IDENTIFICATION AND ASSESSMENT OF TUMOR-PROMOTING AND COCARCINOGENIC AGENTS: STATE-OF-THE-ART IN VITRO METHODS

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I. INTRODUCTION

The tremendous increase in the number of environmental chemicals that may play a role in chemical carcinogenesis has brought about a proliferation in the development of carcinogenesis models for evaluating chemicals. These models are needed to assess, analyze, and compare these chemicals for their possible human carcinogenic risks. Strong epidemiological evidence indicates that exposure to environmental agents is related to most human cancers. Approximately 80% of human cancers are believed to result from exogenous environmental factors.[1] Although in vivo animal models are still considered the best available for assessing chemical carcinogens and tumor promoters, as well as cocarcinogens, the large number of chemicals to be tested makes this an almost impossible task. Recent efforts to develop short-term in vitro tests may help solve this problem. The increasing number of chemical agents to be assessed make such short-term tests a necessity.

Carcinogenesis has been known to occur in stages since Rous,[2,3] Berenblum,[4] Mottram,[5] Boutwell,[6] and their associates demonstrated that hyperplastic agents produced tumors in skin following subcarcinogenic exposure to polycyclic aromatic hydrocarbons. Berenblum discovered that croton oil was a very active hyperplasigenic tumor promoting agent. Later, Hecker[7] and Van Duuren[8] isolated the most active skin tumor-promoting components from croton oil and characterized them as phorbol esters.

The two general stages of skin carcinogenesis are initiation and promotion. The initiation stage involves only one step, whereas the promotional stage includes several steps as elucidated in skin.[9,10] An initiator is a substance that can cause rapid and permanent (irreversible) induction of benign and malignant tumors after a single dose, followed by repeated applications of promoting agents.[11,12] When an initiator is given once at a subcarcinogenic dose, it does not produce tumors during the lifespan of animals; however, repeated doses of an initiator may eventually elicit tumors.[12] A promoting agent (and/or cocarcinogen) is one which, when applied repeatedly after a single subcarcinogenic dose of an initiator, results in benign and malignant tumors.[11,12] A tumor promoter also may occasionally produce a tumor when applied repeatedly without previous exposure to an initiator. The terms *tumor promoter* and *cocarcinogen* should not be used synonymously, however. A cocarcinogen is an agent, which, when administered concomitantly with a carcinogen results in a significantly higher tumor

Table 1
COMPARISON BETWEEN INITIATORS, PROMOTERS, AND COCARCINOGENS

Initiator[a]	Promoter[a]	Cocarcinogen
1. Carcinogenic by themselves; "solitary" carcinogen	1. Not carcinogenic alone	1. Can be carcinogenic
2. Must be given before a promoter	2. Must be given after an initiator	2. Can be given along with carcinogen or promoter
3. Single and usually subcarcinogenic exposure is sufficient	3. Requires long and repeated exposure	3. Requires long and repeated exposure
4. Action is irreversible and additive	4. Action is reversible at early stage and is not additive	4. Action is sometimes reversible but appears not to be additive
5. No apparent threshold	5. Probable threshold	5. Probable threshold
6. Yields electrophiles that bind covalently to cell macromolecules (DNA, RNA, etc.)	6. No evidence of covalent binding	6. May have covalent binding
7. Mutagenic	7. Not mutagenic	7. Can be either mutagenic or nonmutagenic

[a] Adapted from Reference 13.

yield than with the carcinogen alone. It must be emphasized, however, that not all cocarcinogens are tumor promoters and vice versa. The distinguishing features of initiators, promoters, and cocarcinogens are summarized in Table 1.

Although not included in the scope of this review, another factor regarding the initiation phase should be mentioned. Table 1 notes among the properties of initiators that all chemical carcinogens which are not electrophiles must be converted metabolically to active forms. These will then react with some critical macromolecule to start the carcinogenic process.[14]

The essential characteristic function of a tumor promoter is to complete the carcinogenic process already started by the initiators.[6,14-17] By keeping the total dose of a tumor promoter constant during an experiment, an optimal frequency of application can be determined for maximal tumor yield.[8] Either decreasing the frequency of application to once per month (with a corresponding increase in amount of each application) or decreasing the quantity each time (with an appropriate increase in the frequency of application), eliminates the effectiveness of the promoter.[6] The final tumor yield is quantitatively related to the initiating doses, whereas the speed of tumor growth depends on the effectiveness of the promoting action.[18] If the initiating dose is modified in any manner, the incidence of tumors is likely to be changed.[19,20] With the same initiating dose, however, both the rate of tumor development and the yield can be modified with different promoters,[20] or with inadequate frequency and quantity of promoters.[6]

Both initiators and promoters cause qualitative differences in the biological effects of the target molecules within the cells.[21] Two particularly compatible models in the two-stage system provide a good working hypothesis for future experiments with various animal tissues. The first is the interaction of the carcinogen with DNA which results in a heritable defect in the genome known as somatic mutation.[22] The second is the interaction of the carcinogen with a specific RNA, protein, or membrane site which results in a permanent change in genome expression.[23,24] Boutwell's working hypothesis of carcinogenesis states that treatment with an initiator eventually can result in the formation of permanent and heritable, yet unexpressed, changes in the cell genome.[21] In addition, this hypothesis has indicated that tumor promoters can be regarded as activators. The promoters, which cause the phenotypic expressions of changes in

genotype, alter the cellular metabolism, which is followed by altered morphology and, ultimately, a tumor.

In addition to chemical agents, hormones, viruses, immunological factors, nutritional factors, and physical trauma also can assume the role of tumor promoters and/or cocarcinogens. Since some hormones regulate the growth and maturation from the preneonatal period to the termination of these processes in aging of gonadal organs,[25-30] it is reasonable to consider this family of hormonal molecules as prototype cocarcinogens. Besides tumors resulting from hormonal influences in mammary glands, ovaries, prostate, testes, etc., carcinogenesis occurring through the influences of hormones are found in the skin, liver, urinary tract, and lymphoid system.[25] Since the risk of human breast cancers is high,[26] many animal models have been developed to study this process.[27-30] Because the breast is a hormone-dependent organ, it has been inferred that hormone imbalance can be sufficient stimulus to induce tumors,[26] and that this principle may be applied to other tissues as well.

Rous and his associates were among the first to investigate the possibility of viruses acting as cocarcinogens[2,3] and their prototype experiments remain contemporary. A broad range of both viral types and organ systems are involved in this phenomenon. Although viruses can be classified as cocarcinogens, this area of study is not within the realm of this review. Also beyond the scope of this review is the area of immunological cocarcinogens, in which most of the work has dealt with the response of the immune system to cancer through various modes by which neoplastic lesions are prevented or inhibited.[31] The role of nutrition in carcinogenesis has been recognized for a long time, and various dietary factors are known to influence tumor promotion in various organs. For example, riboflavin can partially prevent hepatocarcinogenesis induced by dimethylaminoazobenzene in rats;[32] caloric restricted diets in mice can reduce the tumor yield and an increase latency period for tumors;[33] a zinc deficient diet can cause higher incidences of esophageal tumors in humans;[34] and suboptimal levels of dietary vitamin A, in the presence of a carcinogen, can increase tumor yield.[35]

An excellent example of a physical promoter is repeated skin abrasion. Argyris et al. reported that an increase in epidermal cell regeneration, as a result of repeated skin abrasion, can provide stimulus in the promotion of both benign and malignant neoplasma on the skin of mice.[36-38]

This review begins with a general discussion of "markers" of tumor formation as a result of exposure to tumor promoters/cocarcinogens. Following this, a section on contrasts and comparisons of in vitro and in vivo systems as employed today is presented. Screening methodologies, both in vivo and in vitro, are reviewed specifically in the following subsequent sections: skin, liver, respiratory tract, bladder, gastrointestinal tract, and mammary glands. In addition, a section of cell cultures is included which describes specific cell lines that are in standard use in chemical carcinogenesis. Finally, promising state-of-the-art screening systems for the identification and assessment of tumor promoters and/or cocarcinogens are presented.

II. TUMOR PROMOTION "MARKERS"

The importance of the identification of critical morphological and biochemical events in tumor promotion must be stressed to identify and assess the potential of tumor promoters. A stimulation of macromolecular synthesis by tumor promoters is an important and basic component, since all promoters accomplish this; however, not all agents that increase macromolecular synthesis are promoters. Macromolecular synthesis includes DNA, RNA, protein, plasminogen activator (PA),[9] and most recently, ornithine decarboxylase (ODC) synthesis.[9,49]

It is evident that one factor alone cannot determine the tumor promoting ability of an agent, and that several factors are needed to establish the potential of a tumor promoter. For example, an enhanced DNA synthesis in cells is also apparent for many nonpromoting irritants.[39] Thus, it may be concluded that the ability to stimulate DNA synthesis and cellular division is an essential, but not a sufficient property of tumor promoters. In addition, several investigators reported that the tumor promoters do not always stimulate cell proliferation during in vitro studies.[21,40-42] Peterson et al.[43] discovered that the doubling time of previously initiated 10T½ cells was unaffected by their exposure to a tumor promoter under circumstances that lead to malignant transformation. Nor was the plateau density increased significantly by the same tumor promoter. Total protein synthesis, in general, has been found to be stimulated in the presence of tumor promoters.[21] By measuring specific enzymatic activities, which provide a sensitive measure of specific proteins, the promoting capability of an agent can be studied. This type of study is particularly useful in hepatic cancer research.[44-46]

An interesting enzyme, ODC, was discovered in 1940 when Gale reported this inducible enzyme catalyzing the conversion of L-ornithine into putrescine in *E. coli,*[46a] and Morris and Pardee later found that *E. coli* contained two enzymes, the "inducible" and the "biosynthetic" L-ornithine decarboxylase.[49b,49c] In 1968, two reports further discussed the importance of this enzyme in the growth of rat prostate gland[47] and in the rapid growth of tissues such as regenerating rat liver, chick embryo, and various tumors.[48] O'Brien and his associates found an excellent correlation between the tumor promoting ability of phorbol esters as well as nonphorbol ester compounds and their ability to induce ODC activity in mouse skin.[49] O'Brien went on to propose that the induction of ODC might be an obligatory event in skin carcinogenesis.[50] While the data indicated that ODC induction and the accumulation of putrescine by TPA, it was not clear cut and sufficient[51] since it was reported that the application of indomethacin[51a] or retionic acid[51b,51c] to the skins of mice had inhibited both the induction of the ODC activity and tumor formation. Recently, Takigawa et al. reported that α-difluoromethyl-ornithine, an irreversible inhibitor of ODC, either applied topically to mouse skin or administered in drinking water in conjunction with the applications of TPA to the DMBA-initiated mouse skin, inhibited the formation of mouse skin papillomas significantly.[51d] Therefore, almost complete inhibition of both TPA-induced ODC activity and the accumulation of putrescine were absorbed. This observation brings forth the evidence that the products of the ODC enzyme play an essential role in tumor formation by TPA.

Many of the properties of tumor promoting agents and cocarcinogens are well-illustrated in skin carcinogenesis; some examples are the formation of skin papillomas,[8,9,11,21] induction of ODC activity[49,50] and dark basal keratinocytes,[9,10,52] and stimulation of DNA activity.[41,53] One of the best studied tumor promoters is 12-0-tetradecanoylphorbol-13-acetate (TPA), particularly in skin carcinogenesis. Recent studies have illustrated that tumor promotion is a general phenomenon that extends to a number of other tissues including liver,[54] lungs,[55] colon,[56] bladder,[57] and mammary glands.[58] Various classes of tumor promoters are active for specific tissues, i.e., phenobarbital in rat liver,[54] butylated hydroxytoluene (BHT) in mouse lung,[55] bile acids in rat colon,[56] and sodium cyclamate and saccharin in rat bladder.[57] The formation of tumors as the end point is the characteristic marker in the determination of potential activity of tumor promoters. Except for studies in skin and hepatic carcinogenesis, the biochemical mechanisms in other tissues still remain very poorly delineated.

The markers for in vitro carcinogenesis can be classified into two general properties, i.e., transformation potential and biochemical alterations. An example of cellular transformation in the presence of tumor promoters is the increase in formation of Type III foci as demonstrated in 3T3 cells[59-61] and 10T½ cells.[62-65] Other examples of cellular

transformation by tumor promoting agents are synthesis of new proteins in mouse epidermal cells;[66,67] formation of new phenotypes (anchorage independence) in mouse epidermal cells;[68,69] and criss-crossing and piling up of cells in hamster embryo cells.[70–77]

In addition to the macromolecular changes seen in skin and liver, in vivo changes in ODC and DNA activities are seen in mouse epidermal cell cultures.[78,79] Other examples of markers in vitro systems in the presence of tumor promoters are: inhibition of metabolic cooperation in Chinese V-79 cells[80–83] and in between mouse epidermal cells (HEL/37) and mouse fibroblast cells (PG-19);[83a] inhibition of cellular differentiation in FEL cells;[84–86] and stimulation of cellular differentiation in HL-60 cells.[87–90]

III. IN VIVO VERSUS IN VITRO METHODS

For decades, in vivo animal models have been used to screen chemicals for carcinogenicity. Such models are relatively simple and practical, particularly when rodents are used, which are plentiful and inexpensive. Until a few years ago, tests included giving a test substance to rodents and observing throughout their life span for tumor development. The facilities throughout the world that conduct these long term tests are becoming increasingly inadequate for testing the vast numbers of synthetic chemicals going into large-scale production each year. Thus, increasing efforts in the development of short-term tests, which can predict the carcinogenic and tumor promoting properties of such agents, are needed.

One of the major achievements in cancer research in the past 15 years has been the development of refined cell culture systems, in which normal cells can be converted into tumorigenic cells by single in vitro exposure to chemical carcinogens.[91,92] These in vitro systems show promise as a rapid and simplified bioassay for such agents, and they should also make it possible to analyze more precisely the important cellular and molecular mechanisms involved in chemical carcinogenesis. In 1965, Berwald and Sachs[93] pioneered a quantitative system for the transformation of cell cultures by chemical carcinogens, and since then numerous other investigators have reported the transforming potential of cells in culture systems by a wide variety of carcinogens plus tumor promoters.[91,92]

For many years, the skin has been one of the best organs for study of chemical carcinogenesis in vivo. Some of the advantages of using such a system are:[21]

1. The two-stage system works well; aspects of each stage can be well defined and studied specifically.
2. Tumors develop rapidly and are readily observed, thus making the kinetics of their appearance easily measured.
3. The tumor responses of initiators and tumor promoters can be varied greatly by changes in dose, susceptibility of test animal, isomeric variations in the test agents.
4. The agents causing tumors are likely to act directly on the skin, which may implicate the skin's ability to metabolize the agents.
5. Effective measurements can be made within minutes after chemical application.
6. It is possible to correlate the variations in tumor incidence with the nature and extent of chemical interaction of promoters with cellular and macromolecular, as well as the metabolic, consequences.

In vivo organ systems have become prominent in recent years for the study of cancer causing agents, tumor promoters, and cocarcinogens. It is important to note that organ and tissue specificity for carcinogens, cocarcinogens, and tumor promoters vary greatly with different species and exposure conditions.[94] Thus, one can not predict that a tissue

or cell type will respond in a given way to a chemical agent simply on the basis of how that same tissue responds in a different species or set of conditions.

The demonstration that in vivo initiating-promoting carcinogenesis can work in cell culture systems as well as in organ culture systems has yielded some useful information on specific effects of carcinogens and modulators (promoters and cocarcinogens).[95] In addition, cultivation of epithelial cell types in vitro as possible and offers a set of complementary experimental tools. Thus our understanding of these basic biological processes can be advanced by careful use of an appropriate mixture of tools (culture conditions).

Unfortunately, most of the cell culture systems employed today are either from cell lines having predominantly fibroblastic morphology or they are fibroblastic.[91,92] Since it is well known that at least 80% of human cancers are of epithelial origin, the fibroblastic character of such cells represents a serious limitation.[96,97] Moreover, most tissue culture studies of the aspects of the tumor phenotype have also employed fibroblastic cell cultures. Thus, it is not clear if the phenotypic properties of these cells and the obtained results can be applied to epithelial cells. Although the development of epithelial cell culture systems has advanced greatly in recent years for the assessment of chemical carcinogens, cocarcinogens, and tumor promoters,[98] as yet no practical epithelial cell culture system exists that can be used for the routine bioassay of these potential chemical agents; i.e., transformation of normal epithelial cells to tumorigenic cells.

A few reports have indicated success in the transformation of epithelial cell cultures by chemical carcinogens,[93,99-103] but there are several major limitations, namely: (a) a long lag of 8 to 36 weeks occurs between exposure to a carcinogen and the demonstration that the cells are transformed and (b) transformation is not associated with a distinctive change in morphology.[98] In contrast to such epithelial cell lines, with both the hamster embryo cell system and the mouse fibroblast system, transformation occurs with a short lag time (1 to 6 weeks), has a high efficiency, and can be easily scored quantitatively by morphological detection of altered foci. Table 2 presents transformation properties of fibroblastic and epithelial cell lines in culture. These criteria cannot be used reliably, however, for epithelial cell cultures.[104,105] Weinstein et al. believed that the criterion of growth in agar can be the most reliable one for the transformation of the fibroblasts and epithelial cell cultures.[98]

In a study of biological effects, in vitro systems offer a number of advantages over those conducted in vivo. These are:

1. Sensitivity due to the homogeneity of the cells
2. Rapidity
3. Ease of quantitation[98]
4. Economy
5. Ability to study response of human tissues
6. Allow biological events to be studied under controlled conditions with fewer variabilities than in vivo[92,98]
7. Relatively high yields of transformed cells in contrast with low yield of similar cells in the in vivo models

With this in mind, new insights into mechanisms of action and the molecular biology of spontaneous and virus-induced transformation have resulted from the application of in vitro techniques.[106]

The use of in vitro systems also has some disadvantages as well. The first and probably most important problem deals with the difficulty in mimicking in vitro the reaction that occurs in vivo in response to injury.[98] In other words, one cannot be certain that cells

Table 2
TRANSFORMATION PROPERTIES OF FIBROBLASTIC AND EPITHELIAL CELLS IN CULTURE TO CARCINOGENIC TREATMENT[a]

Cell properties	Cell lines: Fibroblasts	Cell lines: Epithelial cells
Morphological changes (refractiles, criss-cross, rounded) altered foci	+[b]	−
Increased saturation density and piling up	+	±
Decreased serum requirements	+	?
Growth in soft agar	+	+
Tumorigenicity	+	+
Altered cell-surface glycoproteins and glycolipids	+	?
Lectin agglutination	+	+
Decreased c-AMP	+	?
Increased protease production	+	?
Decreased microfilament sheaths	+	?
Increased membrane transport	+	?
Decrease Ca^{++} requirements	+	+

[a] Adapted from Reference 98.
[b] + = positive effect; − = negative effect; ± = some effect; ? = unknown effect.

exposed in vitro to a toxic chemical agent respond in the same manner as cells exposed in vivo. Moreover, some agents require enzymatic conversion to form active metabolites; thus, some factors other than the target cells may be needed to convert such agents.[98] Another possibility is that one agent can prove to be toxic to cells in vitro but fail to show its toxic property in an in vivo model. For instance, dimethylnitrosamine (DMN) is toxic and tumorigenic in vivo[107,108] but it shows great variability in the in vitro tests. Sanders and Burford[109] failed to show any evidence of transformation in hamster cells treated in vitro with DMN, whereas Huberman et al.,[110] were able to transform the cells with an increase in cell multiplication and life span using the same procedure.

IV. SKIN

Although the initiation-promotion model for skin carcinogenesis has been studied for more than 40 years,[4] specific cellular and biochemical occurrences needed for the formation of tumors and their progression to malignancy still are not understood fully. Initiation of mouse skin tumor formation is basically an irreversible step, which may involve a somatic cell mutation.[111,112] Good correlations have been demonstrated between the various carcinogens and their corresponding mutagenic activities. These agents either exist as, or are metabolically converted to, active electrophilic reactants which then bind covalently to cellular DNA and other macromolecules.[14] Direct evidence from tumorigenicity studies has shown that "bay region" diol epoxides of benzo(a) pyrene[113,114] and benzo(a)anthracene[115,116] are the ultimate carcinogens in mouse skins.

Promotion of tumors in mouse skins has been well documented in the literature, but the precise mechanism involved is far from being fully understood. Morphological and biochemical responses of mouse skin to phorbol esters, the best studied of the tumor promoters, are characterized as follows:

1. Induction of inflammation and hyperplasia[6,117]
2. Induction of dark basal cells[10,41,118]
3. Increase in DNA, RNA, and protein synthesis[53]
4. Increase in phospholipid synthesis[119]
5. Increase in histone synthesis and phosphorylation[120,121]
6. Increase in ODC activity followed by an increase in polyamine production[49,122]
7. Decrease in histidase activity[123]
8. Induction of embryonic proteases in adult mouse skin[124]
9. Increase in protease activity[125]
10. Decrease in the isoproternol stimulation of c-AMP[126]
11. A transient increase in c-GMP with no change in c-AMP levels[127]
12. An increase in c-AMP phosphodiesterase activity[128,128a]

Of all the above-mentioned criteria, the induction of epidermal cell proliferation, the ODC activity, and dark basal cells correlate the best with phorbol ester tumor promotion.[10,41,42,49,118] It also has been demonstrated that the tumor promoting potency in vivo of a series of phorbol esters correlates well with their capability to increase the ODC activity and DNA synthesis in the in vitro epidermal cell cultures.[78]

To select one or even two important events in phorbol ester tumor promotion is quite difficult. Nonphorbol ester tumor promoters may not exhibit any of the mentioned properties, whereas some agents that induce hyperplasia in mouse skins are not true promoters.[39] An excellent correlation between the promoting ability of various compounds, both phorbol esters and nonphorbol esters, and their ability to induce the ODC activity, has been reported by O'Brien and his associates.[49] In addition, it was discovered that phorbol ester tumor promoters induced the appearance of dark cells in the epidermis whereas ethylphenylpropiolate, a nonpromoting epidermal hyperplasinogenic agent, did not.[129] A large number of these dark cells have been found in papillomas and carcinomas.[41,42] Slaga et al. found that flucinolone acetonide, a potent anti-inflammatory agent that inhibits the TPA-induced tumors, has very little effect on the TPA-induced ODC activity but counteracts the induction of the dark cells. Thus, the importance of dark basal keratinocytes in tumor promotion is shown. Recently, mezerein, a diterpene similar to TPA, was found to be capable of inducing most of the morphological and biochemical changes in the skin epidermal and in cells in culture that TPA does; yet TPA was at least 50 times more active as a tumor promoter.[51] Since TPA and mezerein are about equipotent in the induction of the epidermal ODC activity, the ODC induction does not appear to be a critical factor in tumor promotion. It has been shown that TPA induced approximately two to three times more dark cells than mezerein in the interfollicular epidermis as well as in the infundibular portion of the hair follicles.[52] Therefore, the induction of dark cells may be the critical factor in tumor promotion.

Recent years have brought many new developments in culturing epidermal cells in an attempt to mimic the growth patterns of biochemical events of in vivo mouse epidermis treated with tumor promoters. Since it is known that most of the tumors and papillomas are of epithelial origin, the need for homogenous epidermal cell cultures is further emphasized. The general effects of tumor-promoting phorbol esters on mouse epidermal cell cultures are as follows:

1. Increased RNA synthesis[130]
2. Early delayed DNA synthetic activity[131,132] followed by an increase in later stage[130-132]
3. Increased PA production[130,133,134]
4. Decreased large-external transformation-sensitive (LETS) protein production[130]

5. Increased cellular proliferation[130]
6. Increased phospholipid metabolism[130]
7. Rapid rise of c-GMP and c-AMP[135-138]
8. An irreversible shift in anchorage independence[139-141]

The last criterion mentioned may be one of the most useful markers in the in vitro studies. In addition, it may be analogous to a later stage of tumor promotion in vivo,[139] since mezerein showed many of the characteristics above, as well as inducing anchorage independence.

An interesting paradoxical observation was reported recently that compared DNA and ODC activities of tumor promoters on skins of newborn mice in vivo and newborn epidermal cells in vitro.[142] In this case, TPA was found to stimulate DNA and ODC activities in adult mouse skin in vivo, as well as in newborn epidermal cell cultures; however, these same biochemical activities were not altered in newborn mice in vivo receiving the same regimen as those for adult mice. These investigators concluded that specialized functions in the skin may be associated with changes in the membrane and do not appear to be mediated through the dermis. In other words, dermis is not included in the newborn epidermal cell cultures that respond to DNA and ODC activity changes upon exposure to phorbol ester tumor promoters and mimic the effects seen in adult mouse skins in vivo. Additionally, the newborn mouse skin appears to be in a natural state of maximum stimulation which is unresponsive to growth-controlling influences as in more quiescent and more sensitive adult skins.

One of the latest tissue culture techniques for mouse epidermal cells which also may be applied to other mammalian cell cultures was recently reported by Fusenig et al. This successful microtechnique determines the rapid analysis of macromolecule synthesis in mini-cultures of mouse epidermal cells.[143] This method, known as the Stanzen technique, can be employed for determination of DNA and protein content, as well as a few other bioassays. Reproducible results using only minimal cell numbers per sample are useful in studying both primary or early passaged cell cultures and malignantly transformed cell lines. Several biochemical tests can be performed at one time using the same pool of cells (the biochemistry of cells sometimes can vary from one batch of animals to another despite the usage of the same species and strain). This technique is also inexpensive, is a great timesaver, and may be used for many potential chemicals over a short time.

Other mammalian skins such as the rat, rabbit, guinea pig, and human samples have been studied to assess the activity of chemical carcinogens and tumor-promoting chemicals. The cellular structures of mouse and rabbit skins are not similar to human skin samples especially in number of cells, epidermal thickness and follicular apparatus.[144-146] The guinea pig skin is most analogous to the human samples in terms of morphology,[145,146] hair cycle, and permeability of extraneous compounds.[147] The relative sensitivity of the different animal species and strains compared with man is not well known.[148]

In contrast to Berenblum's[149] and Shubik's[150] reports, Stenback[144] recently reported that two-stage carcinogenesis can occur in rabbit skins. A plausible reason for this success is that a different strain of rabbits (New Zealand) was employed by Stenback instead of another unspecified strain used by the other investigators.

This basic premise can be applied to different mouse strains; SENCAR (originally from Boutwell's laboratory) is very sensitive to tumor promotion, whereas AKR strain is not very sensitive.[144] The SENCAR mice are derived from specially bred CD-1 mice.[144a] These CD-1 mice are treated with DMBA and TPA by the usual two stagewise skin carcinogenesis procedure, and those responding most favorably by exhibiting a high number of skin papillomas at the end of a 16-week treatment period are employed for

breeding. In addition, contrary to Shubik's report,[150] Goettler et al. reported the success of the initiation-promotion concept to the female Sprague-Dawley rats.[151] Using intragastrically administered DMBA as a carcinogen and the topically applied TPA as a promoter, tumors of the skin at the site of application were observed. The method of carcinogen administration was different from those mouse studies where topical applications of carcinogen were made; this study has given evidence that the initiation can take place in the skin when the initiation is given intragastrically.[6,151,152]

Goettler also experimented with the dorsal skins of Syrian golden hamsters by using the same technique as for the rats.[151,152] The dorsal epidermis did not exhibit any morphological changes, nor did any hyperplastic effect occur; yet high incidence of benign melanomas appeared in the perifollicular dermal melanocytes. The pigment system in the hair follicles remained unaffected in mice[153] with the same treatment regimen, however, and after the 26-week treatment with TPA alone, the melanocyte network retained their structural integrity without progressing to tumor formation. Therefore, the perifollicular melanoses consisted not only of a reactivation of otherwise nonfunctional dermal melanocytes, but also of a greatly enhanced proliferation of this cell population.[154–158] Similar to epithelial two-stage carcinogenesis, the cutaneous melanoma formation, therefore, is ultimately associated with a hyperplastic response of corresponding target cells during the promotional phase. It can be assumed with some uncertainty that TPA-induced/enhanced melanosis is responsible for the reduction of the latent period of the tumor formation.[152]

Much interest in environmental chemicals exists, in particular for those that are potential tumor promoters and/or cocarcinogens, especially those that may affect humans. Further, since a number of recent investigators have been using human cells, it seems logical to discuss this aspect in detail. Thus, the use of human cells can be a valuable tool to link the experimental animal studies with human cancers, as well as assess the validity of extrapolating carcinogenicity data from animals to humans.[159,161a,161b] As mentioned earlier, human cells differ from rodent cells; specifically, these differences are their much shorter in vitro life span,[160] stable karotype,[160] and unusually low incidence of spontaneous and induced transformed cells in humans.[161] The human cells do, however, offer a unique opportunity for studies on the cellular mechanisms of aging, growth, regulation, terminal differentiation, and chemical transformation.[162]

Green and Rheinwald[163,164] established a useful technique for culturing human epidermal keratinocytes obtained from neonatal foreskins. Kitani and his associates successfully cultured the same cells from the skins of normal adults.[165,166] It should be noted that because of environmental factors, normal individuals do differ in their metabolic capacity to activate chemicals, which may result in individual differences to the susceptibility of these chemicals.[167] Kuroki et al.[167] have reported, for instance, that exposure to B(a)P caused different metabolites to be formed in keratinocytes derived from three different individuals.

Other cells in the epidermal cell layers may be a detrimental factor in chemical carcinogenesis. Prunieras presented two excellent reviews on this subject.[168,169] Most of the epidermal cells employed in tissue culture studies are keratinocytes that have the role of synthetizing keratin. These cells are eventually lost by desquamation in vitro and the loss is then compensated by permanent proliferation of the basal cells present in culture. Because the biology of epidermis is dominated by keratinization and regulation of growth, Prunieras proposed two mechanisms:[169] (a) since the epidermis is immediately above the dermis, the growth and keratinization of the epidermis are controlled by connective tissue factors passing through the basement membrane between epidermis and dermis; and (b) maturing keratinocytes, which control the activity of the basal cells, emit signals to block the activity at various stages of cell cycle.

Other cells found in the epidermis are melanocytes and Langerhorn cells which depend on keratinocytes to be biologically active. Prunieras advocates using two types of culture systems in the in vitro studies. One culture would be keratinocytes alone, and the other would be a combination of all epidermal cells. Three useful cell culture models were proposed.[169] One would grow keratinocytes on previously X-irradiated 3T3 Swiss mouse cells, as developed by Green and his co-workers.[170] These 3T3 cells are feeders to keratinocytes and can act as connective tissue. Moreover, by adding epidermal growth factor (EGF) to cultures, the number of cell passages and cell yield are increased with a corresponding increase in the colony-forming ability of the population. The second proposed model would use guinea pig ear skin as a source of both epidermal keratinocytes and dermal fibroblasts.[171,172] The parallel cultures, one of keratinocytes and the other of fibroblasts, are used to study specifically how pharmacological compounds affect growth and keratinization of epidermal cells. The evidence from adult epidermal keratinocytes grown in culture shows that TPA does increase the DNA synthetic activity[168] compared with the control growth. Finally, the third model concept uses skin explant cultures, which have been well studied in recent years by a number of investigators. The method discussed by Prunieras employs small pieces of human neonatal foreskin containing epidermis and the superficial dermis, which are placed on lyophilized pig skin dermis as the culture substrate.[173] As the human keratinocytes use the substrate dermis, the dermis becomes transparent; this makes it easy to measure the surface of growth area and plot the curve of expansion. Moreover, human fibroblasts do not thrive on pig skin.

Recently, Fischer et al. reported success in using skin explant methodology for epidermal cell culture studies.[174] This method produces a large number of cells from these small samples of skin. In addition, no feeder layer is required, which means that the effects of such growth factors can be studied using pharmacological agents without any metabolic contribution from a feeder layer. By maintaining normal skin architecture, the initiating phase in epidermis may mimic the growth and maturation status of the same cells in vivo. TPA was used to determined the toxic and mitogenic characteristics. Unlike the responses seen with mouse epidermal cells, human explant cultures treated with TPA for 3 weeks showed a decrease in growth in a dose-related manner. Yet, it should be noted that the neonatal foreskins used might be unacceptable since it was shown in studies of neonatal and adult mice that these cultures respond in an inverse manner to phorbol ester tumor promoters[142] This activity in adult humans as compared with newborns may be analogous to the report of Slaga et al. which demonstrated that DNA and ODC activities were not stimulated in newborn mice in vivo but were seen in adult mouse skins.[142] A recent report documents that TPA increases DNA incorporation by ^{3}H-thymidine and induces ODC in adult mouse skin explants following TPA treatments.[175]

Tumor promoters other than phorbol esters are reported in studies of skin carcinogenesis. Anthralin (1,8-dihydroxy-9-anthrone) was established to be a tumor promoter.[176,177] Moreover, like TPA, anthralin can act as a cocarcinogen.[178] Of all analogs of anthralin tested for their tumor promoting ability and cocarcinogenicity, the parent compound was the most active in eliciting tumors on mouse skins.[179] Mineral oil and n-dodecane act as cocarcinogens when applied repeatedly on mouse skins concomitantly with various doses of B(a)P.[180,181] In 1976, Horton et al. documented the cocarcinogenic activity among a series of n-alkanes, from C_{12} to C_{25}.[182] It was discovered that octadecone (C_{18}-alkane) and eicosane (C_{20}-alkane) were cocarcinogenic in these mouse skin experiments. In addition, mineral oil was a cocarcinogen with varying doses of DMBA.[183] Pyrene and fluoranthracene, noncarcinogenic aromatic hydrocarbons, exhibit cocarcinogenic activity with a low dose of B(a)P on mouse skin.[184] Van Duuren et al. found that linalyl oleate and linalyl acetate were also weak cocarinogens.[185] In their report, it was noted that phenol, earlier shown to be a weak tumor promoter,[186,187]

Table 3
COMPARISON OF COCARCINOGENIC AND TUMOR PROMOTING ACTIVITIES OF SOME AGENTS IN SKIN CARCINOGENESIS[a]

Compound	Cocarcinogen	Tumor promoter
Phenols		
Phenol	±[b]	+
Esculin	−	−
Eugenol	−	−
Quercitin	−	−
Catechol	+	−
Resorcinol	−	−
Hydroquinone	−	−
Pyrogallol	+	−
Anthralin	+	+
Alphatic and olefinic hydrocarbons		
Squalene	−	−
n-Dodecane	+	±
Tetradecane	+	+
Hexadecane	−	±
Aromatic hydrocarbons		
Pyrene	+	±
Fluoranthrene	+	±
Miscellaneous		
Linalyl oleate	±	−
Linalyl acetate	±	−

[a] Adapted from Reference 179.
[b] + = positive effect; − = negative effect; ± = borderline between positive and negative effect.

was an inhibitor when applied concurrently with repeated applications of B(a)P. On the basis of the premise of the preceding statement, it is not necessarily true that a tumor promoter is a cocarcinogen or vice versa. Table 3 illustrates examples of skin tumor promoters and cocarcinogens.

V. LIVER

In the study of two-stage chemical carcinogenesis, the liver is probably the most investigated organ next to the skin. With the use of experimental hepatocarcinogenesis as a model system, a wealth of background information can be obtained on morphological aspects of hepatocellular carcinoma[188,189] in addition to biochemical and physiological properties of liver.[190]

It has been known for a long time that administering hepatocarcinogens to a rodent causes several distinctive focal and nodular hyperplastic lesions to develop in the liver before the appearance of hepatocarcinoma. Such biochemical "markers" for distinguishing these preneoplastic lesions from liver parenchyma are:[44]

1. Altered metabolism of glycogen
2. Deficiency in the enzyme activities of glucose-6-phosphotase (G-6-P), adenosine triphosphatase, β-glucuronidase, serine dehydratase, acid phosphatase, and glycogen phosphorylase
3. Increased activity of the fetal hepatocyte enzyme, γ-glutamyl transpeptidase

4. Expression of prenoplastic antigen
5. Deficiency of stored iron
6. Resistance to cytotoxic action of hepatotoxins and carcinogens
7. Elevated DNA synthesis and mitosis
8. "Phenotypic heterogeneity" with respect to the above characteristics

In 1959, Farber and his associates were among the first to stress the importance of the hyperplastic node as a possible precursor to hepatocellular carcinoma,[191] and this was further emphasized by Goldfarb and Zak.[192] Such studies were based not only on morphology, but also on the deficiency in G-6-P activity of these nodules.

Because Teebor and Becker reported that many, but not all, hyperplastic nodules regressed after the animal was removed from carcinogenic stimulus,[193] Farber and his co-workers investigated this "reversibility" in rats.[45,194] They found that upon regression perhaps the most important hepatic cells of hyperplastic nodules were remodeled into normal morphological structures of liver, yet they did not show a concomitant loss of the preneoplastic antigen. Butler and Hempsall also demonstrated that structures of lesions occur in mice[195] comparable to those in rats used by Farber and his associates.

Another important biochemical parameter that can be investigated in preneoplastic lesions of liver is enzyme-altered foci, which are mediated by hepatocarcinogens. Some specific examples are dimethylnitrosamine (DMN), which causes small foci of cells devoid of G-6-P activity,[196] and deficient of canalicular adenosine triphosphotase activity[197] and β-glucuronidase.[198] Such enzyme deficiencies can be demonstrated by histochemical studies and these enzyme-altered foci are likely to be clonal in origin.[199] The enzyme, γ-glutamyl transpeptidase, is interesting because minute amounts of the enzyme occur in normal adult hepatocytes[200,201] and hepatocytes of regenerating rat liver,[202] yet it was found in relatively high amounts in hepatocytes of neonatal rodents.[203,204] This enzyme, however, is elevated markedly in hepatocellular carcinomas,[202,205,206] as well as in "prenoplastic" liver lesions.[203,207-211]

Not only are there chemical modifiers of hepatocarcinomas, but also dietary (exogenous) and hormonal (endogenous) factors that can cause such alterations. For example, goitrogens can inhibit hepatocarcinogenesis caused by 2-acetylaminofluorene (2-AAF), a hepatocarcinogen.[212] Riboflavin content of the diet for rats undergoing azo-induced hepatocarcinogenesis influences the incidence of hepatomas markedly.[213] In addition, thyroidectomy and adrenalectomy can inhibit hepatoma production caused by 2-AAF in male rats,[214] and hypophysectomy can inhibit the action of this carcinogen to induce hepatomas in both sexes of rats.[215] On the other hand, weanling rats of both sexes receiving a subcarcinogenic dose of 2-AAF, as well as high levels of pituitary hormones, exhibit high incidence of tumors; rats receiving the carcinogen alone had only precancerous lesions.[216] Pituitary hormones in these studies can be classified as cocarcinogens. Male rats, as a general rule, are much more susceptible to the hepatocarcinogenic action of 2-AAF than the female rats.[44] Thus, hormones can act as cocarcinogens or as anticarcinogens in the process of altering the initiation of hepatic cells. In contrast to the above, promoting agents can exert their effects only when they are administered after carcinogen instillation.

Although much work has been done on liver carcinogenesis for a long time, it was not until 1973 that Peraino and his associates were able to demonstrate clearly that hepatocarcinogenesis could be separated into stages.[217] First, the carcinogen was 2-AAF fed at the level of 0.02% for 18 days and then it was followed by 0.05% phenobarbital as the promoter given at various times up to 30 days after termination of the initiating period. The male rats that received both 2-AAF and phenobarbital had a much higher incidence of hepatic neoplasms than those receiving the carcinogen alone. Moreover, this same research group later demonstrated that DDT, the common insecticide, showed a

promoting effect similar to that exhibited by phenobarbital.[54] Both phenobarbital and DDT, like TPA on mouse skin, caused increases in DNA synthesis and the weight of liver. These investigators also demonstrated that the final yield of neoplasms was much more influenced by the duration of phenobarbital treatment than by the length of intervals between carcinogen and tumor promoter administrations.[218] In two recent reports by Peraino et al., the effects on hepatic tumorigenesis were tested on the following three schedules: (a) duration of 2-AAF feeding period; (b) duration of phenobarbital feeding schedule; and (c) the length of intervals between termination of the 2-AAF feeding and the onset of phenobarbital feeding.[219,220] The results revealed that the classical two-stage mouse skin carcinogenesis phenomenon (or model) can be applied to the rat hepatocarcinogenetic process. They also found that phenobarbital was definitely not a carcinogen, and that the tumor-promoting property of phenobarbital was characterized by an increase in the probability of expression of the neoplastic phenotype by the initiated hepatocytes. In addition, the character of this phenotype and kinetics of its expression are not influenced by phenobarbital.

The work of Peraino and his group was extended by other investigators. Using DMN as the carcinogen, phenobarbital caused a five-fold increase in the production of hepatic tumors over those receiving the carcinogen alone.[221] Moreover, another group reported the utilization of 3'-methyl-4-(dimethylamino)azo-benzene as a hepatocarcinogen and found promotion with phenobarbital.[222]

The two-stage hepatocarcinogenesis model discussed thus far has required more than one dose of hepatocarcinogen to bring about initiation. Recently, Pitot et al. clearly showed the effectiveness of the promoting action of phenobarbital after only one oral dose of DMN within 24 hr of a 70% hepatectomy.[223] In addition, they demonstrated that no carcinomas appeared in rats for at least 24 months in the absence of phenobarbital.[224,225] The mechanism by which the promoter actually increases the number of the initiated cells, which express themselves as enzyme altered foci, is not well understood.[226]

In addition to phenobarbital and DDT as hepatic tumor promoting agents, other compounds such as butylated hydroxytoluene,[227] polychlorinated biphenyls,[228,229] 3-(3,5-dichlorophenyl)5, 5'dimethyloxazoline-2,4-dione,[229] estradiol-17-phenyl propionate and estradiol benzoate[230] are also effective promoters. Polychlorinated biphenyls, DDT, and estradiol may be carcinogens as well.[231-234] Other hepato-promoters have been reported as well, but induction of hepatocarcinomas occurs only after a long period of administration (at least 22 months).[44] A powerful environmental toxicant, 2,3,7,8 tetrachloridibenzo-p-dioxin (TCDD) is an interesting promoter for DMN-induced carcinogenesis in that when TCDD is administered orally, the same effects occur as with rats treated with phenobarbital administration; yet, when TCDD is injected subcutaneously twice weekly, the promoting effects are greatly enhanced.[44] The TCDD may not act as a carcinogen, however, since recently Poland and Glover were not able to show any covalent interaction of this agent with cellular DNA in vivo.[235] Unfortunately, very few studies have been directed towards an understanding of the mechanism of action of effective promoters in hepatocarcinogenesis. Many of the effects caused by TPA in mouse skins do not compare well to those (phenobarbital, DDT, etc.) found in liver.[44] A strong doubt exists as to whether phenobarbital has a significant effect on cell replication in relation to the promotion of neoplasia in liver.[44,217] Nevertheless, one common effect is shared by all tumor promoters in liver — the induction of xenobiotic metabolism is accompanied by a proliferation of smooth endoplasmic reticulum.[44] In view of studies by Poland and Glover on the TCDD receptors in liver,[236] it may be inferred that all promoters exert their organo-specific promoting effects through some type of receptor molecules.[44] It is of some interest that in 1972, Armuth and Berenblum studied the tumor

promoting effects of phorbol in liver of the mice.[237] Phorbol can promote hepatomas in mice after a low dose of DMN, whereas a higher dose of DMN administered before phorbol treatment caused lower incidence of hepatomas. No one else, however, has obtained a biological response to phorbol, either in cultured cells or the intact cells; therefore, administration of phorbol as a promoting agent to newborn mice (and not to older mice which produced negative results) is questionable.

Sequential analysis of liver cancer induction is more than just a simple two-stage hepatocarcinogenesis process. In a recent review, Farber pointed out that hepatic carcinogenesis involves many overlapping sequential events, which produce a wide spectrum of many steps appearing simultaneously at any one moment in time.[46] This same concept may be applied to skin carcinogenesis and other tissues. Farber based his views on many years of work by Foulds,[238,239] as well as his own work.[240,241] He generally believes that most cancer developments are multistepped processes over a long period of time. In other words, a series of sequences, each one consisting of a discrete, discontinuous change known as a "rare event," constitute carcinogenesis, and are followed by the selective growth of one or more types of altered cells.[46] A "rare event," at present, stands for an unknown mechanism that may be a mutation, may be analogous to irreversible change that is a part of normal development and differentiation, or a change of a new type. What is seen and what occurs between the rare events may be the selection of certain altered cells growing to produce focal areas of proliferation.

Relatively few in vitro models of hepatocarcinogenesis have been employed and reported. One of the major reasons for the failure of hepatocytes in vitro to parallel the effects of the same cells in vivo is that the profile of hepatocyte metabolism begins to change immediately upon removal of the cells from the animal.[242] In addition, certain aspects of whole body metabolism (i.e., bioactivation by gut flora and induction of metabolic pathways) are very important; thus, some classes of potent carcinogens are not detected in the primary hepatocyte cultures.[243] Williams and others using primary rat hepatocytes have reported a substantial advance in the development of an assay, the detection of unscheduled DNA synthesis (UDS).[244–247] This assay offers the advantage of metabolically competent target cells. Such liver carcinogens, as DMN, 4-dimethyl-aminoazobenzene, and fluorenylacetamide are metabolized to reactive electrophiles, which in turn alter DNA, and are thereby classified as genotoxic.[244] Promoters such as phenobarbital and DDT, on the other hand, do not alter DNA and are suggested to be epigenetic in nature; they may act on the initiated cells arising genetically or from exposure to genotoxic carcinogens. Hence, the primary hepatocyte culture/DNA repair test[245,246] gave a positive result for every hepatocarcinogen tested, whereas phenobarbital and DDT gave negative results. Because of the possible loss of hepatocyte metabolism in cells in vitro,[242] Mirsalis and Butterworth proposed a new in vivo/in vitro assay for potential hepatocarcinogens.[248] With this assay, rats are treated with a carcinogen in vivo, and then hepatocytes are removed for the measurement of UDS. Dose-related increases in UDS were observed for these hepatocarcinogens, but no responses were seen for the controls. This method may not be able to predict the carcinogenicity of chemicals that (a) are epigenetic, (b) produce irreparable DNA damage, and (c) are metabolized and cause organo-specific damage as sites other than liver enhance the growth of normal liver epithelial cells in the presence of low extracellular calcium.[249] In contrast to this such cells grew better in higher concentrations of calcium in the absence of promoters. This evidence moved investigators to suggest that low concentrations of calcium should be employed in the media of cultures being used for study of the effects of tumor promoters. Yuspa and his co-workers also reported tht mouse epidermal cells responded more favorably to tumor promoters and to cellular growth in the presence of a lower concentration of calcium in the media.[249a,249b] Fluorescence studies have shown that the

primary interaction of the promoting phorbol esters occurs at the plasma membrane of rat liver cells and that the calcium ion has a pronounced effect on this interaction.[249] Therefore, the plasma membrane is altered in the presence of tumor promoters. Cell membranes of other cell types are also affected in the same manner.[120,250-253]

VI. RESPIRATORY TRACT

This review of chemical carcinogenesis in the respiratory tract includes a discussion on lungs, bronchi, and trachea. Most of the studies have employed tobacco smoke condensates as carcinogens and promoters. Some of these chemical agents have been shown to have tumor promoting potential on mouse skin,[178] which adds additional support to the well-known epidemiological findings.

Witschi and his group have investigated the promoting effect of butylated hydroxytoluene (BHT) on urethane-initiated lung tumors in mice.[55,254] When tumor-promoting BHT was given to mice in vivo by intraperitoneal injection[254] or orally (fed at 0.75% in diet),[255] it was found to induce an increased fraction of proliferating cells that are specifically type-2 pneumocytes. These specific cells are the precursor cells for the promotion of lung tumors; this evidence was supported by a later report.[256] Contrary to this, the weekly injections of BHT into mice pretreated with saline reduced the number of spontaneous pulmonary tumors.[254] Time-related experiments have not been performed yet, however, as previously done in skin and liver studies. The BHT also has been shown to accelerate the growth of tumors, rather than to increase the number of tumors. In a later report, it was discovered that perhaps reactive metabolites of BHT, rather than the parent compound, did the damage to the lung cells.[257]

Since the main exposure to the respiratory tract of man and animals is by inhalation, it is not surprising that respiratory cancers occur in this way.[258] The induction of lung cancers results from the interplay of multiple environmental factors.[259] Modification of the manner in which the lung may dispose of inhaled material may be induced by a complex mixture of potentially dangerous materials, which are then capable of being metabolized and bound to cells in the lung.[260-262] In addition to the exposure by inhaled agents, the lungs are perfused by the entire cardiac output, with its supply of chemical compounds absorbed from the gut and liver. The lymphatic systems also empty their absorbed agents into the venous return perfusing the lungs.[263] On the basis of the preceding information, it appears that the isolated perfused lung model is a practical one in investigating the pulmonary metabolism of foreign compounds[261,262] without interference from other organs. The ubiquitous carcinogen B(a)P in tobacco smoke, air pollution, and occupation environments has been studied in isolated perfused lungs for metabolism, along with other air contaminants, as possible cocarcinogens or promoters. One group studied the metabolic effects of B(a)P in the lungs of rabbits pretreated with n-dodecane, a long-chain aliphatic hydrocarbon known to have cocarcinogenic and tumor-promoting potential on mouse skin.[264] The results revealed that the rate of metabolism and the formation of 7,8 and 9,10-diols were increased, and formation of diones and monohydroxylated compounds was decreased. An increase in epoxide hydrase activity was observed, which indicates that diol epoxides may be formed by the lungs as reported for mouse skins, where they bind covalently to DNA in cells.[265] Thus, n-dodecane, which has been proven to be noncarcinogenic, may be a cocarcinogen because it influences the metabolic activity of B(a)P in the lungs and possibly in the skin.

A number of other cocarcinogens have been investigated in respiratory tracts of various animals species. In one study, the following dusts, silicon dioxide (SiO_2), manganese dioxide (MnO_2), and ferric oxide (Fe_2O_3) were administered along with B(a)P in Syrian golden hamsters.[266] When these dusts were given alone, no respiratory tract tumors were formed. The SiO_2 in combination with B(a)P proved to be

cocarcinogenic because it induced papillomas and carcinomas of the larynx, trachea, and lungs. The MnO_2 in combination with B(a)P or dibenz(a, j) acridine (DBA), and Fe_2O_3 in combination with DBA failed to show any significant neoplastic response. The study of Nettesheim et al. which reported, however, that Fe_2O_3 dust was cocarcinogenic in hamsters because it enhances the DENA tumorigenicity in the peripheral lung but not in the larynx and trachea.[267] Acetaldehyde, a major compound of cigarette smoke, was tested for cocarcinogenicity with B(a)P or DENA in Syrian golden hamster and was found not to be cocarcinogenic[268] despite its severe irritating effect. In an earlier report, Feron investigated the cocarcinogenicity of furfural vapor with B(a)P and DENA as the carcinogens; the carcinogenic effect of these chemicals was in no way influenced by furfural vapor.[269]

Since airborne particulates can be inhaled and trapped in the distal airways and most tumors are of epithelial origin, the organ culture system of tracheal tissue has been a useful tool for studying stagewise carcinogenesis in the respiratory system. Several investigators have reported some success in using rat and hamster tracheas in vitro during studies of carcinogens, cocarcinogens, and tumor promoters. One group has performed three studies: (a) neoplastic transformation of tracheal epithelium after exposure to a complete carcinogen N-methyl-N′-nitro-Nnitrosoquanidine (MNNG), (b) stimulation of DNA synthesis in cultures exposed to TPA, and (c) two-stage carcinogenesis.[270] The last study revealed that epithelial cells were morphologically altered after a very low initiation dose of MNNG followed by 2 higher dose of TPA. TPA exposure to the culture for 1 hr every 6 days gave greater DNA stimulation than "continuous" treatment of TPA in cultures. This organ-cell culture model offers many advantages such as (a) tissues mimic closely the tracheal mucosa in vivo, (b) cultures of this type can be successfully maintained for many months under appropriate conditions, which may be necessary for transformation of the epithelial cells, (c) spontaneous transformations, as seen in vivo models, have not been observed, and (d) most importantly, the time needed to observe the rapid proliferation of morphologically altered cells is very short.

Mossman and Craighead have performed several experiments with a methodology[271] similar to the one reported by Steele et al.[270] Whereas Steele's group used rat trachea, Mossman and Craighead used tracheas of Syrian hamsters. In this case, 3-methylcholanthrene (3-MC) was absorbed onto inorganic particulates (Fe_2O_3 and crocidolite asbestos) before they were added to organ cultures.[271] Controls were employed by using these particulates minus 3-MC, in which hyperplasia of the epithelial cells was observed, but no tumor developments. Lesser amounts of 3-MC were needed with asbestos than with Fe_2O_3 to induce tumors in the explants, and both Fe_2O_3 and asbestos are classified as cocarcinogens or as "carriers" as they are often called in research with respiratory tracts. These "carriers" are used to increase the retention of polycyclic aromatic hydrocarbons. This method has been useful in gaining insight into the mechanism of transformation of these respiratory epithelial cells. In addition, since 3-MC is not a water-insoluble carcinogen, the usage of "carrier" particles has proven to be practical. An earlier study by the same investigators reported the successful use of lycra fibers that had been coated with 3-MC.[272]

To clarify the role of carriers in the respiratory tract further, a report by Warshawsky et al.[273] discusses the influence of Fe_2O_3 on metabolism of B(a)P in isolated perfused lung, a method mentioned earlier.[260–264] The Fe_2O_3 was found to act as a physical agent by decreasing the biological availability or slowing the release of B(a)P over a period of time when Fe_2O_3 is administered on the lung with B(a)P, in comparison with the pretreatment of Fe_2O_3 alone. The data presented revealed that two mechanisms may exist for the B(a)P metabolism in lungs in presence of the carrier, Fe_2O_3: (1) the long term effect may be due to the pretreatment of Fe_2O_3 which causes an increase in the total metabolic activity, and (2) the short term effect may be due to the administration of

Fe_2O_3 to the isolated perfused lung which, in turn, causes a decrease in total metabolic activity and inhibits the effects of pretreatment. Further evidence is shown by the slower release of Fe_2O_3, as measured by the appearance of metabolites in the blood by the isolated perfused lung, and significant changes in the metabolic pathway.

Radiation may be a cocarcinogen in the lungs of mice[256] and Syrian golden hamsters.[274] In the latter report, lung tumors increased after the concomitant administration of B(a)P and polonium-210 and when administration of B(a)P occurred 4 months after polonium-210 exposure. Most of the synergistic effects were seen at the latter test regimen and may be attributed to a potential effect of subsequent saline instillations on polonium-210 carcinogenesis. The investigators could not offer an explanation for this. Since epidemiological investigations have observed that the excess number of deaths from lung cancer among uranium miners were only in those who were cigarette smokers,[275,276] the two influences together, uranium exposure and cigarette smoke condensates, may have synergistic effects on the lungs. Meyer et al. reported that a greater increase in labeling index, as well as DNA synthetic activity occurs in mouse lung tumor cells 6 days after BHT administration followed by 200 rads of X-rays, than in those cells receiving BHT alone.[256]

VII. BLADDER

Hicks has shown that the classical two-stage carcinogenesis does exist in the development or bladder tumors.[57,277] This report is an excellent detailed review on the experimental induction and histology of hyperplasia and neoplasia in urinary bladder epithelium.[277] One of the first carcinogens to be studied and proven to initiate urinary bladder cancer was 2-naphthylamine, an aromatic amine.[278] Subsequently, benzidine,[278] 4-aminobiphenyl,[278] 2-AAF,[277] N-butyl-N-(-hydroxy-butyl) nitrosamine (first bladder-specific carcinogen to be studied.)[279] N-(4-(5-nitro-2-furyl)-2-thiazolyl) formamide (another bladder-specific carcinogen), (FANFT,[280] and N-methyl-N-nitrosourea (MNU)[281] have been studied as well. The sequential progression of events that occur after the instillation of a carcinogen into the bladder is as follows: simple hyperplasia, progressing to nodular and papillary hyperplasia, leading to benign-appearing tumors (noninvasive), and ending as invasive carcinomas.[282] The carcinogens mentioned are "complete carcinogens" that have both initiating and promoting properties, although the process is slow and takes years for tumors to be formed.

In 1975, Hicks et al. reported success in using the two-stage carcinogenesis system in rat bladders.[283,284] After a subcarcinogenic intravesical dose of MNU as the initiator, oral administration of sodium saccharin or cyclamate, was found to induce a high incidence of tumors. These promoters, used alone, were very weak carcinogens even at high doses. In addition to these promoters, caffeine has been shown to induce tumors in bladders of rats pretreated with MNU.[57] It is of interest to note that brewed coffee extract incorporated into the water did not promote the initiated cells in any manner; perhaps some other chemical agents in coffee are inhibitors of tumor promotion. Although Hicks et al. did not investigate the specific biochemical and other criteria in this bladder model, they hypothesized that the mechanism is similar to that studied in skin carcinogenesis.[57,285]

In another investigation using a model similar to the one employed by Hicks et al., Cohen studied rat bladders for their carcinogenic response to FANFT as the initiator and either sodium saccharin or DL-tryptophan as the promoter.[282] Saccharin fed after FANFT pretreatment increased the incidence of tumor formation greatly, as DL-tryptophan did, but to a lesser extent. DL-tryptophan has also been studied for its cocarcinogenicity (tumor promoting potential as well) by Radomski et al., who used 4-aminobiphenyl and 2-napthylamine as the carcinogens in dogs.[286] In this very limited

study (3 or 4 dogs were used for each experiment), bladder tumors occurred in those receiving DL-tryptophan for 4 years following single administration of 4-aminobiphenyl and in those receiving DL-tryptophan for 3 years after a 30-day treatment with 2-naphthylamine. No tumors were observed in dogs receiving each of the three agents alone, although the dogs receiving DL-tryptophan developed darkly stained mucosa with white areas of focal hyperplasia. One can suggest that DL-tryptophan is a true promoter since TPA has been shown to develop dark cells in skin.[10]

The in vivo method described in this section does pose several serious limitations. First of all, changes in urinary bladder (epithelium and underlying layers as well) are not visibly seen; one has to kill the animals at various time points to determine the morphological changes. It is unfortunate to report that no in vitro methods have been employed to study the mechanism of two-stage carcinogenesis in urinary bladders; this is probably because the methodology of inducing urinary bladder cancer is relatively new. In addition, Hodges et al. reported that urothelium cell cultures failed to maintain normal differentiation as observed in in vivo studies in the presence of MNU.[287] Hashimoto and his associates, however, found that by adding urea to the medium, cell growth and transformation could be observed in the urinary epithelial cell cultures in presence of MNU.[288,289]

VIII. GASTROINTESTINAL TRACT (GI)

Chemical agents from the environment have been known to induce tumors in the GI tract. Nutritional influences also can be excellent tumor promoters and/or cocarcinogens in experimental animal models. For example, a dietary zinc deficiency can induce and increase the incidence of tumors of the esophagus in rats treated with methylbenzylnitrosamine (MBN).[290] In addition, the lag time for induction of esophageal tumors was shortened in the presence of both a zinc-deficient diet and MBN. It has been demonstrated that MBN is a complete carcinogen. Evidence indicates that it causes tumors and is covalently bound to DNA, a common characteristic of a carcinogen. This occurs because, with diminishing zinc reserves, the thickened epithelium were deranged keratinization of the esophagus becomes more susceptible to penetration of these chemical carcinogens. This is not entirely the case, however, because it has since also been found that no matter how MBN is administered in the body, MBN selectively seeks out the esophagus as a target organ. This was demonstrated by studies with (^{14}C) MBN, where DNA and RNA labeling were highest in the liver and esophagus of all tissues tested. Since it was shown that four different citrus oils and promoted tumor development in mouse skin pretreated with DMBA,[291] Pierce investigated the model of two-stage carcinogenesis in the forestomachs of mice treated with DMBA or B(a)P as the carcinogen and lime oil as the promoter.[292] Tumors of forestomachs were observed in mice receiving either of the two carcinogens and lime oil. In addition, lime oil given alone did induce tumors and may be classified as a weak carcinogen, or the stomachs could have been initiated previously as a result of dietary constituents. Diets deficient in fats will be discussed later.

Because of a phenomenon that exists in classical two-stage mouse skin, Berenblum and Haran tested the two-stage carcinogenesis model by giving a single oral dose of polycyclic aromatic hydrocarbon as the carcinogen, followed by weekly administration of croton oil for 3 weeks.[19] Tumors developed in the forestomach in the group receiving the above regimen, as well as in the groups receiving either of the agents alone. Thus, no clear cut distinction in the mechanism could be made. Recently, Goerttler et al. reported a successful modified two-stage carcinogenesis model in the epithelium of the forestomach of mice receiving DMBA as the initiator and TPA as the promoter.[293] The

initiated/promoted group of mice demonstrated the highest tumor incidence in the epithelium. Whereas no tumors were found in the group that received only the promoter, some mice that received only the initiator developed tumors. The forestomach is, thus, a useful model.[293]

Since dimethylhydrazine (DMH) has been demonstrated to be a complete carcinogen in the rat colon,[294,295] and since phenobarbital is an effective tumor promoter in rodent liver,[54] Pollard and Luckert investigated the promoting effect of phenobarbital on DMH-initiated rat intestine.[296] The results revealed a higher incidence of tumors in intestines of rats receiving both treatments than in those receiving the carcinogen alone. The hypothesis here is that DMH is metabolized by liver to the ultimate carcinogenic metabolite which then acts on the epithelium of the intestines. To test intestinal mucosal cells for carcinogens, Freeman and San used UDS methodology in samples from humans.[297] In the presence of MNNG, these cells have the capacity to undergo DNA repair synthesis, and the UDS method has been proven useful to detect cell-specific, direct-acting, and activation-dependent chemical carcinogens. This method is also advantageous because these cells have limited viability, and only a very short time is required for the entire testing procedure.

The colon is an interesting organ in regard to the mechanism of chemical carcinogenesis. Large bowel contents are suspected to contain initiating and promoting agents[298] and these contents are not excreted from the body rapidly enough to prevent carcinogenic activity. Dietary regimen, dietary fat in particular, is an important factor to be considered in colon cancer[297] where high intake of dietary fat[299] and beef[300] are strongly associated with carcinogenesis. Reddy et al. has proposed the following hypothesis on the etiology of colon cancer:[56] (a) cholesterol, normally found in colonic contents, is converted to reactive metabolites by electron oxidation, which then interact with some critical macromolecule carcinogen; (b) mucosal cells convert cholesterol to epoxides; (c) dietary fat/meat influences the fecal bile acid concentrations, cholesterol metabolites, and the metabolic activity of colon bacteria, and thus produces tumorigenic compounds from bile acids; and (d) modification of colon carcinogenesis occurs upon influence of mucosal mixed function oxidases. Studies of bile acids have shown them to be tumor promoters and cocarcinogens as well as a weak complete carcinogens.

When rats are fed a high fat diet, they are more prone to azoxymethone-induced tumors than those on a low fat diet.[301] Reddy et al. also found that rats on a high fat diet (consisting of corn oil or lard) were also more susceptible to DMH-induced colon tumor incidence than the group receiving a low fat diet.[56,302] The same group also found that those receiving a diet high in beef protein and fat or high in soybean and corn oil had higher levels of DMH-induced tumors in the colon than those on normal diet.[303] Another tumor-causing agent in the colon is MNU in the presence of a high fat diet.[56,302] In addition to the high fat (and/or protein) diet as cocarcinogens, bile acids have been shown to be cocarcinogens in the presence of high fat diets. These bile acids have been studied for their carcinogenicity by several investigators because: (a) they are structurally similar to carcinogenic polycyclic aromatic hydrocarbons, (b) they may be converted into 3-MC, a carcinogen, and (c) some of them induce sarcomas at the injection site. Rats receiving an initiating dose of MNNG and lithocholic or taurodeoxycholic (both bile) acids as promoters demonstrated higher colon tumor incidence that those who received MNNG alone.[304] These two bile acids did not induce any tumors in colons of rats when they were given alone. Unfortunately, the mechanism by which these bile acids act as promoters is poorly delineated. The intestinal microflora and bile acids in the intestine may affect the mucosal cell kinetics.[56]

Several in vitro studies of colon epithelial cells have been made. In 1975, Lipkin demonstrated the similarity of colonic cells in vitro and in vivo in the sequence of events leading to colon neoplasm as induced by chemical carcinogen.[305] Autrup et al. studied the

metabolic effects of B(a)P and DMH, as well as a few cocarcinogens by using both rat and human colon explants in vitro.[306,307] These carcinogens were metabolically converted by enzymes to active constituents and subsequently bound to colonic DNA. Taurodeoxycholic acid significantly increased the binding level of B(a)P to DNA or rat colon explants, whereas phenobarbital did not alter the level; and disulfiram, an antioxidant, decreased the DNA binding effect.[306]

Many surfactants are used as emulsifiers for food, drugs, cosmetics, and in preparation of the same compounds. These surfactants have been tried as promoters after MNNG initiation of the glandular portion of the stomach of the rats.[308-310] Undifferentiated adenocarcinomas were observed in those rats receiving the combined treatment, whereas those receiving MNNG alone exhibited well-differentiated adenocarcinoma. Two surfactants, polyoxyethylene sorbitan and polyoxyethylene nonylphenyl ether, used with MNNG increased the tumor incidence over the MNNG controls.[310] It is not known presently whether the enhancing property of these surfactants is due to the physiological response of target cells to these agents or to the increased uptake of carcinogen by these cells.

IX. MAMMARY GLANDS

Epidemiological findings of the high incidence of mammary cancers in women appear to have caused an explosion of research in this area in recent years. Dietary and hormonal influences seem to have major effects on the incidence of mammary tumors. Since this review has attempted to address the effects of the environmental promoting or cocarcinogenic chemical agents, detailed descriptions of the hormonal and dietary factors will be limited. The use of mammary glands, especially mammary epithelial cells, has proven to be a practical model in studying the classical two-stage carcinogenesis model.

The best studied carcinogen for the induction of mammary tumors in rats appears to be DMBA. Huggins and his associates were among the earliest workers to study mammary tumor induction by DMBA.[58] An oral administration or an intravenous injection of DMBA will induce mammary tumors in 80 to 100% of female rats. Three factors (i.e., dose of carcinogen, age, and hormonal levels) can alter the incidence of mammary tumors. Age is probably the greatest influence; a number of investigators have pointed out that for the maximal effect, the rats should be around 50 to 55 days old.[311,312] This is a period when the mammary gland is in the earlier stage of its development. The incidence of mammary tumors induced by DMBA decreases after 60 days of age up to 120 days, when these glands were "refractory" to DMBA.[312] The route of administration by which DMBA is given to these rats is another interesting factor. Recently, topical application to mammary gland areas was attempted and the results revealed that age was not a critical factor.[312] Because of this new discovery, the DNA synthetic activity of mammary glands in these rats was investigated. Synthesis of DNA was inhibited in the mammary tissue of rats administered DMBA intravenously at 60 and 150 days, and an increase in DNA synthesis was observed in those receiving topical treatment at the same prescribed times. Janss and Ben reported, however, that DMBA binding to mammary gland DNA, an important criterion in carcinogenicity studies, decreased with age.[313]

A number of reports have indicated that a high-fat diet is a true mammary tumor promoter,[314-320] since tumors can only be formed in mammary glands after DMBA treatment and not beforehand. The most recent report indicated that a certain amount of polyunsaturated fat, in addition to a high level of dietary fat, may give its maximal promoting effect.[320]

Since prolactin, a peptide hormone, has been found to be highly elevated in breast cancers,[321,322] Chan and Cohen hypothesized that the effects of this hormone and high-fat

diet are related to one mechanism of action.[316,318] High-fat diet intake can stimulate the prolactin production, thus increasing the tumor development. This was evidenced by the addition of CB-154, an antiprolactin drug, to the high-fat diet, which then decreased the tumor development.[316] Recently, Ip et al. reported that factors other than prolactin alone may be responsible for the higher tumor incidence in rats fed dietary fat.[323] This was demonstrated by placing bilateral electrolytic lesions in the median eminence 10 days after DMBA administration for both rats fed low and high-fat diet which influenced prolactin levels. The tumor incidence in the low-fat group increased almost threefold but failed to do so in the high-fat group. The tumor incidence in the low-fat group was, however, lower than that in the high-fat group. It was found that prolactin has a selective proliferating effect on mammary epithelial cells which is a common characteristic of a tumor promoter.[324]

Although estrogens have no copromoting effect with high dietary fat on mammary tissues of the rats pretreated with DMBA,[316] this class of estrogens, nonetheless, does have an important role in mammary tumor development. By giving progesterone to the female rats 15 days after DMBA-initiated treatment, an increase in mammary tumor incidence and a decrease in latency were observed.[27] In addition, Huggins et al. induced pregnancy 15 days after initiation of DMBA, and the results were similar. In contrast, 17-β-estradiol reduced the tumor incidence and lengthened the latent period. Recently, Yoshida et al. tested the regimen of DMBA and progesterone in androgenized rats (accomplished by administration of testosterone), and they found that mammary tumors did not appear in the absence of ovaries.[325] The regimen, thus, is dependent on estrogens produced by the ovaries. Since these steroid hormones are mediated by specific steroid-binding components modulating intracellular events, it is thought that estrogen target tissue depends on the action of estrogen.[326]

Phorbol has also been demonstrated to be a successful promoter of DMBA-initiated mammary tumors in virgin female rats.[327] Hormonal factors may play a role as copromoters in this case.

In vivo methodology described above (using mammary glands for tumor induction) has been a good epithelial carcinogenesis model. Disadvantages in this system have previously been pointed out in the skin section of this review, as well as in the discussion on the need for in vitro system of epithelial cells. Reasonable success in developing primary mammary cell cultures have been worked out since 1958.[328–332] As seen in the in vivo studies, the most viable mammary epithelial cells would be from rats 50 to 60 days old, because these cells are maximally sensitive to chemical carcinogens.[58,334] Thus, these are the cells used in culture studies. Under appropriate culture conditions, these cells can be successfully transformed by chemical carcinogens. In addition, the cells need to be actively growing and synthetizing DNA; for primary mammary epithelial cell cultures, the peak period was days 2 and 3 and carcinogens were added to the media at that time.[333] As indicated in the in vivo studies, the concentrations of hormones, prolactin, and estrogen in the media are the important factors in aiding the tumor promotion of these cells in vitro.[318] A high ratio of prolactin/estogren must be used in order to observe the maximal cell proliferation in the presence of chemical carcinogens. In addition to these dispersed cell cultures, organ cultures of mammary tissues have been successfully employed.[335,336] Again, hormonal factors are important; with DMBA in the culture, the maximal cellular proliferation and DNA synthesis can occur in the presence of insulin, estrogen, prolactin and progesterone.[335] Progesterone used in combination with insulin induced lobular-alveolar development.

Unfortunately, there appears to be no literature available on the effects of chemical tumor promoters and cocarcinogens on mammary epithelial cells in vitro except for

Table 4
COMPARISON OF PROPERTIES OF CONTACT-SENSITIVE MOUSE EMBRYO CELL LINES[a]

Cell line	Morphology	Chromosome mode	Plating efficiency (%)	Generation time (hr)	Saturation density (60 mm dish)	Stability in tissue culture (days)
Swiss/3T3	Epitheloid	Hypotetraploid	30—50	18	1×10^6	not determined
BALB/c 3T3	Epitheloid	Hypotetraploid	30—50	22	$5—8 \times 10^5$	270
C3H/10T½	Epitheloid	Hypertetraploid	13—50	15.5	$6—8 \times 10^5$	450

[a] Adapted from Reference 64.

hormones. It is presumed, however, that the mechanism by which the promoters exert their effects on mammary tissues might be similar to that previously discussed for other organs.

X. CELL CULTURES

Throughout this review some major disadvantages of various organ cell cultures have been presented. Clear cut, two-stage carcinogenesis is difficult to define; transformation is often long, and many cell passages are needed to identify these potential tumor promoters. In addition, the phenomena that exists in the in vivo systems do not always parallel the morphological and biochemical events in the in vitro systems. With an increasing number of environmental factors confronting us, short-term in vitro assays, which are relatively inexpensive, are now more vitally needed than ever. Fibroblasts multiply rapidly, thus making it possible to perform quick in vitro assays of chemical agents. Of the fibroblasts studied, cell lines 3T3 and 10T½ have recently become important tools in chemical carcinogenesis. These cell lines are contact-sensitive mouse embryo fibroblasts. Table 4 presents comparative properties of these cell lines. The factor that makes this type of system very useful in the study of tumor promotion is the short response time.

The first workers to report the effects of a tumor-promoting compound on cells in culture were Sivak and Van Duuren. In 1967, they demonstrated that in a mixed cell population containing contact-inhibited Swiss/3T3 fibroblasts, and spontaneously or SV40-transformed cells showed enhancement in the presence of a purified fraction of croton oil.[59] Later investigations revealed that a good correlation existed between the relative potencies of various substances (including some agricultural chemicals and food additives in this culture system) and in two-stage carcinogenesis studies using the mouse skin model.[60,61,337] The characteristic marker that determines the potency of tumor promoters in this culture system is the development and observation of Type III-transformed foci, which can be recorded quantitatively and qualitatively. Several investigations have presented evidence that phorbol ester tumor promoters exert their action in cooperation with several cell membrane functions,[338-340] and some activities may be nuclear dependent.[340] Whereas DMBA has been found to be more carcinogenic than 3-MC in mouse skin, the reverse appears true for these 3T3 cells; moreover, TPA enhances the Type III-transformed foci in the presence of 3-MC.

Recently, Blumberg and his associates presented evidence that the effects on chicken embryo fibroblasts (CEF) in culture (transformed in the presence of phorbol ester tumor

promoting agents) were similar to those observed with 3T3 cell cultures.[341-344] As for 3T3 cell cultures, there is an excellent correlation between various phorbol esters in CEF and mouse skin assays.

In 1976, Mondal, Heidelberger, and others demonstrated the similarity between C3H-10T½-CL8 (10T½) cell lines and the classical in vivo initiation promotion model in the mouse skin system.[62,63] Using the cloned lines developed by Reznikoff,[64] and subcarcinogenic doses of polycyclic aromatic hydrocarbons, or low-UV irradiation, transformation was demonstrated in the presence of continuous treatment with TPA. Neither the carcinogen, UV irradiation, nor TPA at the given dose level, alone, gave rise to Type III transformed foci. Nesnow et al. recently tested other nonphorbol tumor promoters and found that 10T½ cells were transformed in the presence of these promoters active in other organs, i.e., phenobarbital and DDT in liver.[65] An interesting difference between 3T3 and 10T½ cell systems was made apparent recently when saccharin, a known tumor promoter in bladder epithelium,[284] enhanced transformation of 10T½ cells initiated with 3-MC,[345] but failed to do so in 3T3 cell system under the same conditions.[337] This shows that any agent shown to have tumor-promoting ability in one organ (tissue) does not necessarily have that property in another organ (tissue).

Before the work of two-stage carcinogenesis in 10T½ cells appeared in the literature, Lasne et al. reported in 1974 the decreased amount of time needed for the transformation of rat embryo fibroblasts by TPA after pretreatment with B(a)P.[346] In a later report, however, this group found that the cells were transformed in the presence of the carcinogen alone, TPA alone, a combination of the two and those untreated.[347] One must look into the time factor in this case because transformation occurred earliest in the cells receiving both initiator and tumor promoter.

The use of Syrian hamster embryo cells (SHE) in culture for tumor promotion studies has also been examined. In the presence of TPA, the induction of ODC activity was observed in both normal and chemically transformed SHE cultures.[348,349] When a series of phorbol esters (tested for a range of promoting activity by using mouse skin assays in vivo) was employed, only the known active ones were able to induce ODC activity. In addition, DNA synthesis was not enhanced in the presence of TPA in normal or transformed SHE cultures. Radioactivity measurements revealed that the metabolism of TPA resulted in the loss of its biological activity in culture.[348] The SHE culture system for tumor promotion studies is relatively new, and although these cultures possess an advantage because of their stable diploid karyotype similar to human cells, tumor promoters other than phorbol esters have not been tested in this system.

While the TPA treated SHE cultures induced ODC activity with no change in DNA activity, TPA-treated BALB/c 3T3 cultures induced both ODC and DNA activities.[349] Changes in ODC and DNA activities were not, however, observed in rat embryo fibroblasts cultures treated with TPA. This partly explains why TPA is not entirely responsible in forming transformed foci in rat embryo fibroblast cultures as Lasne et al. reported. Untreated cells eventually became transformed after many cell passages.[347]

In addition to the ODC and DNA activity alterations in SHE cultures as markers of tumor promotion, Barrett et al. recently demonstrated a promising study of transformation in these cultures.[70-75] Using B(a)P as the carcinogen, they found that morphological transformation gave rise to enhanced fibrinolytic activity, anchorage independent growth, growth in soft agar, decreased serum requirement for growth, decreased organization of intracellular actin, and increased cloning efficiency. These properties have been found to correlate well qualitatively and quantitatively with the degree of tumorigenicity.[73] In addition, the events occurred in a sequence with predictable numbers of population doublings.[74] Poiley et al. also reported that aryl hydrocarbon hydroxylase activity, which is demonstrated by the presence of epoxide hydrase (EH), was useful in predicting the transforming potential of SHE cultures

treated with various carcinogens and other chemical agents.[76] One of the chemicals used was sodium phenobarbital, a tumor promoter in hepatocarcinogenesis, which raised the EH levels over the controls. Recently, Rivedal and Sanner reported an increase in morphological transformation of SHE cultures in presence of both B(a)P and cigarette smoke condensates.[77] Cigarette smoke condensate has promoting ability in this instance, although it did not alter the cell morphology when given alone. The combined treatment increased the transformation to a greater degree than when B(a)P was given alone. Morphological transformation was defined as altered colony morphology consisting of crisscrossing and piling up of cells, which was not observed in control culture.

Another type of hamster cell culture uses Chinese V-79 hamster cells that were originally isolated from the lung of male Chinese hamsters.[350] These cell cultures have been employed to study metabolic cooperation of tumor promoters. Metabolic cooperation is another useful characteristic marker in carcinogenesis, and is a type of intracellular communication of cells where the mutant phenotype of enzyme-deficient cells is corrected by normal cells or by different mutant cells. Two types of markers have been observed; one requires cell-to-cell contact, whereas the other one does not. The former type was first illustrated by Shubak-Sharpe et al., who reported on the hypoxanthine-guanine phosphoribosyltransferase system.[351] Other investigators have subsequently reported on thymidine kinase,[352] Na^+- and K^+-activated adenosinetriphosphate,[353] and PA systems.[354] The second type, with no cell-to-cell contact system, is illustrated by different manifestations of the disease, mucopolysaccharidosis.[355,356] The cellular cooperation for the second type can be accomplished by means of a diffusable product. Metabolic cooperation has also been influenced by different chemical analogs,[357] cell lines,[358] immune response,[359] and growth control.[360]

Trosko and his associates have published a number of studies related to the lack of metabolic cooperation in V-79 cells resistant to 6-thioquanine (6-TG) and ouabain by TPA and other tumor promoters.[80-83,361,362] Their studies show that the frequency of mutants resistant to either 6-TG or ouabain increased when TPA was added to these cultures previously treated with UV light. Specifically, they stated that the maximum recovery of these mutants occurred when TPA was introduced after the mutation "expression" time, and after the completion of DNA repair caused by UV light. A hypothesis was proposed that states that initiation in two-stage carcinogenesis is due to a mutagenic effect, whereas the promotional phase is the result of an epigenetic process involving cyclic nucleotide modulation of gene expression.[80,362,363] Thus, carcinogens can be mutagens or gene modulators, which induce DNA lesions. These lesions become mutated (yet unexpressed) transformants, and the promoters depress (or repress) these transforming genes to the transformation stage by the epigenetic mechanism. Yotti et al. found that a good correlation existed between different phorbol esters and the degree of recovery of the 6-TG mutants in mixed cultures containing 8×10^6 6-TG-sensitive cells and 100 6-TG-resistant cells.[81] In addition, phenobarbital, anthralin, BHT, DDT, and saccharin have blocked the metabolic cooperation in the V-79 cell systems.[83,363] Likewise, flucinolone acetonide, a potent inhibitor of skin tumor promotion, was demonstrated to counteract the TPA-blocked metabolic cooperation.[81] This is an interesting observation in that tumor promoters are not "tissue" specific in the V-79 system, and these tissue-specific promoters generally can be tested by this model before trying other animal tissue systems.

Since differentiation, both morphologically and biochemically, was found to be affected by TPA in mouse skins,[41,364] it was suggested that the effects on terminal differentiation in vitro may be part of the mechanism of tumor promotion. The observation that tumor promoters inhibited terminal differentiation of cells in culture was made first by Cohen et al. They demonstrated that TPA inhibited the formation of myoblasts synthetizing muscle-specific myosin, as well as fusion of definitive myoblasts

into myotubes in chick muscle cultures.[365] Subsequently, other reports indicated that TPA inhibited adipocyte formation in 3T3 cells,[366,367] erythroid differentiation in Friend erythroleukemia (FEL) cells[84,85] chondrogenesis in chick chondroblasts,[368] neurite induction in neuroblastoma cells,[369] and melanogenesis in melanoma cells.[370]

Under appropriate culture conditions, BALB/c 3T3 cells[366] and Swiss 3T3 cells[371,372] differentiate into mature adipocytes, and under confluent monolayered situations they eventually have many of the biochemical characteristics of adipocytes seen in the in vivo models.[366,373] TPA in the media prevents these 3T3 cells from differentiating into adipocytes, and the process can be reversed upon removal of TPA from the culture. Spontaneous differentiation can occur even when the media containing fresh TPA is frequently changed. O'Brien et al. reported that the presence of TPA in 3T3 cultures markedly increased lactate production.[373] This marker, therefore, may suggest the importance of TPA's interference with glucose metabolism which, in turn, affects triglyceride accumulation in these cells.

Because of many similarities between 3T3 and 10T½ cell systems, Mondal and Heidelberger investigated the effects of TPA on differentiation of 10T½ cells.[374] They found that the active phorbol ester tumor promoters inhibited the 5-azacytidine-induced differentiation of 10T½ cells. These 10T½ cells normally differentiate into muscle cells. They also reported a good correlation between other phorbol esters in these cells and their skin tumor promoting activities. Tumor promoters of other organ systems (mezerein, anthralin, and Tween 60) were tested and demonstrated to inhibit the 5-azacytidine-induced differentiation of 10T½ cells as well. Phenobarbital failed to inhibit the differentiation. This model system therefore has serious limitations for testing different classes of tumor promoters in terms of differentiation. In addition, it is not known if spontaneous differentiation of 10T½ cells in the presence of TPA can occur as it has in 3T3 cells.

In contrast to spontaneous differentiation seen in 3T3 cells in the presence of TPA,[366,367] FEL cultures do not overcome the TPA-inhibited differentiation.[375,376] A positive correlation exists between the tumor promoting activity of phorbol esters seen in mouse skin and the ability of the same agents to inhibit the spontaneous differentiation of these cells.[376] In the absence of the inducing agents (TPA, etc.), the differentiation is characterized by a high percentage, 40 to 70% benzidine-positive in one clone of FEL cells.[375] After removal of TPA for a long time, the cells retain the high frequency of spontaneous differentiation whereas the control FEL cells revert to a low percentage of differentiation. In other clones of FEL cells with low spontaneous differentiation, the tumor promoters can inhibit the differentiation according to the types of agents and clones used.[84,85,377] The characteristic markers seen in TPA-inhibited FEL cell differentiation are as follows: accumulation of globin mRNA and synthesis of globin chains (TPA may restrict expression of hemoglobin);[375] suppressed expression by the synthesis of spectrin, heme synthesizing enzymes and heme;[86] and inhibition of TPA-induced differentiation, possibly nullified by addition of end products of the polyamine-biosynthetic enzymes (spermine, spermidine, and to a lesser extent putrescine).[378] It is important to stress that the right clone of FEL cells must be used; in one clone, differentiation is inhibited more than 90% by TPA, whereas in another it is not inhibited even in the presence of high concentrations of TPA.[376]

Although some reports show the inhibition of terminal differentiation in cultures treated with TPA, other reports show TPA-stimulated differentiation in other cells. These include Rauscher virus-transformed murine erythroid cells,[379] human melanoma line,[380] and human promyelocytic leukemia cells (HL-60).[87-90,381] The last cell line mentioned, HL-60, was first established in culture by Collins et al.[382] These cells, in the presence of TPA and related phorbol diesters, differentiate into mature cells.[87-89] The

increase in differentiating cells was characterized by irreversible induction of adherence, cessation of DNA synthesis and cell division, and an increase in the percentage of myelocytes and metamyelocytes. These TPA treated cells also resembled macrophages,[88,89] and this differentiation is more similar to the monocytic than to the myeloid series. More importantly, in contrast to what is seen in FEL cultures, the differentiation of HL-60 in TPA-treated cultures (monocytic) differs from these normal differentiating cultures (myeloid formation).

Recent evidence indicates that the increased response of cells to TPA is associated with an increase in the proportion of cells with a membrane-bound enzyme, TPA-reduced nicotinamide adenine dinucleotide phosphate oxidase.[90] In addition, Mendelsohn et al. proposed (as in the skin system) that promoters act by binding to specific cellular receptors and several forms of receptors are present in the HL-60 cells. Moreover, Cabot et al. reported that TPA can alter the metabolism of phospholipids on HL-60 cell membranes, a property shown in other cell types.[381] A weak tumor-promoting phorbol diester (4-0-methyl-tetradecanoylphorbol-13-acetate) was used, which failed to elicit any activity on phospholipid metabolism. This indicates that a good correlation may exist between the tumor-promoting ability of various phorbol esters seen in mouse skin systems and the stimulation of differentiation in HL-60 cell lines. This cell system is limited to a very narrow range of tumor promoters; however, anthralin, phenobarbital, and saccharin do not affect them in any manner.[88] Furthermore, various inhibitors of tumorigenesis caused by the phorbol esters do not affect the HL-60 cell system.

In other cell culture studies, TPA stimulated DNA synthetic activity[383] and PA production in HeLa cells;[384] MDCK (canine kidney)-TPA-treated cells caused cellular proliferation and morphological alterations, as well as formation or increase of PA;[385] DNA synthesis and cellular proliferation were observed in rat thymic lymphoblasts;[386] and mouse peritoneal macrophages stimulated PA activity in the presence of TPA in culture.[387]

XI. PROMISING STATE-OF-THE-ART SYSTEMS

Tables 5 to 7 summarize the most feasible short-term animal systems both in vitro and in vivo to analyze and assess potential tumor promoters and cocarcinogens. Table 5 describes in vitro transformation studies, whereas Table 6 lists other in vitro screening parameters. It must be emphasized, however, that one cannot rely satisfactorily on only any one system for testing all agents. A potential tumor promoters in one system may have no promoting and/or cocarcinogenic activity in another system. It is important to employ the classical two-stage carcinogenesis model to supplement these animal cell culture systems.

The 3T3 and 10T½ cell culture systems are probably the best models to begin screening for possible tumor promoters and carcinogens. Phorbol diesters and other classes of tumor promoters have already been examined by the use of these systems. Since the quantitation of Type III foci in the 10T½ and 3T3 cell culture systems correlates with the oncogenic potential of these cells, the increase in Type III-transformed foci formation in the presence of tumor promoters is a very satisfactory criterion in assessing these agents. The criterion of inhibition of differentiation should not be used in both of these cell cultures.

Morphological transformation of SHE cells appears to be a promising, but not yet well delineated, model. It was demonstrated that in the presence of the carcinogen B(a)P, morphological transformation enhances fibrinolytic activity and an increase occurs in the anchorage independent growth sequence.[70–75] This last criterion is quite significant in that this property has been found in mouse epidermal cell cultures treated with

Table 5
RECOMMENDED IN VITRO TRANSFORMATION MODELS FOR SCREENING AND ASSESSING TUMOR PROMOTERS AND COCARCINOGENS

Cell System	Initiation	Promotion	Results
C3H/10T½	25 ergs/mm^2 UV light	0.1 μg/mℓ TPA 72 hr later[62,63]	Increase in number of Type III transformed foci
	0.25 μg/mℓ 3-MC, DMBA, or B(a)P	0.1 μg/mℓ TPA 5 days later	
	4 μ *M* B(a)P with promoters	Aroclor-1254> Phenobarbital> Benz(a)anthracene> Pregenelone-16-α-Carbonitrile> 5,6-Benzoflavone> trifluralin> Toxaphene[65]	
	0.1 μg/mℓ 3-MC for 24 hr	2 mg/mℓ saccharin (4 days after 3-MC treatment[345]	
3T3	SV-40 virus transformed cells in mixed cultures containing normal cells	1.0—5.0 μg/mℓ TPA[59,61]	Increase in number of type III transformed foci
		Dimethyldodecylamine acetate, and octanol/1-decanol[60]	
		Cigarette tar samples[61]	
	2 μg/mℓ 3-MC	0.1 μg/mℓ TPA[337]	
Hamster embryo cells	0.01 μg/mℓ B(a)P	Cigarette smoke condensates 1 and 5 μg/mℓ[77]	Criss-crossing and piling up of cells
Mouse epidermal cells (JB-6)	None or 1—2 μg/mℓ DMBA	TPA (0.01 μg/mℓ) mezerein (1.6×10^7 *M*) cigarette smoke condensate, (40 μg/mℓ) and detergents (1200 μg/mℓ) — 3 week treatment[68,139,140]	Promotion of anchorage independence (formation of new phenotypes) (change in surface glycoconjugate synthesis)
Mouse epidermal cells (SENCAR)	—	TPA[66,67]	Synthesis of new proteins that includes structural, secretory, and enzymatic functions in basal cells (preneoplastic changes are increased)
Human foreskin cells	Various carcinogens including MNNG, Aflatoxin B_1, 4-nitroquinoline-l-oxide, etc., at various concentrations with promoters	1 μg/mℓ of 17-β-estradiol, anthralin or TPA in media (10 hr after cell plating)[388,389]	Morphologically altered colonies and increased cellular proliferation in soft agar and tumorigenicity when injected into mice

Table 6
OTHER RECOMMENDED IN VITRO MODELS FOR SCREENING AND ASSESSING TUMOR PROMOTERS AND CARCINOGENS

Cell System	Initiation	Promotion	Results
Chinese V-79 hamster cells (6-thioquanine sensitive and ouabain resistant)	200 ergs/mm^2 UV light or none	1 μg/mℓ TPA (2 days later) (and other active phorbol diesters)[81,82,361]	Recovery of 6-TG^r and the number of ouabain resistant mutants is increased
		Phenobarbital, anthralin, BHT, DDT, and saccharin[83]	
FEL cells	—	1.6×10^7 *M* and 1.6×10^5 *M* TPA[84-86]	Inhibition of differentiation into erythrocytes (reversible upon removal of TPA)
HL-60 cells	—	1.6×10^{-6} to 1.6×10^{-8} *M* TPA, phorbol-12,13-dideconoate, mezerein[87,90]	Differentiation into monocytic cells rather than myeloid cells and increase in number of monocytic cells also
Neonatal mouse epidermal cells	—	0.1 μg/mℓ TPA (1.6×10^{-7} *M*)[78,79]	Increase in ODC and DNA activities

TPA.[139,140] In addition, the shift response can be analogous to a later stage of mouse skin tumor promotion in vivo.[139] Another advantage of using SHE cultures is that their stable diploid karyotype cells are similar to human cells.[350]

Mouse epidermal cells show a strong, irreversible, promoter-dependent change in anchorage-independent growth, which is a very useful in vitro marker of neoplastic phenotype.[139,140] In addition to TPA, other classes of tumor promoters such as mezerein, cigarette smoke condensates (containing several polycyclic aromatic hydrocarbons), and detergents induce anchorage-independent growth. Colburn reported that specific changes (alterations in both 180K and 150K molecular weight glycoproteins) in promotion could induce phenotype in JB-6 cells.[69] These glycoproteins were decreased in the presence of promoting phorbol esters. In addition, retinoic acid, a known antitumor promoter, blocked these specific decreases. Gottesman and Yuspa also demonstrated that the synthesis of 35K glycoproteins increased threefold in mouse epidermal cells treated with TPA.[67] They further indicated that the preneoplastic changes commonly seen in these cultures were more rapidly induced in the presence of TPA.[66] An increase in glycoprotein synthesis per se was not sufficient, however, for promotion; evidence of this was that retinoic acid, lidocaine, and flucinolene acetonide failed to block the glycoprotein synthesis in the presence of TPA.[67]

Milo and his associates developed a new transformation method of human epithelial cell cultures from foreskins by aflatoxin B_1, MNNG, β-propiolactone and UV absorbance of 254 nm.[388] A preliminary report that appeared several years ago indicated cells from human foreskins were altered morphologically in the presence of TPA, anthralin, and various carcinogens, and that they were tumorigenic upon injection into

mice.[389] Thus, the employment of human epithelial cell cultures for transformation studies is definitely promising.

For other in vitro studies, the metabolic cooperation system in Chinese V-79 hamster cells is very useful, since several classes of tumor promoters have been successfully employed.[80–83] In addition, this model is evidently analogous to the classical two-stage skin carcinogenesis model in terms of a possible mutational event followed by an epigenetic change.[361,362]

Inhibition or stimulation of cell differentiation is useful only if spontaneous differentiation does not occur in cell cultures treated with tumor promoters. For studies on the inhibition of cellular differentiation, FEL cultures appear to be the best, whereas HL-60 cell lines are suitable for studies on the stimulation of cellular differentiation by tumor promoters. The HL-60 cell lines may be most practical to use, also, because they do not differentiate in the presence of promoters into myeloid cells, but into monocytic cells and most of them are macrophage-like.[88,89] This is a good indication that spontaneous differentiation does not occur in HL-60 cell cultures treated with tumor promoters. Upon removal of TPA from cultures, FEL cells can differentiate into normal cells. Because of the irreversibility of induction of cell adherence and cessation of DNA synthesis, HL-60 cells can continue to differentiate into monocytic cells.[88,89] This cell system is currently limited to phorbol esters, however.

The cell culture systems discussed thus far have shown good correlation between the effects of various phorbol diesters in these cultures and tumor promotion in the mouse skin system. With this in mind, there is one practical biochemical study that should be employed in mouse epidermal cells in vitro. Since it was reported recently that the ODC induction and the appearance of dark cells are two of the most useful criteria in mouse skin tumor promotion in vivo,[9,10] it appears quite logical to employ ODC and possibly dark cell induction (not yet studied) techniques in future epidermal cell culture investigations.

Although the in vitro models discussed thus far can be successfully employed for screening of tumor-promoting agents, a few in vivo models should be considered to confirm the potential of these agents further. In vitro cell culture methods lack the involvement of other cells in the body for tumor promotion. Perfect tissue culture conditions exactly analogous to the same type of cells in vivo do not exist at this time. Table 7 shows three particularly important in vivo systems: mouse skin, mouse lung, and rat liver. These in vivo methods have been selected because they are rapid and easy to perform. The appearance of the first skin papillomas caused by tumor promoters can take several weeks. The test of various agents in the hepatic system as illustrated in Table 7 is a new method, which was very recently reported.[390–392] Normally, it takes months for hepatic tumors to be formed in the presence of hepatopromoters, but this new system indicates the increase in preneoplastic lesions a few weeks after exposure to these agents and an initiating dose of 2-AAF. In other words, the appearance of these lesions occurs earlier than if the rats were treated with the hepatocarcinogens alone. Ito et al. further concluded that the hepatopromoters are thought to be organ-specific.

Slaga et al. recently stressed that the ODC induction and appearance of the dark basal keratinocytes are among the best biochemical markers in two-stage skin carcinogenesis since other hyperplastic, stimulatory agents had failed to alter these two criteria.[9,10] Therefore, one may determine true skin tumor promoter and/or cocarcinogenic activity this way along with other markers as previously discussed.

As methods become more well defined and developed, the following in vitro methods could be useful in the future: liver cells (hepatocytes and epithelial), lung cells (both epithelial and aveolar), bladder epithelial cells, and colon epithelial cells. Also, organ explant (tracheal, skin, esophagus, bladder, and colon) methodology could be useful in

Table 7
RECOMMENDED SHORT-TERM IN VIVO METHODS FOR SCREENING AND ASSESSING TUMOR PROMOTERS AND CARCINOGENS

Animal model	Initiation	Promotion	Results
Mouse skin	Various, including DMBA, B(a)P and 3-MC (one initiating topical dose)	Various promoters including active phorbol diesters (TPA, etc.) (2—3 times per week, 1 week after initiation); mezerein with TPA; anthralin and other phenols; cigarette smoke condensates; limonene and citrus oils; flame retardants, etc. (Several reviews including Refs. 8, 9, 11, 19)	Increase in number of skin papillomas
Mouse lung	Intraperitoneal injection of 1 mg/g body weight urethan	Weekly injection of 300 mg/kg BHT[55]	Increase in number of tumors in lungs
Fischer F344 rat liver	2-AAF	Phenobarbital, 3-MC, and polychlorinated biphenyls (dose response relationships)[390–392]	Appearance of preneoplastic liver hyperplastic lesions earlier than when initiator given alone. Also an increase in liver microsomal enzyme levels
Mouse skin	DMBA, B(a)P, etc.	TPA, Mezerein, etc.[9,10]	Induction of ODC activity and appearance of dark basal keratinocytes

the assessment of tumor promoters. Transformation of rat embryo fibroblasts is a good model, but spontaneous transformation occurs; perhaps different culture conditions need to be investigated. More importantly, the time needed for the first change to occur in biochemistry and/or morphology of these cells treated with tumor promoters needs to be determined.

XII. CONCLUSIONS

From the literature surveyed in this review, it can be concluded that each "established" tumor promoter or cocarcinogen of one specific organ does not necessarily exhibit the same biochemical and morphological events and consequences in another specific tissue. Therefore, to test such an agent will definitely require the testing of several organs, in addition to different animal species and different strains. Moreover, potential tumor-promoting agents seen in animals may not be potentially dangerous in humans and vice versa. Furthermore, epidemiological findings should be considered when investigating a particular agent; e.g., the agent may be hazardous to skin but may have no effect on lungs.

By using either cell cultures or organ cultures, the conditions under which transformation occurs can be well controlled. Bioassays are simplified without

consideration of other cell types being involved; yet, this should not be ignored entirely as it is well known that the biochemical consequences of one cell type system can influence another. For example, in the skin system, the dermal layer may play an important role in carcinogenesis if affected by dangerous chemicals. More importantly, one should investigate the best analogy between in vitro and in vivo systems of the same organ. This has been difficult to accomplish for many years but recently has been investigated carefully. Under appropriate culture conditions, in vitro methodology can be used successfully in the screening of tumor promoters as well as cocarcinogens.

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