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Studies on the Mechanisms Involved in Multistage Carcinogenesis in Mouse Skin

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Skin tumors can be effectively induced in mice by the repetitive application of a carcinogen. The relative order of sensitivity to complete carcinogenesis is Sencar > CD-1 > C57BL/6 ≥ BALB/c ≥ ICR/Ha Swiss > C3H. Skin tumors in mice can also be induced by the sequential application of a subthreshold dose of a carcinogen (initiation phase) followed by repetitive treatment with a weak or noncarcinogenic tumor promoter (promotion phase). The relative order of sensitivity to initiation-promotion is Sencar>> CD-1 > ICR/Ha Swiss ≥ Balb/c > C57BL/6 ≥C3H ≥DBA/2. The initiation phase requires only a single application of a carcinogen and is essentially an irreversible step, which probably involves a somatic cell mutation as is evidenced by a good correlation between the carcinogenicity of many chemical carcinogens and their mutagenic activities; the promotion stage, however, is initially reversible, later becoming irreversible. For strains and stocks of mice which respond to initiation-promotion, there is a good correlation between the tumor-initiating activities of polycyclic aromatic hydrocarbons (PAH) and their abilities to bind covalently to DNA. Potent inhibitors and stimulators of PAH tumor initiation appear to effect the level of the PAH diol epoxide bound to specific DNA adducts. However, when the binding of a given PAH to DNA is compared in various stocks and strains of mice, there is no correlation, since in those mice which are able to metabolize PAH, the amounts of carcinogen bound to DNA are similar.

The phorbol ester tumor promoters have been shown to have several cellular and biochemical effects on the skin. Of all the observed phorbol ester related effects on the skin, the induction of epidermal cell proliferation, polyamines, prostagladins, and dark basal keratinocytes as well as other embryonic conditions appear to correlate the best with promotion. Mezerein, a

Abbreviations used: PAH, polycyclic aromatic hydrocarbon; BA, benz(a)anthracene; DB(a,c)A, dibenz(a,c)anthracene; BP-diolepoxide; benzo(a)pyrene 7,8-dihydrodiol-9,10-epoxide; BA-diolepoxide, BA-3,4-dihydrodiol-1,2-epoxide; DMBA, 7,12-dimethylbenz(a)anthracene; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PCB, polychlorobiphenyls; Poly I:C, polyinosinic:polycytidylic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; ODC, ornithine decarboxylase; FA, fluocinolone acetonide; DMSO, dimethyl sulfoxide; BCG, Bacillus Calmette-Guerin; DFMO, α-difluoromethylornithine; IBMX, isobutylmethylxanthine; αMO, α-methylornithine; ETYA, 5,8,11-14-eicosatetraynoic acid; EPP, ethylphenylpropiolate; TPCK, tosyl phenylalanine chloromethylketone; RA, retinoic acid.

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weak promoter, was found to induce many cellular and biochemical changes similar to 12-O-tetradecanoylphorbol-13 acetate (TPA), especially epidermal hyperplasia and polyamines; however, it was not a potent inducer of dark cells. We recently found that promotion could be divided into at least two stages. The first stage (I) can be accomplished by limited treatment with TPA or the nonpromoting agents, 4-O-methyl TPA and the calcium ionophore A23187, and the second stage (II) by repetitive applications of mezerein. The dark basal cells appear to be important in the first stage of promotion, since TPA, 4-0-methyl TPA, and A23187 are potent inducers of dark cells. Fluocinolone acetonide (FA) was found to be a potent inhibitor of stage I and II. Retinoic acid (RA) was ineffective in Stage I but was a potent inhibitor of Stage II promotion, whereas tosyl phenylalanine chloromethylketone (TPCK) specifically inhibited Stage I. In addition, FA and TPCK effectively counteracted the appearance of dark basal keratinocytes but had very little effect on polyamines, whereas RA had no effect on dark cells but is a potent inhibitor of TPA-induced ornithine decarboxylase activity and subsequent putrescine formation. These results provide additional evidence for the importance of dark basal keratinocytes (primitive stem cells) in Stage I of promotion and indicate that most of the other cellular and biochemical responses normally associated with promotion (such as polyamines) are actually associated with Stage II of promotion.

Although C57BL/6 mice are relatively resistant to initiation-promotion by PAH initiation and phorbol ester promotion, they are fairly sensitive to complete carcinogenesis by PAH. This suggests that the C57BL/6 mice are resistant to phorbol ester tumor promotion. Preliminary experiments suggest that C57BL/6 and Sencar mice respond qualitatively but not quantitatively to a single treatment with TPA.

Key words: carcinogenesis, DNA alkylation, DNA repair, O^6-methylguanine, nitrosamines

This report is not intended to be an exhaustive review of skin carcinogenesis but only to be a summary of some important data which relates to the multistage nature of skin carcinogenesis. Topical application of some chemical carcinogens will induce skin tumors on mice. In general, most chemical carcinogens have to be given repetitively in order to induce a large number of tumors (complete carcinogenesis). Alternatively, skin tumors can be induced by the sequential application of single subthreshold dose of a carcinogen (initiation phase) followed by the repetitive treatment with a noncarcinogenic promoter (promotion phase). This second procedure employing initiation and promotion is referred to as two-stage carcinogenesis which has been extensively reviewed [1,2].

The primary aims of this report on skin carcinogenesis are to (1) provide evidence that an important aspect of tumor initiation by polycyclic aromatic hydrocarbons (PAH) is their metabolism to an electrophilic intermediate(s) which covalently interacts with epidermal DNA; (2) provide evidence for the multistage nature of skin tumor promotion; (3) correlate promotion associated morphological and biochemical responses with specific stages of promotion; and (4) compare data on complete and two-stage carcinogenesis in various stocks and strains of mice in order to determine if tumor initiation and/or promotion is responsible for their varying sensitivities to skin cancer induction.

TUMOR INITIATION

Whenever a known skin carcinogen has been appropriately tested, it has shown skin tumor initiating activity [2–16]. In a two-stage mouse skin system,

initiation is the only stage that requires the presence of the carcinogen, and the measured carcinogenic potency of a chemical reflects its capacity for tumor initiation. There is both a good qualitative and a good quantitative correlation between the complete carcinogenic and tumor initiating activities of several chemical carcinogens in mouse skin [17]. This is true when one considers the number of papillomas per mouse at early times (10 to 20 weeks) or the final carcinoma incidence after tumor initiation [17].

It is possible that a carcinogen lacking promoting ability would not be detected when tested as a complete carcinogen. In this regard, we have found a number of chemical compounds such as benz(a)anthracene (BA), dibenz(a,c)anthracene (DB(a,c)A), chrysene, urethan, benzo(a)pyrene 7,8-dihydrodiol-9,10-epoxide (BP-diol-epoxide), and BA-3,4-dihydrodiol-1,2-epoxide (BA-diol-epoxide) that have tumor initiating activity but either lack or have very weak complete carcinogenic activity [4,9–11,14].

There is a good dose–response relationship of many carcinogens used as tumor initiators in the two-stage carcinogenesis system using Sencar mice. This is illustrated in Table I. A good dose–response relationship exists for 7,12-dimethylbenz(a)anthracene (DMBA) and BP to initiate skin tumors in Sencar mice. As can be seen a good correlation exists between the number of papillomas per mouse at 15 weeks and the final carcinoma incidence at 50 weeks. The percentage of mice with papillomas has also a reasonable correlation but the dose–response is very narrow. The Sencar mouse was derived from crossing Charles River CD-1 mice with skin tumor sensitive mice (originally derived from Rockland mice) and selecting for sensitivity to DMBA-phorbol ester tumor promoter two-stage carcinogenesis for eight generations starting with the F_1 cross [2]. The mice developing the earliest and most papillomas after initiation-promotion treatment were selected for each breeding. The Sencar mice are between 10 and 20 times more sensitive to DMBA tumor initiation than the CD-1 mice, whereas the Sencar mice are only between three and five times more

TABLE I. Dose–Response Studies on the Ability of DMBA and BP to Initiate Skin Tumors in SENCAR Mice*

Initiator	Dose (nmol)	Papillomas per mouse at 15 weeks (No.)	Mice with papillomas at 15 weeks (%)	Mice with carcinomas at 50 weeks (%)
DMBA	100	22	100	100
DMBA	10	6.8	100	40
DMBA	1	3.2	93	22
DMBA	0.1	0.5	20	5
BP	200	7.5	100	55
BP	100	3.2	78	30
BP	50	1.4	60	18

*See [17]. The mice were treated 1 week after initiation with twice weekly applications of 5 μg of TPA.

sensitive to BP tumor initiation than the CD-1 mice [18]. In addition, the Sencar mice are two to three times more sensitive to TPA promotion than the CD-1 [18].

The tumor initiation phase appears to be an irreversible step which probably involves a somatic cell mutation as evidenced by a good correlation between the carcinogenicity of many chemical carcinogens and their mutagenic activities [19,20]. Most tumor initiating agents either generate or are metabolically converted to electrophilic reactants, which bind covalently to cellular DNA and other macromolecules [21]. Previous studies have demonstrated a good correlation between the carcinogenicity of several polycyclic aromatic hydrocarbons (PAHs) and their ability to bind covalently to DNA [21-23]. Table II summarizes our data which show the strong correlation between the covalent binding of PAH to DNA and their tumor initiating activities.

In order to help us better understand the mechanism of PAH carcinogenesis, we have been studying many compounds with the capacity to inhibit PAH tumor initiation. Table III summarizes various potent inhibitors of skin tumor initiation in mice. In most of our studies we have used PAH

TABLE II. Correlation of Polycyclic Aromatic Hydrocarbons' (PAHs) Abilities to Bind Covalently to Epidermal DNA With Their Tumor Initiating Activities*

PAHs	Relative ability to covalently bind to epidermal DNA[a]	Relative tumor initiating activity[b]
DMBA	10.0	10.0
MC	6.5	6.0
BP	3.3	2.0
DB(a,h)A	1.7	1.5
DB(a,c)A	0.8	0.2

*DMBA was given a value of 10 since it gave the maximum response in binding and to initiate tumors in a two-stage system of tumorigenesis. All the other PAHs are expressed as values relative to DMBA's response.

[a]The relative abilities of various PAHs to bind covalently to epidermal DNA are based on dose-response binding studies. See [7,17,23,29] for details of actual binding levels.

[b]The relative tumor initiating activities are based on dose-response studies in Charles River CD-1 mice. See [7,8,29] for details.

TABLE III. Inhibitors of Tumor Initiation

Inhibitors	References
1. Antioxidants: butylated hydroxytoluene (BHT), butlylated hydroxyanisole (BHA), and selenium	[26,27]
2. Flavones: 7,8-benzoflavone, 5,6-benzoflavone,and quercetin	[7,24,25,28]
3. Vitamins: A, C, and E	[26]
4. Certain noncarcinogenic polycyclic aromatic hydrocarbons: dibenz(a,c)anthracene, benz(a)anthracene, benzo(a)pyrene, and pyrene	[29-31]
5. Environmental contaminants: 2,3,7,8-tetrachlorodibenzo-p-dioxin (RCDD) and polychlorobiphenyls (PCB)	[32-34]
6. Sulfur mustard	[35]
7. Polyriboinosinic-polyribocytidylic acid (Poly I:C)	[36]
8. Anti-inflammatory steroid	[37]

carcinogens which must be metabolized by the mixed-function oxidases to active form(s) before they are carcinogenic. Some of the flavones and antioxidants appear to inhibit carcinogenesis by inhibiting the metabolism of the carcinogen to its ultimate carcinogenic form [7,24–27]. 5,6-Benzoflavone and quercetin have been found to be inhibitory to skin, lung, and mammary carcinogenesis whereas 7,8-benzoflavone inhibits skin carcinogenesis by some PAHs and enhances carcinogenesis by others [7,27,28]. The antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are widely used as food preservatives and have been shown to inhibit skin, lung, mammary, forestomach, colon, and liver cancer in experimental animals induced by a wide range of chemicals [27]. Similar inhibitory results have been noted for selenium and vitamins C and E [27]. The noncarcinogenic PAHs and the environmental contaminants appear to inhibit skin carcinogenesis by inducing the metabolism of the carcinogen to detoxified products, thereby decreasing the binding of the PAH to DNA [29–33]. This is epitomized by the environmental contaminants 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polychlorobiphenyls (PCB) which are extremely potent inducers of PAH carcinogen metabolism and potent inhibitors of their carcinogenic effect [32–34]. Although TCDD is one of the most toxic agents known, its inhibitory effect on PAH carcinogenesis is at nontoxic dose levels.

Sulfur mustard inhibits tumor initiation by actually killing the initiated cells [35]. The polyinosinic:polycytidylic acid (Poly I:C) and the anti-inflammatory steroids appear to inhibit tumor initiation by slowing down carcinogen metabolism by their anti-growth effect [36,37]. Some of the agents listed in Table III have been shown to inhibit carcinogenesis in a number of tissues and by a variety of chemical carcinogens indicating they may be useful agents in the chemoprevention of cancer in man [28]. In general, the inhibitors of skin tumor initiation shown in Table III inhibit by either (1) alteration of the metabolism of the carcinogen (decreased activation and/or increased detoxification); (2) scavenging of active molecular species of carcinogens to prevent their reaching the critical target site(s) in the cells; or (3) competitive inhibition. In all cases this leads to a decrease in covalent binding to critical targets such as DNA. Table IV reveals a good correlation between the ability of a number of compounds to inhibit tumorigenesis and their ability to inhibit the binding of the PAH to DNA.

TUMOR PROMOTION

Although the phorbol esters are the most potent of the mouse skin tumor promoters, a wide variety of other compounds have been shown to have skin tumor promoting activity, as shown in Table V. After the phorbol esters and dihydroteleocidin B, anthralin is the most potent tumor promoter known of the compounds listed in Table V. Van Duuren and coworkers have reported fairly extensive structure-activity study with anthralin and derivatives [38]. Likewise, Boutwell and co-workers [39] have reported a structure-activity study of a number of phenolic compounds which are as weak promoters in comparison to the phorbol esters and anthralin. Although several of the other compounds shown in Table V have moderate to weak activity as tumor promoters, there have not been any extensive structure-activity studies performed.

TABLE IV. Correlation of Various Compounds to Inhibit Tumor Initiation by DMBA With Their Abilities to Inhibit Covalent Binding of DMBA to Epidermal DNA*

Inhibitors	Relative ability to inhibit DMBA tumor initiation by at least 50%	Relative ability to inhibit DMBA binding to DNA by at least 50%
TCDD	100.0	100.0
DB(a,c)A	10.0	15.0
7,8-BF	5.0	8.0
B(e)P	5.0	3.0
BHA	0.2	0.1
BHT	0.1	0.1
Vitamin C	0.1	0.1

*TCDD was given a value of 100 since it gave the greatest inhibition of tumor initiation and DMBA binding to epidermal DNA. For example, TCDD at a 1-μg dose level almost completely inhibited DMBA tumorigenesis and DMBA binding to DNA. All the other compounds are expressed as values relative to TCDD's response. For example, BHA at a 1000-μg dose level inhibited DMBA tumor initiation and binding by at least 50%. See [24–26, 29–34] for details.

TABLE V. Skin Tumor Promoters

Promoters	Potency	References
Croton oil	Strong	[2]
Certain phorbol esters found in croton oil	Strong	[1,2,5,51]
Some synthetic phorbol esters	Strong	[1,51]
Certain euphorbia latices	Strong	[1]
Anthralin	Moderate	[38]
Certain fatty acids and fatty acid mehtyl esters	Weak	[42]
Certain long chain alkanes	Weak	[38]
A number of phenolic compounds	Weak	[39]
Surface active agents (sodium lauryl sulfate, Tween 60)	Weak	[2,43]
Citrus oils	Weak	[44]
Extracts of unburned tobacco	Moderate	[45]
Tobacco smoke condensate	Moderate	[46]
Iodoacetic acid	Weak	[47]
1-Fluoro-2,4-dinitrobenzene	Moderate	[48]
Benzo(e)pyrene	Moderate	[31]
Benzoyl peroxide	Moderate	[40]
7-Bromomethylbenz(a)anthracene	Strong	[41]
Dihydroteleocidin B	Strong[a]	–

[a]Dihydroteleocidin B has promoting activity at doses similar to TPA (Slaga and Sugimura, unpublished data).

The dose-response ability of 12-O-tetradecanoylphorbol-13-acetate (TPA) to promote tumors after DMBA initiation is shown in Table VI. As was the case for tumor initiation, there is also a very good dose-response relationship for tumor promotion when considering either the number of papillomas per mouse at 15 weeks or the percentage of mice with squamous cell carcinomas at 50 weeks. Similar results have also been reported using SENCAR mice [49], Charles River CD-1 mice [50] or ICR/Ha Swiss mice [51,52].

In addition to causing inflammation and epidermal hyperplasia, the phorbol ester and other tumor promoters produce several other morphological and biochemical changes in skin as listed in Table VII. Of the observed phorbol ester related effects on the skin, the induction of epidermal cell proliferation, ornithine decarboxylase (ODC), and dark basal keratinocytes have the best correlation with promoting activity [53–58]. In addition to the induction of dark cells, which are normally present in large numbers in embryonic skin, there are many other embryonic conditions which appear in adult skin after treatment with tumor promoters (Table VII).

It is difficult to determine which of the many effects associated with phorbol ester tumor promotion are, in fact, essential components of the promotion pro-

TABLE VI. Dose-Response Studies on the Ability of TPA to Promote Tumors After DMBA Initiation*

Promoter	Dose (μg)	Time to first papilloma (wk)	Papillomas per mouse at 15 weeks	Papillomas at 15 weeks	Carcinomas at 50 weeks
TPA	10	8	3.0	100	32
TPA	5	6	7.2	100	46
TPA	2	7	6.5	100	45
TPA	1	8	3.6	80	25
TPA	0.1	11	0.4	5	8

*See [82] for details. The mice were initiated with 10 nmol of DMBA and promoted 1 week later with twice weekly applications of various dose levels of TPA.

TABLE VII. Morphological and Biochemical Responses of Mouse Skin to Phorbol Ester and Other Tumor Promoters

Responses	References
Induction of inflammation and hyperplasia[a]	[2,5]
Increase in DNA, RNA, and protein synthesis	[63]
An initial increase in keratinization followed by a decrease	[55–57]
Increase in phospholipid synthesis	[64]
Increase in prostaglandin synthesis	[65]
Increase in histone synthesis and phosphorylation	[66,67]
Increase in ornithine decarboxylase activity followed by increase in polyamines[a]	[53]
Decrease in the isoproterenol stimulation of cAMP	[68]
Decrease in the number of dexamethasone receptors[b]	–
Induction of embryonic state in adult skin[a]	[62]
1. Induction of dark cells (primitive stem cells)	[55–58]
2. Induction of embryonic proteins in adult skin	[69]
3. Induction of morphological changes in adult skin resembling papillomas, carcionmas, and embryonic skin	[55–58]
4. Decrease in histidase activity	[70]
5. Increase in protease activity	[71]
6. Decrease response of G1 chalone in adult skin	[72]
7. Increase in cAMP independent protein kinase in adult skin resembling tumors and embryonic skin	[73]

[a]Events which appear to show a reasonable correlation with promotion.
[b]Davidson and Slaga, submitted for publication.

cess. A good correlation appears to exist between promotion and epidermal hyperplasia when induced by phorbol esters [54]. However, other agents that induce epidermal cell proliferation do not necessarily promote carcinogenesis [59]. O'Brien et al [53] have reported an excellent correlation between the tumor promoting ability of various compounds (phorbol esters as well as nonphorbol ester compounds) and their ability to induce ODC activity in mouse skin. However, mezerein, a diterpene similar to TPA but with weak promoting activity, was found to induce ODC to levels that were comparable to those induced by TPA [60]. Raick found that phorbol ester tumor promoters induced the appearance of "dark basal cells" in the epidermis, whereas ethylphenylpropiolate (EPP), a non-promoting epidermal hyperplasic agent, did not [55–57,61]. Wounding induced a few dark cells which seemed to correlate with its ability to be a weak promoter [55–57]. In addition, a large number of these dark cells are found in papillomas and carcinomas [56,57]. Slaga et al [58,62] reported that TPA induced about three to five times the number of dark cells as mezerein which was the first major difference found between these compounds.

Inhibitors and Modifiers of Tumor Promotion

Various modifiers of the tumor promotion process have been very useful in our understanding of the mechanism(s) of tumor promotion. Table VIII lists the potent inhibitors of mouse skin tumor promotion by TPA. The anti-inflammatory steroid fluocinolone acetonide (FA) was an extremely potent inhibitor of phorbol ester tumor promotion in mouse skin [74]. Repeated applications of as little as

TABLE VIII. Inhibitors of Phorbol Ester Skin Tumor Promotion

Inhibitors	References
1. Anti-inflammatory steroids: cortisol, dexamethasone, and fluocionolone acetonide (FA)	[74]
2. Vitamin A derivatives	[75]
3. Combination of retinoids and anti-inflammatory agents	[76]
4. Protease inhibitors: Tosyl lysine chloromethyl ketone, (TLCK); tosyl arginine methyl ester, (TAME); tosyl phenylalanine chloromethyl ketone (TPCK); antipain and leupeptin	[78]
5. Cyclic nucleotides	[78]
6. Phosphodiesterase inhibitors; isobutylmethylxanthine (IBMX)[a]	
7. Dimethyl sulfoxide (DMSO)[b]	
8. Butyrate, acetic acid	[78]
9. Bacillus Calmette-Guerin (BCG)	[79]
10. Polyriboinosinic: polyribocytidylic acid (Poly I:C	[80]
11. Prostaglandin synthesis inhibitors 5,8,11,14-eicosatetraynoic acid (ETYA) and RO-22-3582	[81]
12. Arachidonic acid	[81]
13. Polyamine synthesis inhibitor difluoromethylornithine (DFMO)	[82]
14. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)[c]	

[a]IBMX at a dose of greater than 100 μg is a potent inhibitor of TPA promotion (Slaga and Weeks, submitted for publication).
[b]TPA has very weak promoting activity when applied in DMSO as the solvent, unpublished data.
[c]BHT and BHA at doses greater than 1 mg are potent inhibitors of TPA and benzoyl peroxide promotion, unpublished data.

0.01 μg almost completely counteracted skin tumorigenesis. FA also effectively counteracts the induced cellular proliferation associated with application of phorbol ester tumor promoters. Certain retinoids are also potent inhibitors of mouse skin tumor promotion [75]. In addition, Sporn and co-workers have found that retinoids are potent inhibitors of lung, mammary, bladder, and colon carcinogenesis [76]. Verma and co-workers [75] have shown that the retinoids that inhibit skin tumor promotion are potent inhibitors of phorbol ester induced epidermal ODC activity. We have recently found that a combination of FA and retinoids produces an inhibitory effect on skin tumor promotion greater than that produced by each separately [77].

The work of Belman and Troll also indicates that protease inhibitors, cyclic nucleotides, dimethyl sulfoxide (DMSO), and butyrate also inhibit mouse skin tumor promotion by phorbol esters [78]. In addition to butyric acid, acetic acid also inhibits tumor promotion [59,78]. The phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) was also found to inhibit tumor promotion which gives further support to the inhibitory effect of cyclic nucleotides (Slaga and Weeks, unpublished results). Schinitsky and co-workers [79] reported the inhibitory effect of Bacillus Calmette-Guerin (BCG) vaccination on skin tumor promotion. It has been shown that Poly I:C has an inhibitory effect on carcinogenesis and tumor promotion [80]. This appears to be mediated by its inhibition of promoter and carcinogen induced cell proliferation [80]. Certain prostaglandin synthesis inhibitors also inhibit skin tumor promotion which suggests that prostaglandins may be important in tumor promotion [81]. Although the mechanism is not presently understood, arachidonic acid at high doses is a potent inhibitor of tumor promotion [81]. α-Difloruomethylornithine (DFMO), an inhibitor of polyamine synthesis also inhibits tumor promotion which suggests that polyamines are also important [82]. Although both BHA and BHT are potent inhibitors of skin tumor promotion, their mechanism of action is currently not known (T.J. Slaga, unpublished results). It is possible that free radicals are important in tumor promotion and thus these agents may prevent promotion by their free radical scavenging ability.

Table IX lists a number of compounds that we have tested as modifiers of tumor promotion. Most of these compounds were examined because of their effect on either cellular polyamines, prostaglandins, or cyclic nucleotide levels. Although DFMO, an irreversible inhibitor of ODC inhibited tumor promotion, α-methylornithine (αMO), a reversible inhibitor either had no effect or a slight stimulatory effect [82]. Putrescine, spermidine, and spermine were found to be inactive as tumor promoters, but putrescine consistently was found to enhance TPA promotion, whereas spermine inhibited TPA promotion [82]. The cyclooygenase inhibitors, indomethacin and flurbiprofen, increased TPA tumor promotion [83], whereas 5,8,11,14-eicosatetraynoic acid (ETYA) which inhibits both the cyclooxygenase and the lipoxygenase pathways inhibited tumor promotion (S.M. Fischer, unpublished results). The thromboxane synthetase inhibitor RO-22-3382 was also found to inhibit TPA promotion (S.M. Fischer, unpublished results). It is of interest to point out that high doses of arachidonic acid inhibited tumor promotion whereas linoleic acid had no effect [81]. Prostaglandin E_1, E_2, and $F_{2\alpha}$ were inactive as tumor promoters, but E_2 and $F_{2\alpha}$ when given with TPA increased its promoting ability, whereas prostaglandin E_1 inhibited tumor promotion by TPA [81].

Since polyamines have been implicated in the mechanism of tumor promotion, we were interested in determining the effects of various chemicals on epidermal ODC activity, polyamine levels and tumor promotion (Table X). Mezerein is capable of increasing ODC activity and polyamine levels comparable to or greater than TPA but is a weak tumor promoter [82]. EPP, a hyperplastic agent with very

TABLE IX. Modifiers of TPA Promotion in Mouse Skin*

Modifier (dose, μg)	TPA response (% of TPA)	Modifier (dose, μg)	TPA response (% of TPA)
FA (1)	2	Arachidonic acid (500)	15
RA (10)	10	Linoleic acid (500)	92
TPCK (10)	40	Prostaglandin E_2 (10)	140
DFMO (2,000)	65	Prostaglandin E_1 (10)	60
MO (4,000)	125	Prostaglandin F_2 (10)	154
Putrescine (250)	160	IBMX (400)	45
Spermidine (200)	100	BHA, BHT (5,000)	20
Spermine (400)	60	DMSO and ethanol as solvent for TPA	40
Indomethacin (100)	145	Acetic acid (20,000)	10
Flurbiprofen (10)	140	A23187 (80)	180
ETYA (100)	55	Benzoyl peroxide (10,000)	200
RO-22-3582 (100)	45	Mellitin (50)	120

*See [81-83] for details concerning the skin tumor promotion-modifying activity of most of these agents. Although only one dose is presented for each agent, in most cases, several doses were investigated in order to determine if these agents had no effect or either an enhancing or inhibiting effect. The effects of IBMX, BHA, BHT, A23187, mellitin, ETYA, and RO-22-3582 on TPA promotion are unpublished results to be submitted for publication.

TABLE X. Effects of Various Chemicals on Epidermal ODC Activity, Polyamine Levels, and Tumor Formation*

Compound (dose)	ODC activity (6 hr) (% TPA)	Putrescine (treated/control)	Tumor (% TPA)
TPA 1 μg	100[a]	3.2	100
2 μg	125	4.5	180
MEZ 1 μg	100	2.6	<2
2 μg	150	3.7	4
5 μg	–	6.5	8
EPP 3 mg	<5	1.2	1
30 mg	20	1.6	2
TPA 1 μg + FA 1 μg	60	2.7	5
TPA 1 μg + αMO 4 mg	200	2.1	125
TPA 1 μg + DFMO 2 mg	<10	0.7	65
TPA 2 μg + TPCK 10 μg	70	–	40
TPA 2 μg + RA 10 μg	10	0.8	20
TPA 1 μg + indomethacin 100 μg	40	–	145
TPA 1 μg + RO 22-3582 100 μg	95	–	45
TPA 1 μg + ETYA 100 μg	110	–	55
TPA 1 μg + IBMX 200 μg	100	–	45

*See [82] for details concerning the effects of these agents on ODC, polyamines, and tumor promotion. promotion.

[a]Values are ratios 9 to 12 hr post-treatment.

weak promoting activity, increased ODC activity and polyamine levels, but to a much lesser degree than TPA or mezerein [82]. FA, the very potent inhibitor of TPA promotion, only slightly decreased the TPA increased ODC activity and polyamine levels [82]. Although αMO caused a paradoxical increase in ODC activity induced by TPA the level of putrescine was decreased [82]. αMO did not decrease TPA promotion, but the irreversible inhibitor on ODC (DFMO), decreased the TPA increased ODC activity, polyamine levels, and TPA promotion [81]. The protease inhibitor tosyl phenylalanine chloromethylketone (TPCK) effectively inhibited tumor promotion but had very little effect on TPA increased ODC activity [82]. As previously shown by other investigators [75], retinoic acid (RA) inhibits TPA promotion as well as TPA increased ODC activity and polyamine levels. Indomethacin was found to increase TPA promotion and to decrease TPA increased ODC activity whereas ETYA and RO-22-3382 inhibited TPA promotion but had no effect on TPA increased ODC activity [82]. IBMX was found to decrease TPA promotion but had no effect on TPA increased ODC activity. If all the data on the effects of the above compounds on ODC activity and polyamine levels are taken into consideration, one would have to conclude that there is no direct relationship between changes in ODC activity and subsequent polyamine levels and tumor promotion.

Multistage Promotion

As previously discussed, mezerein, a diterpene similar to TPA (Fig. 1) was capable of causing most of the morphological and biochemical changes in skin and in cells in culture that TPA does, but TPA was at least 50 times more active as a tumor promoter [60]. A comparison of these TPA and mezerein responses are shown in Table XI. Clearly, mezerein is as potent than TPA. This is especially true regarding the induction of epidermal ODC and epidermal hyperplasia. The effect of mezerein on ODC activity suggests that ODC induction is not a critical event in tumor promotion [60]. It should be emphasized that this conclusion is also true for the other morphological and biochemical responses to mezerein.

Because of the many similarities in morphological and biochemical responses induced by TPA and mezerein, we felt that mezerein, although a weak promoter, would be a good candidate as a compound to be used in the second stage of a two-stage promotion protocol as originally reported by Boutwell [2]. We recently reported that mezerein was a potent Stage II promoter [62,88]. Before these experiments are discussed in detail, a discussion of the original two-stage promotion protocol as reported by Boutwell [2] is needed. His results showed that promotion could be divided into two steps, conversion and propagation [2]. After initiation, the conversion stage was accomplished by a limited number of croton oil treatments which, with no further treatment, only produced a few tumors. The propagation stage was accomplished by repeated treatment with turpentine, a nonpromoting hyperplasic agent [2]. The three-stage protocol (initiation-conversion-propagation) produced a significant tumor response but less than that observed when croton oil was given for the complete promotion stage [2]. However, although the above experiments were repeatable at that time, recent results suggest that nonpromoting hyperplastic agents such as turpentine, EPP, and acetic acid when given repetitively after a few treatments with TPA are not able to complete the promotion process as reported by Boutwell [56,59,61]. In fact, Raick reported

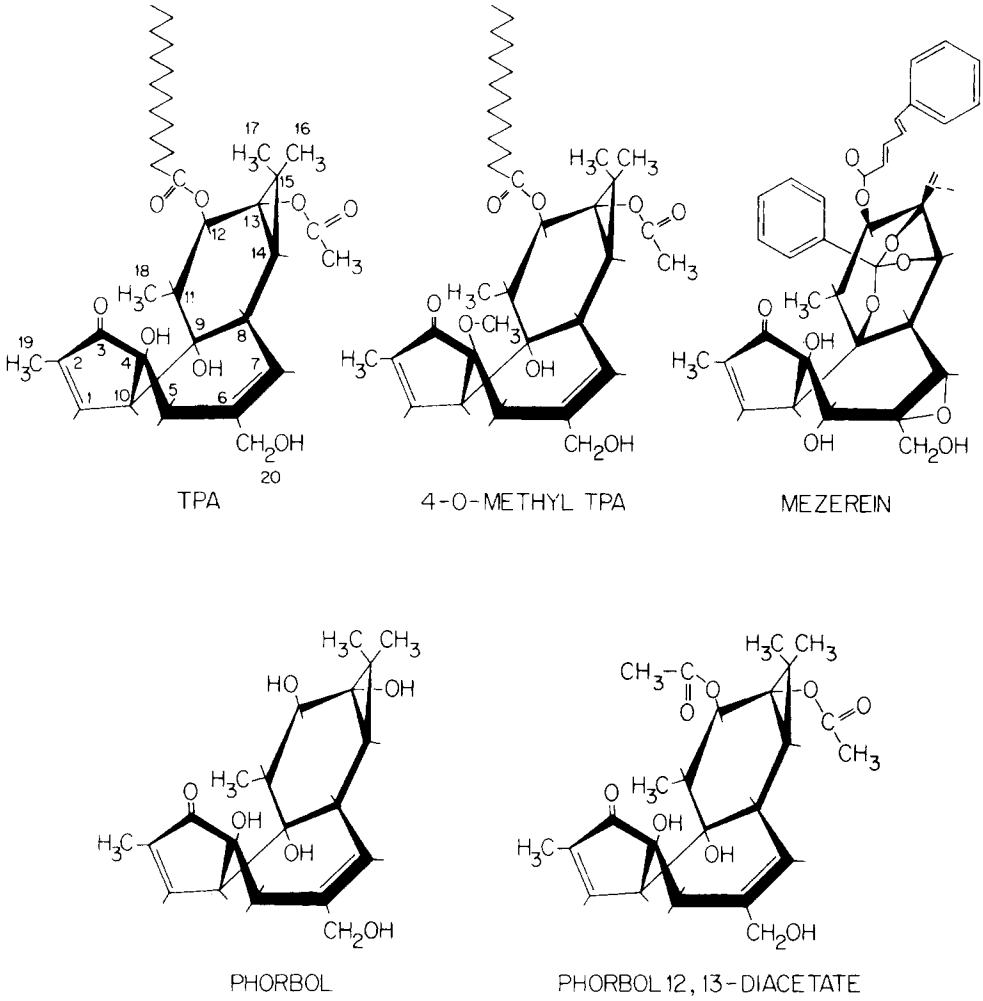

Fig. 1. A comparison of the structures of TPA, 4-0-me TPA, phorbol, phorbol 12,13-diacetate, and mezerein.

that turpentine and EPP gave fewer tumors in a three-stage system than when DMBA was followed only by limited TPA treatment [56,61]. Similar results were reported by Slaga et al [59] using acetic acid as a second stage promoter. It should be pointed out that turpentine, EPP, and acetic acid do not induce many of the biochemical responses induced by TPA and mezerein even though they are hyperplastic agents. It is possible that the variable response of turpentine as a Stage II promoter may be related to the fact that it is a complex mixture which can vary from batch to batch (Boutwell, personal communication).

A summary of the results on the use of mezerein as a second stage promoter in two-stage promotion are shown in Table XII. As illustrated TPA is about 50 times more active as a promoter then mezerein. When 2 μg of TPA are given twice weekly for only 2 weeks after DMBA initiation, no tumors are induced compared

TABLE XI. Comparison of Cellular and Biochemical Responses to TPA and Mezerein

	Relative response[a]		
	TPA	Mezerein	References
1. Enhancement of neoplastic phenotype	100	100	[84]
2. Promotion of neoplastic transformation (C3H-10T ½)	100	80	[85][b]
3. Induction of epidermal cellular proliferation	50	100	[5,60]
4. Comitogenesis in lymphocytes	100	100	[86]
5. Inhibition of differentiation in Friend erythroleukemia cells	100	100	[84,87]
6. Stimulation of DNA synthesis	50	100	[60][c]
7. Stimulation of ODC activity	80	100	[60][c]
8. Stimulation of plasminogen activator production	20	100	[84]
9. Skin tumor promotion	100	2	60

[a]For a comparative purpose the maximum response of mezerein or TPA is expressed as a 100. The values should only be considered as an approximation.
[b]Personal communication from S. Mondal and C. Heidelberger.
[c]Manuscript in preparation by C.E. Weeks, S.M. Fischer, and T.J. Slaga: "Comparative study on the effects of TPA and mezerein to induce epidermal DNA synthesis, ornithine decarboxylase, and polyamine in vivo and in vitro."

TABLE XII. Two-Stage Promotion*

	Initiation	Promotion		Relative tumor response
1	DMBA 1 wk	TPA	32×	100
2	DMBA 1 wk	Mezerein (4 μg)	32×	2
		Stage I	Stage II	
3	DMBA 1 wk	TPA 4×	Acetone 28×	0
4	DMBA 1 wk	TPA 4×	Mezerein (1 μg) 28×	35
5	DMBA 1 wk	TPA 4×	Mezerein (2 μg) 28×	50
6	DMBA 1 wk	TPA 4×	Mezerein (4 μg) 28×	85
7	DMBA 1 wk	TPA 4×	Mezerein (6 μg) 28×	120
8	DMBA 1 wk	4-O-methyl TPA (80 μg) 4×	Mezerein (2 μg) 28×	40
9	DMBA 1wk	TPA 4×	4-O-methyl TPA (80 μg) 28×	0
10	DMBA 1 wk	A23187 (80 μg) 4×	Mezerein (2 μg) 28×	60
11	DMBA 1 wk	TPA 4×	A23187 (80 μg) 28×	0
12	DMBA 1 wk	EPP (14 mg) 32×		1
13	DMBA 1 wk	TPA 4×	EPP (14 mg) 28×	2

*The mice were initiated with 10 nmol of DMBA and promoted with 2 μg of TPA or as shown above. See [88] for details concerning these experiments.

to twice weekly treatments for 18 weeks. However, when mezerein is given at a dose of either 1, 2, 4, or 6 μg twice weekly after the limited TPA treatment, it induced a significant tumor response in a dose-dependent manner. The ability of mezerein to act as a potent second stage promoter was repeated in 10 separate experiments [62,88,89]. Also shown in Table XII is the ineffectiveness of EPP as a complete promoter and as a second stage promoter. In addition, we recently found that 4-0-methyl TPA and the calcium ionophore A23187 which do not promote are effective first stage promoters (Table XII). These compounds induce epidermal hyperplasia and increase the number of dark basal keratinocytes (Klein-Szanto et al, unpublished data). Table XIII shows that a good dose–response exists for Stage I of promotion. In addition, only a single application of TPA is necessary for Stage I of promotion to be expressed after repeated applications of mezerein.

The effectiveness of some of the inhibitors of tumor promotion on two-stage promotion was recently reported by this laboratory [89]. The effects of FA, RA, and TPCK on two-stage promotion are shown in Table XIV. FA was a potent inhibitor of Stages I and II of promotion but to a greater degree for Stage I than Stage II. It should be emphasized that only four applications of FA with TPA were necessary to counteract the tumor response. RA was ineffective in Stage I but was a potent inhibitor of Stage II promotion whereas TPCK specifically inhibited Stage I but not Stage II. These experiments were repeated several times and were very reproducible [62,89].

TABLE XIII. Characteristics of Two-Stage Promotion*

Stage 1 (No. of applications of TPA)	Dose (ug)	Stage II (No. of applications of mezerein)	Tumor response (papillomas/mouse)
0	0	36	0
0	0	36[a]	0.2
1×	1	35	0.42
1×	2	35	1.40
1×	4	35	1.80
1×	6	35	2.20
1×	6	35[a]	3.40
2×	1	34	1.80
2×	2	34	2.50
2×	4	34	3.20
2×	6	34	3.60
2×	6	34[a]	4.60
4×	1	32	2.80
4×	2	32	4.10
4×	4	32	4.60
4×	4	–	0
4×	6	32	6.1
4×	6	–	0.2
4×	6	32[a]	8.4

*Mainly unpublished results. Thirty mice per group were used. All the mice were initiated with 10 nmol of DMBA followed 1 week later by various dose levels and number of applications of TPA (Stage I). Stage II was accomplished by twice weekly applications of 2 μg of mezerein after the last TPA treatment. Total promotion was continued for 18 weeks (36 applications).
[a]Mezerein was applied twice weekly at 4 μg per application.

Since the only major morphological or biochemical difference between the effects of TPA and mezerein on the skin is the ability of TPA to induce a large number of dark basal keratinocytes [58,89], we were interested in determining the effects of various inhibitors of promotion on the appearance of these dark cells. We reasoned that if these dark cells are critical in the first stage of promotion and if FA and TPCK are potent inhibitors of Stage I and RA of Stage II, then FA and TPCK should counteract the appearance of these cells, whereas RA should not. The results of FA, RA, and TPCK on the induction of dark basal keratinocytes by TPA are summarized in Table XV. As hypothesized, FA and TPCK were found to effectively counteract the appearance of the dark cells induced by TPA, whereas RA had no effect [58].

Since TPCK inhibited Stage I of promotion but not Stage II, and since TPCK counteracted the TPA induced increase in the dark basal keratinocytes but did not have any effect on TPA induced hyperplasia, we were interested in determining the effect of TPCK on TPA induced ODC activity. As shown in Table XV, TPCK had very little effect on TPA and mezerein induced epidermal ODC activity.

TABLE XIV. The Effects of Tumor Promotion Inhibitors on Two-Stage Promotion*

	Promotion		
Initiation	Stage I	Stage II	Tumor response (% of control)
1. DMBA 1 wk →	TPA 4× →	Mezerein 28× →	100
2. DMBA 1 wk →	TPA + FA4× →	Mezerein 28× →	0
3. DMBA 1 wk →	TPA 4× →	Mezerein + FA 28× →	20
4. DMBA 1 wk →	TPA + RA 4× →	Mezerein 28× →	95
5. DMBA 1 wk →	TPA 4× →	Mezerein + RA 28× →	20
6. DMBA 1 wk →	TPA + TPCK 4× →	Mezerein 28× →	25
7. DMBA 1 wk →	TPA 4× →	Mezerein + TPCK 28× →	94

*The mice were initiated with 10 nmol of DMBA and promoted with 2 μg of TPA and 2 μg of mezerein. FA (1 μg), RA (10 μg), and TPCK (10 μg) were applied simultaneously with TPA or mezerein. See [89] for details concerning these experiments.

TABLE XV. Effects of FA, RA, and TPCK on Tumor Promotion and TPA-Induced Epidermal Hyperplasia, Dark Keratinocytes, and Polyamine Levels*

	Relative ability (%) to counteract			
Inhibitor	TPA promotion	TPA-induced hyperplasia	TPA-induced dark cells	TPA-induced ODC and polyamine levels
FA	100	100	100	20
RA	80	0	0	85
TPCK	70	0	70	10

*The abilities of FA, RA, and TPCK to counteract the various TPA responses are expressed from 100% (complete suppression) to 0% (no effect). The effects of the inhibitors were determined from dose-response studies. See [58,82,85] for details concerning these experiments.

The anti-inflammatory steroid, FA, not only counteracted the appearance of dark cells induced by TPA but also suppressed the hyperplasia induced by TPA. In fact, the skins from FA plus TPA treated mice appeared as untreated skin. This is in agreement with our previously reported observations on the inhibitory effect of FA on TPA induced inflammation, hyperplasia, and DNA synthesis [74]. However, FA had little effect on the TPA increased ODC activity (Table XV) as compared to its effect on inhibition of promotion.

It is also of interest to point out that although RA inhibited Stage II of promotion, it had no inhibitory effect on the TPA or mezerein induced hyperplasia (Table XV). However, certain retinoids have been found to be potent inhibitors of TPA and mezerein induced epidermal ODC activity [75]. These data suggest that the induction of epidermal ODC activity followed by increased polyamines may be important in Stage II of promotion. In this regard FA and TPCK have either no effect or only a slight inhibitory effect on TPA or mezerein induced ODC activity [88]. FA does, however, significantly decrease the TPA induced spermidine levels in the epidermis [82,88]. This effect plus FAs inhibitory effect on TPA induced hyperplasia may be responsible for its inhibitory effect on Stage II promotion.

Complete and Two-Stage Carcinogenesis in Different Stocks and Strains of Mice

As previously stated the SENCAR stock was selectively bred for sensitivity to skin tumor induction by DMBA initiation followed by TPA promotion [2,18]. Consequently, the SENCAR mouse is extremely sensitive to two-stage carcinogenesis and coincidentally sensitive to complete carcinogenesis [17]. However, there exist several other stocks and strains of mice that are refractory to promotion or differ in their susceptibility to complete and two-stage carcinogenesis. Table XVI ranks the susceptibility of several mouse strains and stocks to complete and two-stage carcinogenesis. It is important to emphasize the limitiations of these rankings. First, only the response to BP and DMBA were included in the analyses. Second, dose-response data for both the carcinogen and/or promoter were not available for many of the mouse strains and stocks. Although these rankings represent subjective analyses, the differences between mice on the extremes of the rankings are significant.

Complete Carcinogenesis

As previously discussed, for any individual stock or strain of mouse, it has been generally observed that there is an excellent correlation between the amount of PAH bound to DNA and the skin tumor response [62,90,91]. However, this correlation between DNA binding and tumor response breaks down when a comparison is made between mouse strains or stocks that differ in their tumor response to complete carcinogenesis [91]. Sims and co-workers [91] have demonstrated that the kinetics of binding of DMBA to the DNAs of C57BL/6, DBA/2, and Swiss mice were virtually identical. Although there is the possibility that a specific metabolite of the DMBA was responsible for the tumor response and was undetected in this study, recent investigations suggest that the major metabolites of DMBA and BP are qualitatively similar in mouse strains that vary

in their response to complete carcinogenesis with PAHs [18]. Although these data are far from conclusive, they suggest that some aspects of initiation are probably similar in strains of mice that differ in their response to complete carcinogenesis. A priori, complete carcinogenesis probably has built into it the equivalent of a promotion phase. Differences in the promotional phase of complete carcinogenesis might be responsible for the variation in sensitivity to complete carcinogenesis measured in different stocks and strains of mice.

Two-Stage Carcinogenesis

As discussed previously, present evidence suggests that some aspects of initiation with PAHs are qualitatively and quantitatively similar in mouse stocks and strains that differ in their response to complete carcinogenesis. Furthermore, initiation is probably similar or identical in complete and two-stage carcinogenesis. C57BL/6 mice are refractory to two-stage carcinogenesis (BP-TPA) but responsive to complete carcinogenesis (T.J. Slaga et al, unpublished results). ICR/Ha Swiss mice respond poorly to complete carcinogenesis but do respond to initiation-promotion [38]. This unequal susceptibility to complete and two-stage carcinogenesis within a stock or strain of mice strongly suggests that the promotional phases of complete and two-stage carcinogenesis are dissimilar. Second, differences in sensitivity to initiation and promotion between mice may be due to alterations in the promotional phase of two-stage carcinogenesis.

In order to investigate promotion in C57BL/6 mice further, we have examined the biochemical and morphological responses assumed to be markers for multistage promotion (Table XVII). Delclos et al [96] have reported that C57BL/6 mice contain specific receptors for TPA. As in the case of SENCAR mice, TPA

TABLE XVI. Sensitivity to Skin Carcinogenesis in Different Stocks and Strains of Mice*

Complete Carcinogenesis
Sencar > CD-1 > C57BL/6 ≥ BALB/c ≥ ICR/Ha Swiss > C3H

Two-Stage Carcinogenesis (Initiation – Promotion)
Sencar >> CD-1 > ICR/Ha Swiss ≥ BALB/c > C57BL/6 ≥ C3H ≥ DBA/2

*Data represent sensitivities to BP and DMBA. Ranking represent a subjective analysis because dose-response data were not available for many strains. Sources of data: [2,18,62,90–95].

TABLE XVII. Comparison of Morphological and Biological Responses Induced in Sensitive (Sencar) and Resistant (C57BL/6) Mice*

Skin response	Sencar	C57BL/6
1. Hyperplasia	+ + + +	+ +
2. Stimulation of ODC activity	+ + + +	+ +
3. Changes in keratin proteins resembling embryonic pattern	+ + +	+ +
4. Induction of dark cells	+ + + +	+ +
5. Stimulation of protein kinases	+ +	+ +
6. Decrease in glucocorticoid receptors	+ +	+ +
7. Presence of TPA receptor[a]	+	+

*Unpublished results.
[a]Data from [96].

induces hyperplasia, ODC, dark cells and changes in the SDS-DTT extractable proteins of C57BL/6 mice. In addition, we have examined two parameters which could modulate promotion. TPA induces a two-to-three fold increase in a cytoplasmic cAMP independent protein kinase that phosphorylate the stratum corneum basic protein, a structural protein important in the terminal differentiation of skin (Mamrack et al, unpublished data). However, this enzyme is induced to the same level in both Sencar and C57BL/6 mice. In addition, we have examined the effects of TPA on the levels and affinity of the glucocorticoid receptors (Davidson et al, unpublished data). Within 6 hr, TPA treatment inhibits dexamethasone binding to glucocorticoid receptors by 60% in both SENCAR and C57BL/6 mice (Davidson et al, unpublished data). The dissociation constant for dexamethasone binding was not altered by TPA treatment in either stock of mice.

Relative to SENCAR mice, C57BL/6 mice have large adrenal glands. Trainin's [97] observation that adrenalectomy enhances tumor promotion by croton oil suggests that endogenous glucocorticoids can modulate promotion. We do not currently know if the differences in sensitivity to two-stage promotion are related to the size of the adrenal glands.

Based upon the analyses reported in Table XVII, we postulate the following explanations for the failure of the C57BL/6 mice to respond to promotion. First, the data in Table XVII show that some of the responses assayed are qualitatively but not quantitatively similar in the SENCAR and C57BL/6 mice. Possibly there is a threshold level that needs to be exceeded in order to obtain promotion. Second, the data in Table XVII represent the results obtained after only one topical application of TPA. Sisskin and Barrett [98] have shown that the hamster, a species that is refractory to two-stage carcinogenesis, responds to a single treatment of TPA but loses its responsiveness to repetitive treatments. In this respect, we presently do not know if C57BL/6 mice retain their responsiveness to TPA after multiple treatments. Third, although the parameters listed in Table XVII are the best-known markers for promotion, there may exist other critical, but presently cryptic, processes important to promotion.

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