as a percent of the A31 control. Secondary fibroblasts were from newborn BALB/c dermises (mice obtained from R. L. Ullrich, Oak Ridge National Laboratory), minced after removing the epidermises as described by Yuspa and Harris (1974). All feeders were subcultured with 0.05% trypsin--0.02% EDTA; unirradiated cells grew well in enriched Waymouth's medium and were maintained at 37°.

Attempts to Reconstitute the A31 Effect

In Figure 19, (a), (b), (c), (d) refer to paragraphs below. Irradiated, A31 feeder cells were included on the LOWER half of the culture surface (distal to the neck) of all flasks used in the experiments described in this section. They provided continuously conditioned medium and were the positive control for the reconstitution attempts at the UPPER end of the flasks (proximal to the neck).

a. Continuously conditioned medium--1.5 X $10^6$ irradiated A31 cells/flask were plated on the LOWER half of the culture surface of tipped 25 $cm^2$ culture flasks. After these cells had attached, primary epidermal cells were subcultured and uniformly plated over the entire culture surface at 0.5 or 1 X $10^6$ cells/flask. The flasks were then incubated at 31°. In one experiment, additional flasks were sealed and incubated on a covered shaker platform above a 31° water bath. Epidermal colonies were counted in five or ten microscopic fields of

Table 8. Cell Lines Tested as Feeder Layers for Mouse Epidermal Cells

| Line | Origin | Donor[a] | Reference |
|---|---|---|---|
| BALB/c 3T3 Clone A31 | BALB/c mouse embryo | R. W. Tennant | American Type Culture Collection (1975) |
| NIH Swiss 3T3 | Swiss mouse embryo | R. W. Tennant | Jainchill et al. (1969) |
| SIM 3T3 | SIM mouse embryo | R. W. Tennant | Tennant et al. (1979) |
| SIM.R 3T3 | SIM.R mouse embryo | R. W. Tennant | Tennant et al. (1979) |
| BHK-21(C-13) SN cl 10 | Baby Syrian hamster kidney | J. S. Cook | Bouck and diMayorca (1976) |
| V79-4 | Chinese hamster lung | E. Huberman | Chu and Malling (1968) |
| CHO-$K_1$-$BH_4$ | Chinese hamster ovary | A. W. Hsie | Hsie et al. (1975) |
| HeLa | Human cervical carcinoma | J. S. Cook | American Type Culture Collection (1975) |
| Hepa-2 | Mouse hepatoma | J. Papaconstantinou | G. J. Darlington,[b] personal communication |

[a]Cell lines maintained at the Biology Division, Oak Ridge National Laboratory.

[b]Cornell Medical College, Division of Human Genetics, New York, New York.

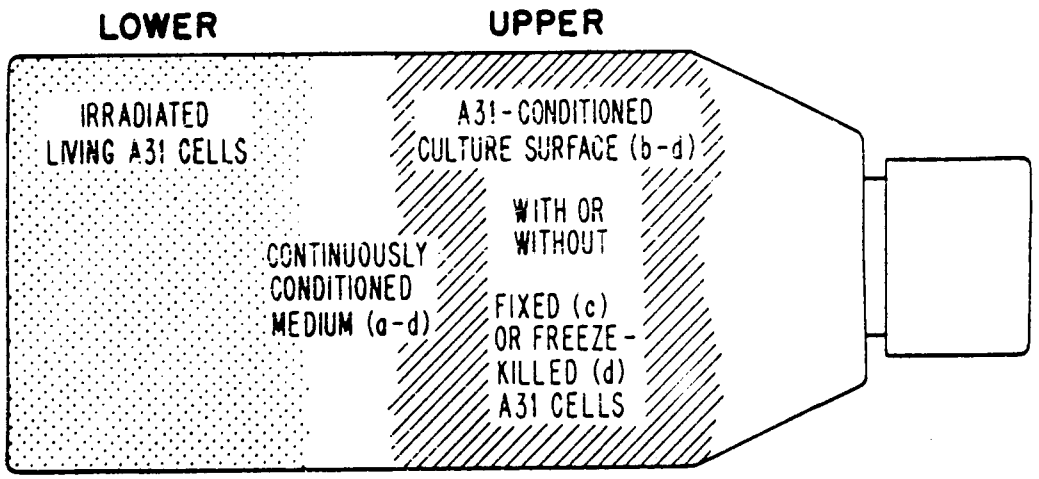



Figure 19. Attempts to reconstitute the A31 feeder effect. For explanation, see text.

view at each end of the flasks two weeks later. Macroscopic colonies at later times were also counted in some experiments.

b. Conditioned medium and culture surface--2 X $10^6$ irradiated A31 cells/flask were uniformly plated in 25 $cm^2$ culture flasks. The cells were removed two days later with 0.02% EDTA. The flasks were then replated with irradiated A31 cells on the LOWER half (to provide conditioned medium), and uniformly plated with epidermal cells as described in paragraph "a." Epidermal colonies were counted at each end two weeks later.

c. Fixed A31 cells with conditioned medium and culture surface--1.5 X $10^6$ A31 cells/25 $cm^2$ flask were plated on the UPPER half of the culture surface. Two days later the cells were washed twice with phosphate buffered saline (PBS), fixed 15 minutes with either 70% ethanol or 4% formaldehyde in PBS, and washed three more times with PBS, 30 minutes each wash. The UPPER surface then had both an A31-conditioned surface and fixed A31 cells. An equal number of irradiated A31 cells were plated on the LOWER half to provide conditioned medium, and epidermal cells uniformly plated as described in paragraph "a." Epidermal colonies at each end were counted at two weeks.

d. Freeze-thawed A31 cells with conditioned medium and culture surface--Secondary epidermal cultures with A31-conditioned medium and culture surface were prepared as described in paragraph "b." The following day (and at each medium change, twice per week), freeze-thawed A31 cells were added to the cultures. The freeze-thawed cells were prepared in the following manner. The culture medium was removed

from a 75 $cm^2$ flask of confluent A31 cells (2-3 X $10^7$ cells). The flask was floated twice on liquid nitrogen until the residual medium had frozen. More medium was then added to the flask and the thawed cells were shaken loose. One-tenth of this preparation was added to each epidermal culture. These cultures (already with conditioned medium and culture surface) were then incubated in a tipped position so that the freeze-thawed cells would settle predominantly in the UPPER end of each flask, opposite the intact A31 cells. Epidermal colonies at each end were counted two weeks later.

TPA Treatment of Epidermal Cultures

TPA was obtained from Dr. Peter Borchert (University of Minnesota, Minneapolis). Secondary epidermal cultures on A31 feeder layers were prepared as described for the "secondary epidermal cloning efficiency assay." Continuous TPA treatment of these cultures was begun the day after epidermal plating. TPA was made up in DMSO and added to the medium at each medium change (final DMSO concentration in treatments as well as controls, 1.1%). Epidermal colonies were counted at two weeks.

Pure cultures of primary epidermal cells (3 X $10^6$/25 $cm^2$ flask) were also treated beginning the day after plating. Two weeks later, the attached, nonterminally differentiating epidermal cells (apparently basal) released by trypsin-EDTA were counted as an indicator of long-term TPA toxicity.

Statistical Analyses

All analyses were done as described by Sokal and Rohlf (1969). Parametric analyses were done on the raw colony counts. Where the number of colonies was so small that the underlying mathematical assumptions of the analyses could not be met, we corrected the data with a square root transformation, but reported the results in the original scale.

## Results

Specificity of the A31 Effect

Other feeders were less effective than the A31 line in stimulating epidermal cell growth (Table 9). The feeders are ranked in order of decreasing effectiveness, although the results varied somewhat from experiment to experiment. Most cell types (SIM.R 3T3 through HeLa) enhanced short-term (two week) colony growth over feederless controls (nonspecific feeding). Epidermal cells growing on feeders were more difficult to detect than those in feederless controls; they also senesced (i.e., the well-defined, stratified epithelial morphology deteriorated) and enlarged earlier in feederless cultures, probably producing artifactual "colonies" not resulting from cell division. Hepa-2 and SIM 3T3 cells therefore probably had no effect on epidermal colony production compared to the feederless controls, instead of an apparent inhibition.

Although no other cell line tested was as effective as the A31 line (all values less than 100%), the SIM.R 3T3 line gave good results even at the more sensitive later time points. CHO cells were less

Table 9. Secondary Epidermal Colony Production on Various Irradiated Feeder Layers (Expressed as Percent of A31 Control, with 95% Confidence Intervals)[a]

| Feeder Layer/ Morphology[b] | Colony Production [%] Weeks After Plating | | | |
|---|---|---|---|---|
| | 2 | 3 | 5, Grossly Visible | 13, Grossly Visible |
| SIM.R 3T3/Fb [5 vs. 5] | 50***[c] (39.4-61.8) | - | - | 48*[c] (23.8-78.4) |
| CHO/Ep [5 vs. 5] | 59*** (45.3-74.6) | - | - | 24*** ( 1.9-60.7) |
| NIH 3T3/Fb [5 vs. 5] | 55*** (47.4-63.2) | - | - | 5*** ( 0.0-13.6) |
| BALB/c Fb [4 vs. 5] | 74**[c] (57.2-90.0) | 27*** (10.4-51.4) | < 3 | - |
| V-79/Fb [4 vs. 4] | 51*** (25.9-83.6) | - | - | - |
| HeLa/Ep [4 vs. 4] | 31*** (17.2-48.2) | - | - | - |
| BHK/Fb [5 vs. 5] | 12*** ( 8.5-16.0) | 0.3*** ( 0.0- 1.4) | - | - |
| Hepa-2/Ep [4 vs. 4] | 6*** ( 1.9-12.3) | - | - | - |
| SIM 3T3/Fb [4 vs. 5] | 1*** ( 0.0- 5.7) | - | - | - |
| No feeder [3 vs. 5] | 15*** ( 6.8-22.8) | - | - | - |

[a]Data from several experiments. Primary epidermal cells were replated onto irradiated feeder layers. Cloning efficiencies (mean ± standard error) at two weeks on A31 feeders varied between 0.243 ± 0.0131 and 0.953 ± 0.0398%. This value dropped as much as 50% by three weeks as most epidermal colonies senesced. Some colonies continued to proliferate, producing about six grossly visible colonies at five weeks per $10^6$ epidermal cells plated, and ten such colonies at thirteen weeks.

[b]Fb, fibroblast-like; Ep, epithelial-like. In brackets, sample size of each listed feeder vs. sample size of A31 control.

[c]Significance [analysis of variance (ANOVA) with a priori comparison of each listed feeder vs. A31 control]: *-5%, **-1%, ***-0.1%.

effective. Epidermal cells on NIH 3T3 and BALB/c fibroblasts grew well for a short time, but senesced earlier than colonies on A31 cells. Epidermal colonies on the remaining feeders never survived long enough to be grossly visible. The specific A31 effect, most apparent after prolonged culture, was not characteristic of a particular feeder morphology, nor of the 3T3 development regimen.

In trying to reconstitute the specific A31 effect (as discussed in the following sections), we did not feel that marginal improvement of epidermal cell growth over feederless controls would have been an important result. Such an effect could have been due to the non-specific feeding discussed in the first paragraph of this section. It could also have been a secondary artifact of the main specific mechanism. For example, if the specific effect were normally transmitted by epidermal-A31 contact, cellular debris from the A31 cells could conceivably have been transported to epidermal cells by the culture medium, possibly producing a slight medium-related effect. For these reasons, we were primarily interested in large effects comparable to that of an intact A31 feeder layer.

A31-Conditioned Medium (Paragraph "a") Does Not Transmit the Effect

A31 cells plated on one end of a culture flask stimulated epidermal colony production at that end but not at the other (Table 10). The UPPER values were very close to feederless control values without conditioned medium. Epidermal colonies without adjacent A31 cells appeared very unhealthy (absence of a well-defined, stratified epithelial morphology) with greatly enlarged cells, as in feederless controls.

Table 10. The Production of Secondary Epidermal Colonies in Medium Continuously Conditioned by Irradiated A31 Cells Plated on the LOWER Half of the Culture Surface (Distal to the Neck of the Flask): Colonies Counted (With 95% Confidence Intervals) at Each End of Flask[a]

| | Colonies Counted Weeks After Plating | | | |
|---|---|---|---|---|
| End of Flask | 2[b] | | 6, Grossly Visible | |
| UPPER[c] | 3.8(2.81- 4.86) | $F_s = 86^{***}$[d] | 0.6( 0.0 - 2.79) | $F_s = 56^{***}$[d] |
| LOWER | 10.2(8.56-11.91) | | 17.0(10.73-24.76) | |

[a]A31 cells were plated on the LOWER end of the culture surfaces of five flasks. After these cells had attached, $10^6$ epidermal cells/flask were replated over the entire culture surface.

[b]Number of colonies two weeks after plating in five microscopic fields of view at each end of flask.

[c]Proximal to the neck.

[d]$F_s$, ANOVA test statistic.

Shaking neither stimulated epidermal colony growth at the UPPER end nor inhibited it at the LOWER end (Table 11, the test of independence), ruling out any short-range diffusible effect. The two groups of flasks were incubated under slightly different conditions (see Materials and Methods), hence the differences between columns are probably not meaningful.

The A31-Conditioned Culture Surface (Paragraph "b") Does Not Transmit the Effect

Conditioned medium plus conditioned flask surfaces did not enhance epidermal colony production (Table 12). These epidermal cells grew somewhat better than previous preparations, hence the differences between UPPER and LOWER were less pronounced. "Colonies" on the UPPER end, however, were much less healthy and senesced earlier. Similar results were obtained if the surfaces were conditioned for two weeks by A31 cells; accumulation of some extruded product was apparently not involved. From these data, we concluded that the A31 effect probably required cell-cell contact.

Fixed or Freeze-Thawed A31 Cells (Paragraphs "c" and "d") Do Not Transmit the Effect

Table 13 shows that neither ethanol- or formalin-fixed nor freeze-thawed A31 cells (metabolically inactive), with conditioned medium and culture surface, were as effective as irradiated A31 cells (metabolizing, but amitotic) in stimulating the growth of mouse epidermal cells. The results with the fixed or freeze-thawed cells were very similar to results with feederless controls. Since the

Table 11. Effect of Shaking on the Production of Secondary Epidermal Colonies in Medium Continuously Conditioned by Irradiated A31 Cells Plated on the LOWER End: Colonies Counted (With 95% Confidence Intervals) at Each End of Flask[a]

| | Colonies Counted | |
|---|---|---|
| End of Flask | Not Shaken | Shaken |
| UPPER | 1.7( 0.48- 3.35) | 1.8( 0.16- 4.30) |
| LOWER | 16.2(14.37-18.08) | 25.1(18.50-32.78) |

[a]Cultures were prepared as described for Table 10, except that one group was continuously shaken after epidermal attachment ($0.5 \times 10^6$ epidermal cells/flask, 5 flasks/group). Colonies were counted in five fields of view at each end of the flasks. In a 2 X 2 test of independence, G = 0.51 ns (not significant).

Table 12. Effect of A31-Conditioned Culture Surfaces at the UPPER End on the Production of Secondary Epidermal Colonies in Continuously Conditioned Medium: Colonies Counted (With 95% Confidence Intervals) at Each End of Flask, Irradiated A31 Feeder Plated Only on LOWER End[a]

| | Colonies Counted | |
|---|---|---|
| End of Flask | Untreated Surface at Upper End[b] | Conditioned Surface at Upper End[c] |
| UPPER | 9.5( 7.03-12.67) | 8.5( 6.53-10.65) |
| LOWER | 20.2(10.55-32.87) | 17.7(15.13-20.52) |

[a]Cultures were prepared as described for Table 10 ($0.5 \times 10^6$ epidermal cells/flask) except that some flasks had previously contained uniformly plated A31 cells later removed with EDTA. Colonies were counted in five fields of view at each end of the flasks. An ANOVA ($F_s$ = 26***) with a posteriori comparisons showed a significant difference (at 5%) within each column, but not within each row.

[b]Sample size, 3.

[c]Sample size, 4.

Table 13. Effect of Nonmetabolizing A31 Cells at the UPPER End on the Production of Secondary Epidermal Colonies with Conditioned Medium and Flask Surfaces: Colonies Counted (With 95% Confidence Intervals) at Each End of Flask, Irradiated A31 Feeder Plated Only on LOWER End[a]

| | Colonies Counted | | | | | |
|---|---|---|---|---|---|---|
| End of Flask | Ethanol-Fixed A31 Cells at the UPPER End[b] | | Formaldehyde-Fixed A31 Cells at the UPPER End[b] | | Freeze-Thawed A31 Cells at the UPPER End[c] | |
| UPPER | 6.7 ( 4.27- 9.60) | $F_s = 68^{***}$ | 3.4 (0.58- 8.57) | $F_s = 20^{**}$ | 0.6 (0.0 -2.51) | $F_s = 32^{**}$ |
| LOWER | 19.2 (15.31-23.49) | | 13.4 (8.68-19.02) | | 6.4 (3.75-9.69) | |

[a]Data from two experiments. Flasks with fixed A31 cells at the UPPER end were replated with irradiated A31 and epidermal cells as described for Table 10. Other flasks with A31-conditioned surfaces were prepared as for Table 12, except that freeze-thawed A31 cells were added initially and at each medium change, and were allowed to settle on the UPPER end during incubation, opposite the irradiated A31 feeder layer. Each group consisted of four flasks with $0.5 \times 10^6$ epidermal cells/flask.

[b]Colonies in ten microscopic fields at each end of flask.

[c]Colonies in five microscopic fields at each end.

nonmetabolizing A31 cells at the UPPER end might have released toxic products that inhibited epidermal growth at that end, we did similar experiments with nonmetabolizing A31 cells uniformly distributed over the entire culture surface. We obtained the same results, arguing that differential toxicity was not involved. Metabolically active A31 cells therefore appeared to be necessary for the effect.

TPA Inhibits A31-Supported Secondary Epidermal Colony Production

Continuous treatment with TPA inhibited epidermal colony production at all concentrations tested (Table 14). We noticed no toxicity in the A31 feeder layer. Optimal concentrations of TPA for most of its *in vitro* effects are around 0.1 μg/ml ($1.6 \times 10^{-7}$ M). This concentration reduced cloning efficiency by 98%. A ten-fold lower concentration reduced it by 49%. In many past experiments (unpublished), we noted that TPA treatment of epidermal cells for up to a week in pure primary culture did not appreciably reduce DNA content. Such cultures without A31 feeders proliferate to a limited extent if at all, with terminal differentiation continually reducing the pool of apparent basal cells (Chapter IV). TPA treatment of such cultures for two weeks did not reduce cell number below that of controls (Table 14), indicating no apparent toxicity. These values were not correlated to the TPA inhibition of secondary cloning efficiency, which depends on epidermal proliferation. TPA, therefore, apparently interfered with A31-stimulated proliferation without killing epidermal cells.

Table 14. Effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) for Two Weeks on Primary Epidermal Cell Number and A31-Supported Secondary Epidermal Cloning Efficiency[a]

| TPA [µg/ml] | Primary Cells/Flask After 2 Weeks In Vitro[b] ($\times 10^{-6}$) | Secondary Epidermal Cloning Efficiency at 2 weeks[c] [%] (95% Confidence Interval) |
|---|---|---|
| 0 | 0.33 | 0.960 (0.8014-1.1331) |
| 0.01 | 0.27 | 0.491 (0.3887-0.6042) |
| 0.05 | 0.52 | 0.094 (0.0203-0.2164) |
| 0.1 | 0.40 | 0.021 (0.0 -0.0707) |
| Correlation Coefficient | r = - 0.66ns | |

[a]Primary and secondary epidermal cultures were treated with TPA beginning the day after plating, each with four flasks per treatment. Primary cultures initially contained 3 X $10^6$ cells/flask. Secondary cells were cloned on irradiated A31 feeder layers.

[b]Average of two counts of the pooled, trypsin-EDTA released nonterminally differentiating cells.

[c]An ANOVA showed that these values were significantly different ($F_s$ = 111***).

## Discussion

Hennings et al. (1980) reported that mouse epidermal cells can be subcultured and cloned in medium with low calcium levels without feeder layers. These cells do not terminally differentiate but continue to produce keratin. We have continued to use A31 feeder layers and standard calcium levels because epidermal cells grown under these conditions stratify and terminally differentiate, analogous to normal skin (Chapter II); this should allow us to study epidermal carcinogenesis and differentiation under more normal conditions. Peehl and Ham (1980a,b) recently were able to clone normal human epidermal cells by using media supplemented with 10 µg/ml hydrocortisone. The cells terminally differentiate under these conditions, although they found that lower calcium improves growth and interferes with normal differentiation as described by Hennings et al. They did not directly compare growth on a 3T3 feeder layer, and whether their techniques will work as well with the less prolific mouse epidermal cells is unclear. What is intriguing, however, is how hydrocortisone, calcium, etc. levels substitute for the cell-cell contact required by epidermal cells cultured under other conditions (discussed below). More work needs to be done before these questions can be answered.

Epidermal cells grown under our conditions are directly dependent upon the A31 feeder layer. The combined effects of conditioned medium, conditioned culture surface, and nonmetabolizing A31 cells did not enhance the growth of secondary mouse epidermal cells. If the A31 effect were transmitted through the medium, then continuously conditioned medium should have produced healthy, proliferating colonies

at both ends of the flask. If it were transmitted by the culture surface (e.g., secreted extracellular matrix, Gospodarowicz and Ill, 1980 and Vlodavsky et al., 1980), then conditioned surfaces should have produced healthy colonies. Explants do not seem to require conditioned surfaces (see below), and we have never noticed any differences in colony formation whether A31 cells were plated before or after dispersed epidermal cells, so prior conditioning of the culture surface would seem to be unimportant. It is possible that some labile factor that must be continually replenished by living A31 cells is left on the surface. While this possibility cannot be ruled out, it would make more sense for a putative labile mesenchymal factor to be freely diffusible to its target epithelium. Nonspecific attachment to other support structures would only further lessen its effect. It seems more likely that cell-cell contact is involved through either surface recognition or modification, or direct transfer of important molecules.

Fixing the A31 cells abolished the effect. Fixation involves stabilization of cellular proteins (Baker, 1958). Ethanol coagulates proteins by making them less hydrophilic, but does not complex with the proteins or fix lipids or carbohydrates. Formaldehyde reacts with side-chain amino groups to crosslink proteins in a more natural configuration, and does fix lipids. Neither fixation, nor freezing, which would seem to do much less overall damage to A31 cells, stimulated epidermal proliferation. Murray and Fitzgerald (1979) recently demonstrated contact-mediated transfer (metabolic cooperation) of labeled uridine between a continuous epidermal line and

Swiss 3T3 cells. TPA inhibits metabolic cooperation in those cells and in V79 cultures (Yotti et al., 1979). That TPA also inhibited A31-stimulated epidermal proliferation may imply that metabolic cooperation mediates the effect. Simple cell killing seems not to be involved since TPA did not decrease cell number in primary epidermal cultures; moreover, TPA in vivo causes epidermal hyperplasia (Scribner and Suss, 1978). In previous studies, TPA did not reduce the incidence of highly differentiated epidermal lines arising from A31 cocultures (Chapter II). The cocultures were allowed to interact for eight days before TPA application, perhaps reducing or eliminating TPA's effect.

If cell contact is required, then this interaction differs from typical epithelial-mesenchymal interactions in vivo. The structure now known as the basement membrane is secreted by epithelial cells (Rhodin, 1967; Vracko, 1974). Cell contact does not occur across it under normal conditions. Briggaman and Wheeler (1968) found that degeneration of human epidermis grafted to the chick chorioallantoic membrane is prevented by either intact or freeze-killed dermis, in contrast to our results. Flaxman et al. (1967) found that epidermal sheets from skin explants generally proliferate ahead of any fibroblasts (ruling out surface conditioning) and grow just as well after removal of the explant. These findings argue that A31 cells may instead be mimicking some cooperative epidermal function. Rheinwald (1979) reported that once human epidermal colonies reach a certain size, the Swiss 3T3 feeder layer can be removed and the colonies will still proliferate. We noted similar results

when mouse epidermal colonies reached a certain size (unpublished observation). Price et al. (1980), using an improved culture medium without feeders, found that human epidermal colonies will arise from small clusters of cells but not single cells. Eisinger et al. (1979) and Milo et al. (1980) could only subculture pure epidermal cells at high cell densities by maximizing cell contact. Contiguous epidermal sheets from explants generally grow better than dispersed epidermal cells (Halprin et al., 1979). Proper cell contact is therefore very important for epidermal cell growth. TPA is known to have membrane effects (Boutwell, 1974; Castagna et al., 1979; Murray and Fusenig, 1979; Shoyab et al., 1979; Van Duuren et al., 1979; Yamasaki et al., 1979; Belman and Garte, 1980; Garte and Belman, 1980). TPA's promoting action may be through an interference of epidermal communication leading to more autonomous proliferation (Murray and Fitzgerald, 1979), perhaps reprogramming the cells (Boutwell, 1974) into a less differentiated (Diamond et al., 1978), but more proliferative state. A31 cells may be substituting for other keratinocytes or even the Langerhans cell that may play a role in epidermal organization (Potten, 1978). While cell lines derived by the 3T3 methodology probably arise from embryonic fibroblasts, phenotypic changes in culture do occur (Montesano et al., 1973; Flaxman, 1974; Borenfreund et al., 1975; Auersperg, 1978; Taylor and Jones, 1978; Schwarz et al., 1979; Parker et al., 1980). The ability to stimulate epidermal proliferation (possibly a quantitative phenomenon) appears not to be characteristic of 3T3 lines or of fibroblast lines in general, and may

only have arisen incidentally during development of the Swiss, BALB/c and SIM.R 3T3 lines.

# CHAPTER VI

## CONCLUSION

Cultured mouse epidermal cells grown in enriched Waymouth's medium require both 31° and a BALB/c 3T3 Clone A31 feeder layer for optimum growth. Human epidermal cells on a Swiss 3T3 feeder layer do not require 31°. The reason for this difference is unknown and has not been studied. The A31-stimulated proliferation of mouse epidermal cells appears to require epidermal-A31 cell contact. If TPA inhibits this interaction as it has been shown to inhibit direct transfer of label between Swiss 3T3 cells and a continuous mouse epidermal cell line (Murray and Fitzgerald, 1979), it may be that TPA's function in promotion is to free initiated cells from the normal growth controls that affect all epidermal cells, allowing the initiated cells to form tumors. Since epidermal cells that have not been initiated require 3T3 support in vitro, TPA isolation of these epidermal cells would prevent colony growth.

Epidermal cells are particularly well suited as an experimental model for epithelial carcinogenesis, partly because of their susceptibility to the polycyclic aromatic hydrocarbons. With benzo[a]pyrene, the 7,8-dihydrodiol is one of the major metabolites formed by mouse epidermal cells, which is not the case with most other cell types. SENCAR epidermal cells in vitro are more susceptible to polycyclic aromatic hydrocarbon toxicity than BALB/c cells, implying that the in vivo sensitivity of SENCAR mice to epidermal carcinogenesis may be due to the higher sensitivity of the target cells. SENCAR cells in

*vitro* also appear to be more autonomous than BALB/c cells, since they proliferate for a longer period of time outside of the organism. Initiated SENCAR cells *in vivo* may thus be more easily isolated from surrounding influences by TPA, resulting in an increased tumorigenicity.

Newborn mouse epidermal cells, grown under my improved culture conditions, will now routinely yield highly differentiated, continuous cell lines that if transformed by chemical carcinogens will produce squamous cell carcinomas in nude mice. These improved culture conditions should permit detailed examination of the cellular processes associated with epidermal carcinogenesis and differentiation *in vitro* and *in vivo*.

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# VITA

Don Ray Miller was born in New Braunfels, Texas on February 15, 1949. He attended elementary school there and graduated from Canyon High School in May 1967. That fall he entered Texas Tech University. While a permanent student at Texas Tech, he attended summer school at Sam Houston State University in 1968 and was a National Science Foundation Undergraduate Research Participant at Trinity University during the summer of 1970. In May 1971 he received a Bachelor of Science degree in physics (minor in mathematics) from Texas Tech.

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He then entered the University of Tennessee--Oak Ridge Graduate School of Biomedical Sciences at the Biology Division, Oak Ridge National Laboratory. He did his doctoral work under a National Cancer Institute traineeship, and in December 1980 he received the Doctor of Philosophy degree in biomedical sciences.

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