Histologic Alterations Produced by Chrysarobin (1,8-dihydroxy-3-methyl-9-anthrone) in SENCAR Mouse Skin: Relationship to Skin Tumor Promoting Activity

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Histologic changes induced in SENCAR skin following a single treatment with chrysarobin (1,8-dihydroxy-3-methyl-9-anthrone) exhibited differences in time course from that observed with 12-O-tetradecanoylphorbol-13-acetate (TPA). Although not significantly different, maximum elevations in epidermal thickness, total number of nucleated epidermal cells, and dark basal keratinocytes (DCs) induced by 220 nmol chrysarobin occurred at 96 h after treatment, while those induced by 3.4 nmol TPA occurred at 48 h. Both compounds elicited comparable inflammatory responses.

Twice-weekly applications of chrysarobin for 2.5 weeks induced a moderate hyperplasia, increase in total nucleated epidermal cells, and increased DCs at 48 and 96 h after the last treatment, with a higher value for these parameters occurring at 48 h. Interestingly, the magnitude of these changes was similar to that observed after a single application. In contrast, twice-weekly applications of TPA induced a dramatic, potentiated induction of epidermal hyperplasia and DCs. Once-weekly applications of chrysarobin led to a potentiated induction of both hyperplasia and DCs compared to the twice-weekly treatment regimen and also more effectively promoted epidermal papillomas in previously initiated SENCAR mice. Skin sections from mice treated with chrysarobin displayed overt signs of epidermal toxicity including altered basal cell morphology and a decreased number of basal cells per 125 μm of basement membrane. Hyperplasia induced by multiple but not single treatments with chrysarobin and TPA correlated quantitatively with their papilloma promoting activity. In addition, the data suggest that epidermal toxicity may play a role in tumor promotion by anthrones. *J Invest Dermatol 92:64–71, 1989*

Anthrone derivatives [e.g., anthralin (1,8-dihydroxy-9-anthrone) and chrysarobin (1,8-dihydroxy-3-methyl-9-anthrone)] have been used in the treatment of psoriasis for many years (reviewed in Ref 1). Earlier studies have shown that anti-psoriatic anthrones are not carcinogenic when applied topically to mouse skin [2,3]; however, they are effective skin irritants [4,5]. In addition, anthrone derivatives such as anthralin and chrysarobin have been shown to be effective skin tumor promoters in mice previously initiated with subcarcinogenic doses of, for example, 7,12-dimethylbenz(a)anthracene (DMBA) [2,3,6]. More recently, studies in our laboratory have shown that both chrysarobin and anthralin are very efficient skin tumor promoters in mice because they produce carcinoma responses similar to those produced by the potent skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) [7,8]. It is of interest, therefore, to fully characterize the cellular, biochemical, and molecular effects produced by this class of compounds in relation to their skin tumor promoting activity and to compare these with the potent tumor promoting phorbol esters.

The phenomenon of tumor promotion in general has been extensively studied using phorbol esters such as TPA [9,10]. While the mechanism of tumor promotion is yet unknown, specific cellular and biochemical responses have been associated with the mechanism of action of TPA in mouse skin. Those events which correlate best with the skin-tumor promoting action of TPA (reviewed in Refs 11 and 12) are the induction of epidermal hyperplasia [13], dark basal keratinocytes (DCs) [13–15], a dermal inflammatory response [16], and epidermal ornithine decarboxylase (ODC, EC 4.1.1.17) activity followed by increased polyamine levels [17,18]. Furthermore, the promotion response to TPA is presumably mediated in part by its interaction with protein kinase C (PKC), the putative TPA receptor [19–22].

In general, there is only limited information on the histologic alterations induced by other classes of tumor promoters in mouse skin. A previous limited study from our laboratory reported on the epidermal hyperplasia and skin edema associated with a single topical application of both anthralin and chrysarobin in SENCAR mice [7]. These data suggested that the hyperplasia induced by anthralin and chrysarobin shared differences in both magnitude and time course when compared to the TPA response. Klein-Szanto and Slaga [15] demonstrated that anthralin was a moderate inducer of

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Abbreviations:
BM: basement membrane
CHR: chrysarobin
DC: dark basal keratinocyte
DMBA: 7,12-dimethylbenz(a)anthracene
EPP: ethylphenyl propriolate
ODC: ornithine decarboxylase
PMN: polymorphonuclear leukocyte
TPA: 12-O-tetradecanoylphorbol-13-acetate

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dark basal keratinocytes compared to TPA in SENCAR mice. Mannisto et al [23] have shown that NMRI mice developed a visible skin irritation in response to a limited number of treatments (three applications per week for 2 weeks) with anthralin or 10-propionyl anthralin. They also observed that these compounds elicited epidermal hyperplasia after a chronic treatment regimen (three applications per week for 50 weeks). In a comparable study using SENCAR mice, Viluksela et al [24] reported that visible skin irritation and histologically observed inflammatory changes were evident after chronic treatment regimens (three applications per week for up to 36 weeks) using anthralin or several 10-acyl analogues.

The present study was designed to provide an in-depth analysis of the histologic changes produced in the skin of SENCAR mice following both single and multiple applications of chrysarobin in comparison with TPA. As part of the experimental design, two multiple application protocols were used with chrysarobin consisting of a series of five treatments given at different application frequencies; twice-weekly for 2.5 weeks or once-weekly for 5 weeks. We have recently demonstrated that the once-weekly application protocol is more efficient for the induction of epidermal ODC [25] and papilloma formation [8] in SENCAR mice. The present data indicate that promoting doses of chrysarobin effectively induced epidermal hyperplasia, DCs, and dermal inflammatory changes. In addition, these responses, which are known to be important components of skin tumor promotion by phorbol esters, varied as a function of treatment protocol in direct relation to skin tumor promoting activity. Finally, evidence is presented to suggest that epidermal toxicity may be an important component of the promotion response to anthrone tumor promoters.

MATERIALS AND METHODS

Chemicals DMBA was obtained from the Eastman Kodak Co. (Rochester, NY). TPA was purchased from Chemicals for Cancer Research, Inc. (Eden Prairie, MN). Chrysarobin was purchased from ICN Pharmaceuticals, Inc. (K and K Laboratories Division, Plainview, NY), and was purified by column chromotography as described in a previous study [6]. All other chemicals and reagents used were of the highest purity deemed necessary.

Animals and Treatments Female SENCAR mice were obtained from the National Cancer Institute (Frederick, MD). At 7 weeks of age, the backs of the mice were carefully shaved using surgical clippers. Mice were allowed to stabilize for 2 days, and only those mice in the resting phase of the hair growth cycle were utilized. All chemicals were applied topically to the shaved area. Animals received either a single or a multiple treatment regimen. The two multiple treatment regimens, consisting of five treatments, were given either twice-weekly for 2.5 weeks or once-weekly for 5 weeks. For the single application and twice-weekly protocols, groups of three mice each were treated with 0.2 ml acetone solutions containing 3.4 nmol TPA or 220 nmol chrysarobin. For the once-weekly protocol, groups of two mice each were treated with 220 nmol chrysarobin in 0.2 ml acetone. All compounds were dissolved in acetone immediately prior to use. Control groups were treated either once or five times with 0.2 ml acetone for the single and multiple treatment protocols, respectively. Mice were then killed at various times after the last application of each promoter.

Histologic Preparation Skin from treated animals was excised, fixed with 3% glutaraldehyde in 0.05 M Sym-collidine buffer (pH 7.4), post-fixed with 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4), and embedded in Poly/Bed 812 [14]. Several blocks were embedded for each animal, and two blocks were selected on the criterion of the best possible orientation. From these blocks, 1-μm-thick sections were prepared and stained with 0.05% toluidine blue.

Morphometric Studies and Quantitative Evaluations The measurements of the epidermal thickness (except the horny layer), the dermal thickness (from the basement membrane to s.c adipose tissue), the number of basal and total epidermal nucleated cells per 125 μm length of basement membrane (BM), and the number of PMNs and mast cells per 250 μm length of BM were performed using an Olympus ocular micrometer at objective lens magnification of 40x. PMNs and mast cells were counted in all areas of the dermis from the BM to the upper surface of the adipose cells along a 250 μm horizontal length. At least 12 areas of a section from each block were measured at random for each parameter. We defined the DCs as cells having darkly stained nuclei and cytoplasm using toluidine blue and without degenerative changes such as large intracytoplasmic vacuoles or wide intercellular spaces. The quantitative histology data presented represents mean $\pm$S.E.M., and these figures were calculated from at least four sections of skin (two mice per group) and in some cases six sections or more (three mice per group). Sections sampled from an individual mouse were taken from different areas of dorsal skin. Statistical significance of the differences between means was evaluated with Student's t-test. The level of significance was set at $p \leq .05$.

Tumor Induction Experiments Each experimental group contained 30 preshaved mice. Mice were initiated with 25 nmol of DMBA and beginning two weeks after initiation, received topical applications of chrysarobin given either once or twice weekly. A third experimental group received twice-weekly applications of 3.4 nmol TPA. The incidence of papillomas was observed and recorded weekly. Papillomas were removed at random for histologic verification. Statistical analyses of the difference between mean papilloma responses (i.e., papillomas per mouse) were performed using students t-test.

RESULTS

Effects of a Single Topical Application For the present study we chose a dose of 220 nmol chrysarobin. We have recently shown that this dose is a maximal skin tumor promoting dose in female SENCAR mice [8]. Figure 1 shows the time course for the induction of epidermal hyperplasia (expressed as epidermal thickness) after a single topical application of 220 nmol chrysarobin. The data show that chrysarobin is a very effective hyperplasiogenic agent. Interestingly, the time course for induction of epidermal hyperplasia after a

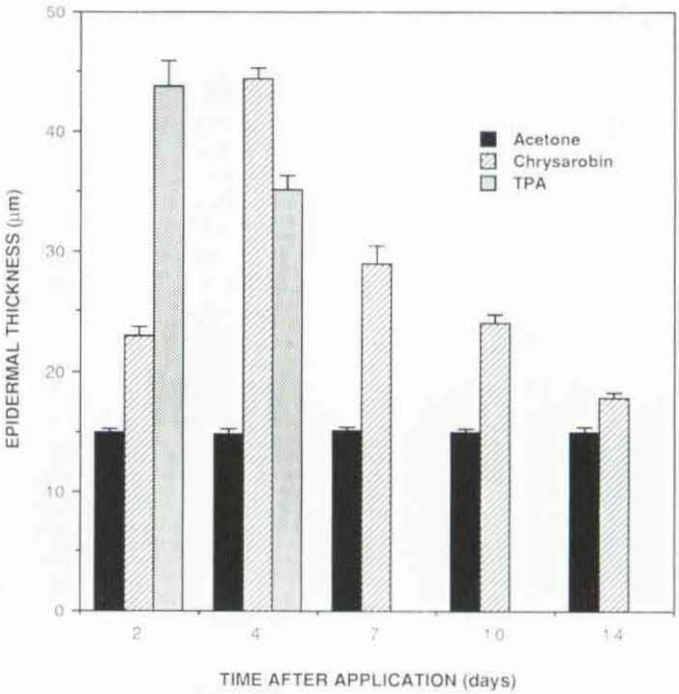

Figure 1. Time course for the induction of epidermal hyperplasia after a single application of chrysarobin (220 nmol per mouse) or acetone (0.2 ml). Also shown for comparative purposes is the epidermal hyperplasia 48 and 96 h after a single application of TPA (3.4 nmol). Three mice were used for each experimental group. Values were calculated from at least two sections from each animal and represent mean $\pm$S.E.M.

single topical application of chrysarobin was different from that observed with TPA. Previous work has shown that the hyperplasia produced in mouse skin by a single topical application of TPA reaches a peak by 48 h [12,26]. In contrast, the hyperplasia produced by a single application of chrysarobin reached a peak by 96 h after treatment. In addition, significant epidermal hyperplasia was evident even 14 d after a single topical application of the anthrone. For comparison, the changes in epidermal thickness after a single topical application of 3.4 nmol TPA are given for both 48 and 96 h after treatment. It should be evident that the epidermal hyperplasia had begun to subside by 96 h after treatment with TPA. It is also interesting to note that the magnitude of the peak increases in epidermal thickness for chrysarobin and TPA were not significantly different after a single treatment.

Table I more clearly demonstrates this latter point, especially when comparing the total number of nucleated epidermal cells per 125 μm of BM and also presents a more detailed analysis of the epidermal and dermal changes observed at 48 and 96 h after a single application of chrysarobin or TPA. In addition, Fig 2 shows the histologic appearance of skins treated with a single application of chrysarobin or TPA. Argyris [26] demonstrated that the suprabasal cells increased significantly after a single treatment with 17 nmol TPA to the skins of CD-1 mice, whereas the basal cells actually decreased in number within 1-2 d after treatment. We noted a consistent and reproducible decrease in the number of nucleated basal cells 48 h after treatment with chrysarobin (Table I) but not with TPA at the dose utilized. In addition, the epidermal basal cells from mice treated with chrysarobin had an unusual morphology, being relatively large and exhibiting both swollen nuclei and cytoplasm (Fig 2, *panel C*). At 96 h after treatment, the number and morphology of epidermal basal cells from mice treated with chrysarobin were comparable to those of the acetone control group (Fig 2, *panels B and D*). TPA (3.4 nmol per mouse) induced only a marginal increase in the number of epidermal basal cells at both 48 and 96 h after treatment, and these cells possessed a "normal-looking" morphology when compared to sections from acetone control mice.

Chrysarobin was an effective inducer of epidermal DCs with a greater number present at 96 h compared to 48 h after a single topical application (Table I). We have previously demonstrated that there is an excellent linear correlation between the magnitude of epidermal hyperplasia produced by a wide variety of chemical promoters and the log percentage of DCs induced by the same agents [27]. The data presented in Table I are consistent with these observations in that the percentage of dark cells was greatest when the hyperplasia was maximal. Although we did not examine the induction of DCs at time points other than 48 and 96 h after treatment we assume, based on our previous work, that the value obtained for chrysarobin at 96 h would be at or very near the maximal response. It should also be pointed out that the maximal response observed for TPA at 48 h (16.4 ± 7%), although higher, was not significantly ($p > .05$) different than that observed for chrysarobin at either 48 or 96 h.

Significant dermal thickening was induced 48 h after treatment with either chrysarobin or TPA; however, at 96 h the values in both treatment groups were comparable to the acetone control. TPA and chrysarobin induced a significant infiltration of inflammatory cells, specifically PMNs, into the dermis at 48 and 96 h after a single treatment. Dermal mast cell numbers were not significantly altered or only slightly altered at 48 and 96 h after treatment with chrysarobin or TPA. While the maximal levels of dermal thickening and PMN infiltration induced by chrysarobin and TPA were significantly different than acetone control values ($p < .05$), they were not significantly different from each other. Also, the time course for the development of these dermal changes following treatment with chrysarobin and TPA were similar (i.e., peak induction at 48 h after treatment).

Effects of Multiple Treatments Skin tumor promotion protocols involve multiple treatment regimens, and in the case of the phorbol esters the optimum treatment is twice weekly. In order to fully understand those histologic changes essential for skin tumor promotion by other classes of promoters it is necessary to examine changes occurring after more than one treatment. Tables II and III and Fig 3 summarize the histologic changes in the skins of SENCAR mice after five applications of chrysarobin (220 nmol) given either twice weekly (Table II) or once weekly (Table III). Included for comparison are the histologic changes in the skins of mice treated with five applications of TPA (3.4 nmol) given twice weekly (Table II). The most striking observation when comparing twice-weekly applications of chrysarobin and TPA is the lack of potentiated hyperplasia with the former compound. In this regard, five applications of TPA, given twice-weekly, induced a significantly greater epidermal hyperplasia in terms of epidermal thickness and DC induction compared to a single application of this promoter (Tables I and II, Fig 2*E,F* and 3*G,H*). In contrast, five applications of chrysarobin given twice weekly did not induce a significantly greater epidermal hyperplasia than observed after a single application (Tables I and II, Fig 2*C,D* and 3*E,F*). The time course for the epidermal changes produced by twice-weekly applications of chrysarobin was shifted earlier compared to a single application and the kinetics were more like that with TPA, where maximal values were obtained 48 h after the last treatment (Table II).

The total number of nucleated cells increased slightly, but the basal cells were again significantly decreased in number 48 h after the last treatment with chrysarobin in this twice-weekly application protocol (Table II). Furthermore, the epidermal basal cells observed in sections 48 h after the last application of chrysarobin again had an unusual morphology, being relatively large and exhibiting both swollen nuclei and cytoplasm (Fig 3, *panel E*). Thus, similar alterations in basal cell number and morphology were observed following both single (Table I, Fig 2) and twice-weekly treatments (Table II, Fig 3) of chrysarobin.

As noted in the Introduction, we have recently demonstrated that chrysarobin is a more effective promoter of papilloma formation

Table I. Changes in the Skin of SENCAR Mice after a Single Application with Various Promoters[a]

Time After Treatment	Promoter	Thickness of (μm)		No. of Nucleated Epidermal Cells per 125 μm of BM		% Dark Basal Keratinocytes	No. of Dermal Inflammatory Cells per 250 μm of BM	
		Epidermis	Dermis	Basal Layer	Total		PMN	Mast Cells
48 h	Acetone	15.8 ± 1.0	153.8 ± 13.0	17.6 ± 0.6	24.7 ± 0.9	0.8 ± 0.2	0.05 ± 0.1	4.5 ± 0.7
	Chrysarobin	20.8 ± 1.2	194.0 ± 23.5	13.0 ± 0.3	24.8 ± 1.4	8.2 ± 2.4	19.6 ± 5.2	5.1 ± 0.9
	TPA	42.8 ± 2.1	223.0 ± 45.0	19.7 ± 1.4	40.4 ± 0.6	16.4 ± 7.0	24.0 ± 7.2	7.6 ± 0.9
96 h	Acetone	16.0 ± 1.0	148.5 ± 17.0	17.7 ± 0.4	25.0 ± 0.8	1.0 ± 0.4	0.08 ± 0.2	4.9 ± 0.8
	Chrysarobin	42.5 ± 1.8	163.5 ± 13.5	18.4 ± 0.8	40.7 ± 1.6	8.2 ± 1.5	3.9 ± 1.7	4.0 ± 0.6
	TPA	35.8 ± 1.2	168.8 ± 12.5	19.6 ± 0.9	39.0 ± 0.9	10.0 ± 3.9	5.9 ± 1.9	5.0 ± 1.3

[a] Three mice were used for each treatment. TPA (3.4 nmol) and chrysarobin (220 nmol) in 0.2 ml acetone or the same volume of acetone were applied once on the backs of mice. Figures were calculated from two sections from each animal and represent mean ± S.E.M.

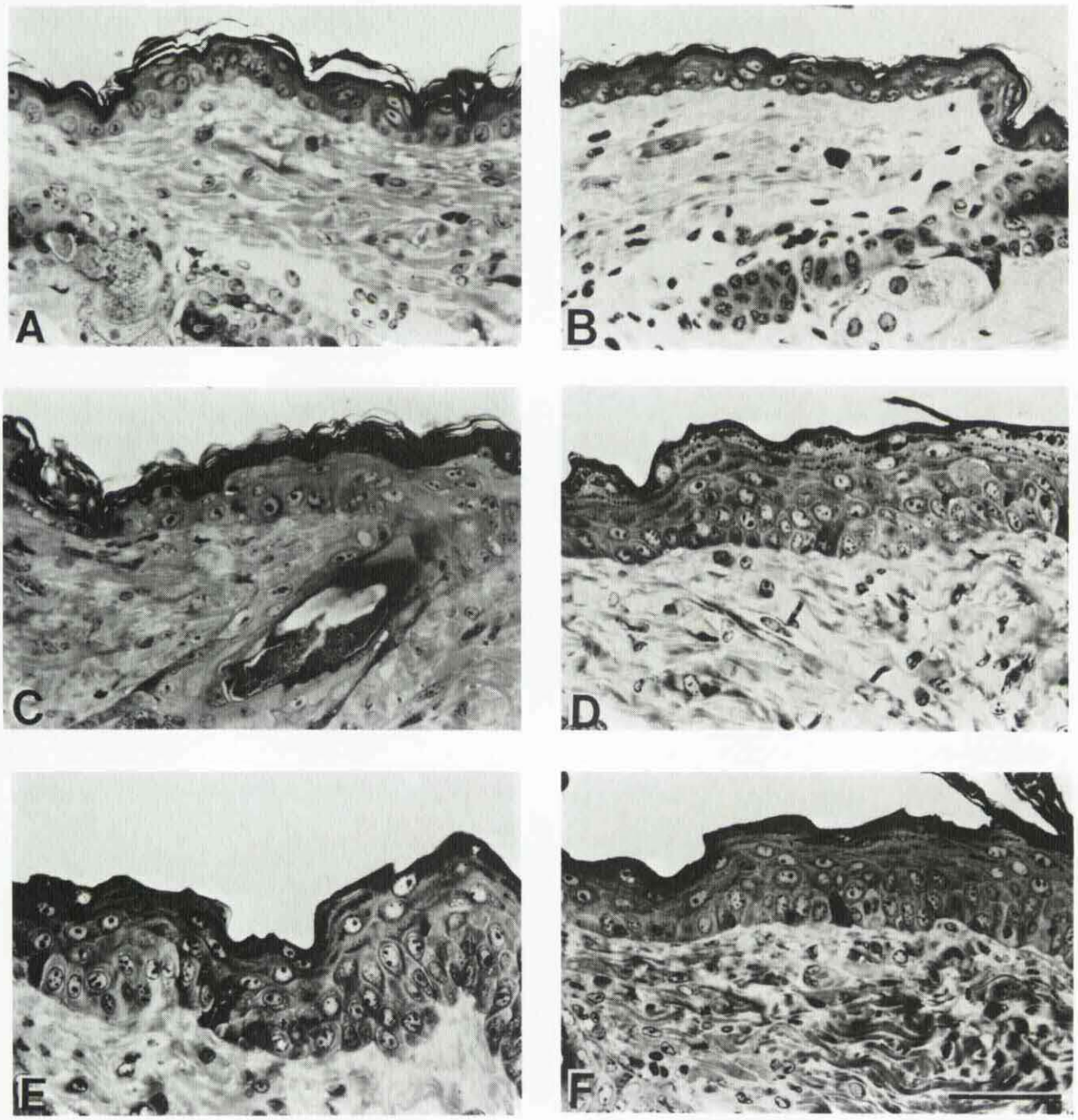

Figure 2. Response of the skin of SENCAR mice 48 and 96 h after a single application of 0.2 ml acetone, 220 nmol chrysarobin, or 3.4 nmol TPA. *Panels A* and *B:* 48 and 96 h, respectively, after treatment with 0.2 ml acetone. *Panels C* and *D:* 48 and 96 h, respectively, after treatment with 220 nmol chrysarobin. The epidermis showed slight hyperplasia and altered cell morphology 48 h after treatment. In addition, the number of basal cells were reduced. At 96 h after treatment, the epidermis showed a good hyperplasia. *Panels E* and *F:* 48 and 96 h after treatment with 3.4 nmol TPA. The epidermis showed a good hyperplasia 48 h after treatment. At 96 h after treatment, the epidermis still showed a good hyperplasia but the magnitude was less than that observed in chrysarobin treated skin. Epon-toludine blue: x420; Bar 50 μm.

when given once-weekly compared to twice-weekly (8). Table IV summarizes the results of a tumor experiment conducted concurrently with our histology studies comparing the papilloma response in mice receiving once- or twice-weekly applications of chrysarobin. A once-weekly application frequency with 220 nmol chrysarobin produced approximately twice the number of papillomas compared with the twice-weekly application frequency ($p<0.5$). The data in Table III and Fig 3, *panels C and D* demonstrate that a once-weekly application of chrysarobin, unlike the twice-weekly treatment, produced a potentiated hyperplasia compared to that observed after a single application of the promoter. In addition, the time course for hyperplasia induction after the last of five twice-weekly applications of chrysarobin was again identical to that observed with once-weekly applications of TPA. The maximum percentage of DCs induced was significantly greater in the once-weekly compared to the twice-weekly application protocol ($p<0.5$). At the time points examined, the number of nucleated cells in the basal layer of mice treated once weekly with chrysarobin was comparable to the values found in the acetone control group. This observation was different than that for the chrysarobin groups

Table II. Changes in the Skin of SENCAR Mice after Multiple Applications of Various Promoters Given Twice Weekly[a]

Time After Last Treatment	Promoter	Thickness of (μm)		No. of Nucleated Epidermal Cells per 125 μm of BM		% Dark Basal Keratinocytes	No. of Dermal Inflammatory Cells per 250 μm of BM	
		Epidermis	Dermis	Basal Layer	Total		PMN	Mast Cells
48 h	Acetone	15.2 ± 0.5	140.5 ± 3.2	17.4 ± 0.2	24.7 ± 0.6	1.2 ± 0.1	0	6.5 ± 0.3
	Chrysarobin	41.3 ± 2.3	224.5 ± 2.5	13.0 ± 0.3	28.5 ± 0.8	8.4 ± 1.3	5.9 ± 1.6	8.0 ± 0.4
	TPA	71.5 ± 4.9	229.0 ± 12.2	18.4 ± 0.3	39.8 ± 1.0	44.2 ± 3.9	11.4 ± 1.8	11.6 ± 1.3
96 h	Acetone	15.0 ± 0.5	134.2 ± 12.5	17.2 ± 0.2	24.3 ± 0.5	1.0 ± 0.2	0	5.9 ± 0.3
	Chrysarobin	36.5 ± 3.5	190.5 ± 4.2	18.9 ± 0.4	28.8 ± 1.2	5.2 ± 0.8	0.4 ± 0.1	7.2 ± 0.6
	TPA	66.3 ± 4.3	208.5 ± 8.0	19.0 ± 0.1	41.4 ± 0.3	31.9 ± 3.9	8.0 ± 0.8	16.4 ± 1.1

[a] Three mice were used for each experiment. TPA (3.4 nmol) and chrysarobin (220 nmol) in 0.2 ml acetone or the same volume of acetone were applied five times on the backs of mice over a 2.5 week period, using a twice-weekly application frequency. Figures were calculated from two sections from each animal and represent mean ±S.E.M.

treated singly or twice weekly, where at 48 h after the last treatment there existed a significantly decreased number of nucleated basal cells.

Thus, the above data demonstrate a good correlation between the magnitude of the induced hyperplasia, DC response, and efficiency for papilloma formation when comparing the once- vs. twice-weekly applications of chrysarobin. Furthermore, neither multiple treatment protocols with chrysarobin produced hyperplasia or induced dark cells to the same extent as twice-weekly applications of TPA. In addition, TPA was much more effective at promoting the development of papillomas in SENCAR mice. When taken together these data support an important role for epidermal hyperplasia and dark cell induction in skin tumor promotion by anthrones.

DISCUSSION

The anthrone class of tumor promoters has been hypothesized to work through an initial mechanism distinct from the phorbol esters (reviewed in Ref 28). This concept is based on various data, including differential papilloma formation in DMBA-initiated mice [8], differential induction of epidermal ODC [25], and the inability of anthrone promoters to interact directly with the phorbol ester receptor [19,28]. The induction of epidermal ODC after a single treatment by chrysarobin is significantly different, both in the magnitude (lower) and time course (delayed) compared with TPA [2,16]. Our present observations, which also support the above hypothesis, indicate that the epidermal hyperplasia and DCs induced in SENCAR skin following a single treatment with chrysarobin also exhibited a time course different from that observed with TPA [12,26]. Whereas significant elevations of epidermal thickness, total number of nucleated epidermal cells, and DCs induced by 220 nmol chrysarobin were maximal at 96 h after a single treatment, those induced by 3.4 nmol TPA were maximal at 48 h. Interestingly, the maximum values for the epidermal changes after a single application of chrysarobin or TPA were found to be very similar.

It has been suggested that the induction of epidermal hyperplasia is not sufficient for epidermal tumorigenesis in mouse skin (for review, see Refs 29 and 30) because of data on a number of agents including acetic acid, cantharidin, mezerein, and ethylphenyl propriolate (EPP). These agents induce an epidermal hyperplasia similar to that produced by TPA after a single application, but unlike TPA they are poor promoters of epidermal tumorigenesis in mice. However, Argyris has demonstrated that both acetic acid [31] and mezerein [32] are less effective hyperplastic agents because they cannot maintain the hyperplasia they initially produce when applied using multiple treatment regimens. The results of our present study, involving chrysarobin and TPA, support the concept that the magnitude of the sustained hyperplasia after multiple treatments correlates very well with the papilloma promoting ability of a given chemical. In this regard, results with the twice-weekly application protocol indicated that TPA induced a sustained hyperplasia which was greater than that produced by chrysarobin in terms of epidermal thickness and total nucleated epidermal cells per 125 μm BM. In addition, the magnitude of the hyperplasia induced by twice-weekly applications of chrysarobin was not significantly different than after a single application. Results obtained using the once-weekly application protocol with chrysarobin indicated that this protocol, which is optimal for skin tumor promotion by anthrone promoters (Ref 8 and Table IV), is also more optimal for induction of a sustained hyperplasia. It was also interesting to find that both the once-weekly and twice-weekly application protocols with chrysarobin yielded a time course for the induction of hyperplasia which was closer to that produced by TPA given twice weekly (i.e., maximum hyperplasia at 48 h).

Argyris [33] has operationally defined a regenerative hyperplasia as an epidermal hyperplasia which is associated with tissue damage. The histologic signs of tissue damage include cell death, karyorrhexis, pyknosis, cytoplasmic swelling, and an inflammatory response. Although we did not measure epidermal toxicity directly, the results of our present study suggest that the induction of epidermal hyperplasia by anthrones may result from chemically-induced toxicity and a subsequent regenerative response. The data from our present study that support the above hypothesis include 1) the de-

Table III. Changes in the Skin of SENCAR Mice after Multiple Applications of Chrysarobin Given Once Weekly[a]

Time After Last Treatment	Promoter	Thickness of (μm)		No. of Nucleated Epidermal Cells per 125 μm of BM		% Dark Basal Keratinocytes	No. of Dermal Inflammatory Cells per 250 μm of BM	
		Epidermis	Dermis	Basal Layer	Total		PMN	Mast Cells
48 h	Acetone	15.0 ± 1.0	147.5 ± 15.5	16.6 ± 0.4	24.5 ± 0.7	0.6 ± 0.1	0	5.0 ± 0.5
	Chrysarobin	54.2 ± 3.2	235.5 ± 4.5	18.7 ± 0.8	45.9 ± 2.8	14.2 ± 3.0	8.5 ± 2.9	7.4 ± 0.5
96 h	Acetone	17.8 ± 0.5	136.5 ± 3.5	18.2 ± 1.1	28.7 ± 2.9	1.9 ± 0.2	0	4.5 ± 0.6
	Chrysarobin	49.5 ± 1.5	225.0 ± 11.8	18.2 ± 0.5	46.0 ± 1.4	10.8 ± 2.2	2.2 ± 1.4	7.2 ± 0.4
168 h	Acetone	16.0 ± 0.8	159.5 ± 7.8	17.7 ± 0.1	25.1 ± 0.3	1.2 ± 0.3	0	5.8 ± 0.2
	Chrysarobin	31.0 ± 0.1	198.5 ± 4.0	18.6 ± 0.3	39.6 ± 1.0	8.3 ± 0.0	0	6.8 ± 0.8

[a] Two mice were used for each experiment. Chrysarobin (220 nmol) in 0.2 ml acetone or the same volume of acetone were applied five times on the backs of mice over a 5-week period, using a once-weekly application frequency. Figures were calculated from two sections from each animal and represent mean ±S.E.M.

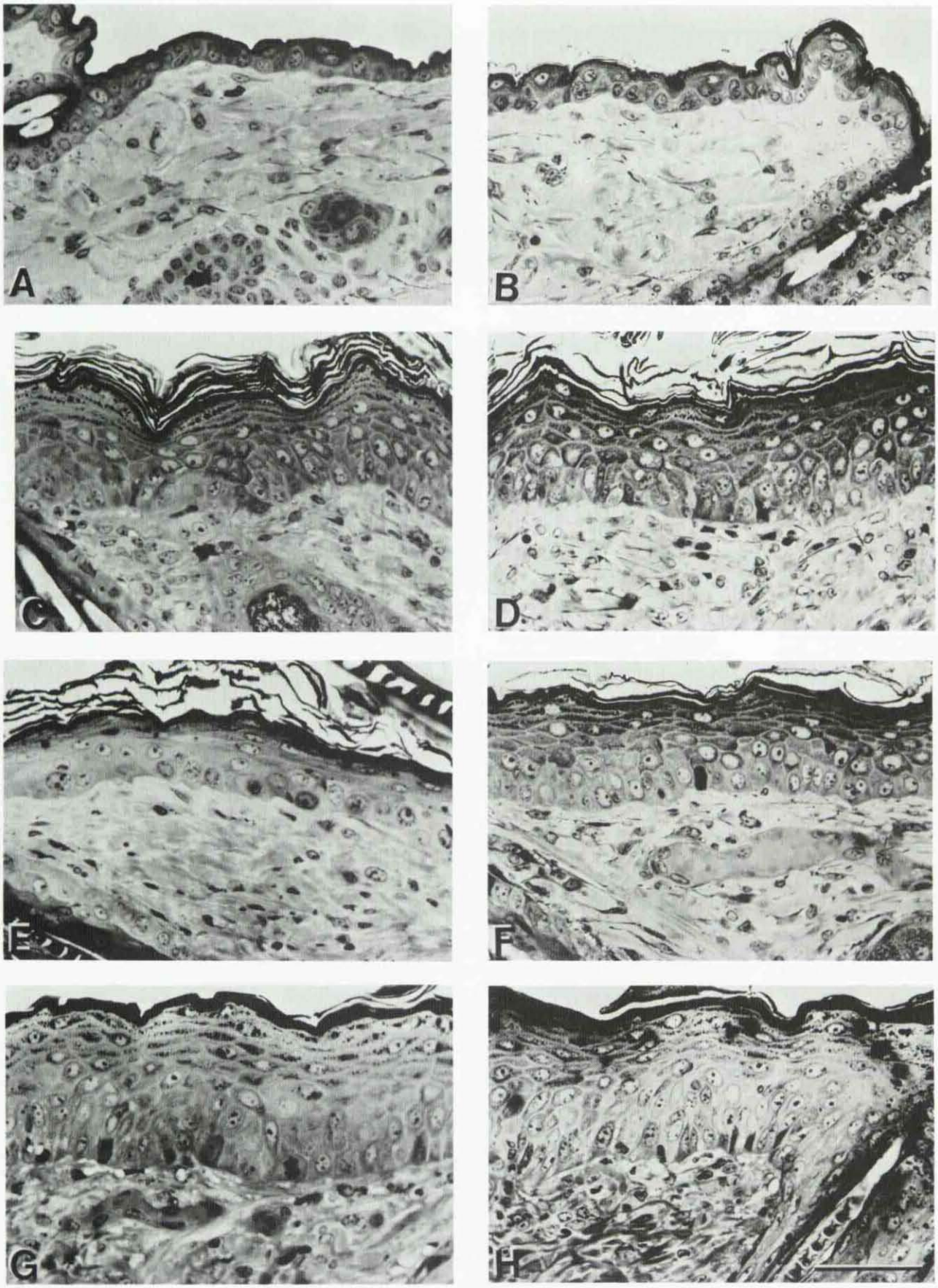

Figure 3. Response of the skin of SENCAR mice 48 and 96 h after multiple treatments with 0.2 ml acetone, 220 nmol chrysarobin, or 3.4 nmol TPA. *Panels A* and *B:* 48 and 96 h, respectively, after the last of five treatments with acetone given once weekly. This response was similar to that occurring after a twice-weekly treatment protocol with acetone; *Panels C* and *D:* 48 and 96 h, respectively, after the last of five treatments with chrysarobin given once weekly. The epidermis showed maximum hyperplasia and DCs 48 h after the last treatment. At 96 h the epidermis still showed a good hyperplasia. *Panels E* and *F:* 48 and 96 h, respectively, after the last of five treatments with chrysarobin given twice weekly. At 48 h after the last treatment, the epidermis showed a moderate hyperplasia and altered morphology of epidermal cells. In addition, the number of the basal cells were reduced. At 96 h after the last treatment, the epidermis also showed a moderate hyperplasia. *Panels G* and *H:* 48 and 96 h, respectively, after the last of five treatments with TPA given twice weekly. At both 48 and 96 h after the last treatment, the epidermis showed good hyperplasia and a large number of DCs. Epon-toluidine blue: x420. Bar: 50 μm.

Table IV. Effect of Application Frequency on Skin Tumor Promotion with Chrysarobin in SENCAR Mice[a]

Application Frequency per Week	Promoter (Dose)	Time to Reach Tumor Response Plateau (Weeks)	Percent of Mice with Papillomas	Papillomas per Mouse[b]
Once	Chrysarobin (220 nmol)	25	93	10.7 ± 0.80
Twice	Chrysarobin (220 nmol)	30	92	5.4 ± 1.86
Twice	TPA (3.4 nmol)	20	100	21.7 ± 2.50

[a] Thirty female SENCAR mice were used for each experimental group. Animals were initiated with 25 nmol DMBA followed 2 weeks later by applications of chrysarobin (220 nmol) or TPA (3.4 nmol).

[b] Average number of papillomas per mouse at the time of the tumor response plateau. All values in the Table are significantly different from one another ($p < .05$).

layed time course for the induction of epidermal hyperplasia after a single treatment which is very similar to the time course of hyperplasia induced by wounding or abrasion (33); 2) a significant reduction in the number of nucleated basal cells per unit length of basement membrane with both a single treatment and twice-weekly applications; 3) the presence of basal cells with altered morphology compared to the acetone-treated control (Fig 2, *panels A* and *C*, and Fig 3, *panels A* and *E);* 4) the presence of cytoplasmic and nuclear swelling in the basal cells; and 5) the presence of a dermal inflammatory response characterized by PMN infiltration and a thickened dermis. Recent work in our laboratory has demonstrated that in DBA/2 and C57BL/6 mice, once-weekly treatments with chrysarobin also produced similar changes. In this regard, chrysarobin-treated skins had fewer nucleated cells per unit length of BM and those cells again had altered morphology [27]. The absence of a reduced number of nucleated basal cells and abnormal cell morphology associated with a once-weekly application protocol in our present study (Fig 3, *panels C* and *D*) may be related to the use of a different mouse stock (i.e., SENCAR) compared to our previous studies, but does not preclude the presence of a toxic response. Rather, toxicity could be operative and present at a lower level. Further work will be necessary to fully substantiate the role of epidermal toxicity in skin tumor promotion by anthrones. It is interesting that in our present study the treatment protocol producing the least amount of observable toxic manifestations in skin sections was more effective at promoting skin papillomas. This finding is also consistent with the observations of Klein-Szanto et al [34], who showed that optimal promoting doses of TPA (i.e., 3.4 nmol) in SENCAR mice produce only moderate, sublethal damage to epidermal keratinocytes.

DC induction, as analyzed by light microscopy, has been used as a morphologic indicator of an early stage of tumor promotion (i.e., Stage I of promotion) [10]. Using electron microscopy, Raick [35,36] first reported that the number of DCs in TPA-induced hyperplasia was greater than that in epidermal hyperplasia induced by skin wounding or the weak tumor promoter, EPP. Chiba et al [37] classified TPA-induced DCs into Type-I and Type-II. Type-I DCs are poorly differentiated or de-differentiated cells and constitute the majority of TPA-induced DCs, while Type-II are involutional or degenerative cells. In the present study we did not strictly classify DCs into Type-I and Type-II. Several recent reports [27,38] have suggested that DCs are proliferating rather than degenerating cells as reported by others [39]. Although the function of DCs is not understood as yet, it has been suggested that DCs may be epidermal stem cells [40]. We observed that TPA induced more DCs than chrysarobin, but this difference was only statistically significant when comparing the multiple treatment protocols. There was also a potentiated induction of DCs following multiple treatments with the phorbol ester. This was also true for chrysarobin but only for the once-weekly application protocol. It is interesting to note that chrysarobin lacks Stage I promoting activity in SENCAR mice [7]. Our current histologic data suggest that the induction of DCs may be a necessary but not sufficient condition for effecting Stage I of promotion in SENCAR mice. Nevertheless, our current data show a direct correlation between the papilloma promoting ability and DC inducing ability of both chrysarobin and TPA.

In summary, the data from the single and multiple treatment protocols demonstrate that chrysarobin and TPA are effective inducers of hyperplasia, total number of nucleated epidermal cells, DCs, and a dermal inflammatory response. Our results with multiple treatment protocols show a direct correlation between the greater ability of TPA to promote papillomas when compared to chrysarobin using a two-stage carcinogenesis protocol in mouse skin (Refs 6 and 7 and Table IV). Also, results from multiple treatment protocols with chrysarobin indicate that the greater ability of the once-weekly protocol to induce hyperplasia and total epidermal cellularity correlates well with greater tumor promoting activity (Refs 8 and 25 and Table IV). These data clearly demonstrate that an analysis of promoter-induced histologic alterations in mouse skin following multiple but not single treatment protocols is more predictive of promoter activity in terms of the papilloma response as suggested by others[31,32]. Finally, a number of observations suggest that anthrone-induced toxicity may be important in the production of epidermal hyperplasia and tumor promotion in mouse skin. Further investigations are currently underway to understand the nature and importance of toxicity in the mechanism of tumor promotion by the anthrones.

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REFERENCES

1. Ashton RE, Andre P, Lowe N, Whitefield M: Anthralin: Historical and current perspectives. Sem Dermatol 2:287–303, 1983
2. Bock FG, Burns R: Tumor-promoting properties of anthralin (1,8,9-anthratriol). J Natl Cancer Inst 30:393–397, 1963
3. Segal A, Katz C, Van Duuren BL: Structure and tumor promoting activity of anthralin (1,8-dihydroxy-9-anthrone) and related compounds. J Med Chem 14:1152–1154, 1971
4. Mustakkallio KK: Irritation, staining, and antipsoriatic activity of 10-acyl analogs of anthralin. Br J Dermatol 105:20–23, 1981
5. Mannisto P, Havas A, Haasio K, Hanhijarui H, Mustakallio K: Skin irritation by dithranol (anthralin) and its 10-acyl analogs in three animal models. Contact Dermatol 10:140–145, 1984
6. DiGiovanni J, Boutwell RK: Tumor promoting activity of 1,8-dihydroxy-3-methyl-9-anthrone (chrysarobin) in female SENCAR mice. Carcinogenesis 4:281–284, 1983
7. DiGiovanni J, Decina PC, Prichett WP, Cantor J, Aalfs KK, Coombs MM: Mechanism of mouse skin tumor promotion by chrysarobin. Cancer Res 45:2584–2589, 1985
8. Kruszewski FH, Conti CJ, DiGiovanni J: Characterization of skin tumor promotion and progression by chrysarobin. Cancer Res 47:3783–3790, 1987
9. Slaga TJ: Mechanisms involved in two-stage carcinogenesis in mouse skin. In: Slaga TJ (ed.). Mechanisms of Tumor Promotion, Vol. 2: Tumor Promotion and Skin Carcinogenesis. CRC Press, Boca Raton, 1984, pp 1–16
10. Diamond L: Tumor promoters and cell transformation. Pharmacol Ther 26:89–145, 1985
11. Slaga TJ, Fischer SM, Weeks CE, Nelson K, Mamrack M, Klein-Szanto AJP: Specificity and mechanism(s) of promoter inhibitors and multistage promotion. Carcinog Surv 7:19–34, 1982
12. Klein-Szanto AJP: Morphological evaluation of tumor promoter ef-

fects on mouse skin. In: Slaga TJ (ed.). Mechanisms of Tumor Promotion, Vol. 2: Tumor Promotion and Skin Carcinogenesis. CRC Press, Boca Raton, 1984, pp. 41–72

13. Raick AN: Ultrastructural, histological, and biochemical alterations produced by 12-O-tetradecanoylphorbol-13-acetate on mouse epidermis and their relevance to skin tumor promotion. Cancer Res 33:269–286, 1973
14. Klein-Szanto AJP, Major SK, Slaga TJ: Induction of dark keratinocytes by 12-O-tetradecanoylphorbol-13-acetate and mezerein as an indicator of tumor-promoting efficiency. Carcinogenesis 1:399–406, 1980
15. Klein-Szanto AJP, Slaga TJ: Numerical variation of dark cells in normal and chemically induced hyperplastic epidermis with age of animal and efficiency of tumor promoter. Cancer Res 41:4437–4440, 1981
16. Stenback F, Garcia H, Shubik P: Present status of the concept of promoting action of cocarcinogenesis in skin. In: Homburger F and Shubik P (eds.). The Physiopathology of Cancer, Vol. I. S. Karger AG, Basel, 1974, pp 155–225
17. O'Brien TG, Simsiman RC, Boutwell RK: Induction of the polyamine-biosynthetic enzymes in mouse epidermis by tumor-promoting agents. Cancer Res 35:1662–1670, 1975
18. O'Brien TG, Simsiman RC, Boutwell RK: Induction of the polyamine-biosynthetic enzymes in mouse epidermis and their specificity for tumor promotion Cancer Res 35:2426–2433, 1975
19. Blumberg PM, Dunn JA, Jaken S, Jeng AY, Keach KL, Sharkey NA, Yeh E: Specific receptors for phorbol ester tumor promoters and their involvement in biological responses. In: Slaga TJ (ed.). Mechanisms of Tumor Promotion, Vol. 3: Tumor Promotion and Carcinogenesis in vitro. CRC Press, Boca Raton, 1984, pp 143–148
20. Niedel JE, Kuhn LJ, Vandenbark GR: Phorbol diester receptor copurifies with protein kinase C. Proc Natl Acad Sci USA 80:36–40, 1983
21. Ashendel CL, Staller JM, Boutwell RK: Solubilization, purification, and reconstitution of a phorbol ester receptor from the particulate protein fraction of mouse brain. Cancer Res 43:4327–4332, 1983
22. Verma AK, Pong R-C, Erickson D: Involvement of protein kinase C activation in ornithine decarboxylase gene expression in primary culture of newborn epidermal cells and in skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. Cancer Res 46:6149–6155, 1986
23. Mannisto PT, Vaissi KK, Mustakallio M, Viluksela M, Kosma V-M, Collan Y: Tumor-producing activity of dithranol (anthralin) and two of its 10-acyl analogs in the dorsal skin of female NMRI mice. J Pharm Exp Ther 229:255–260, 1984
24. Viluksela M, Puotunen E, Newman AJ, Mannisto PT: Tumor-producing and skin-irritating activity of dithranol (anthralin) and its 10-acyl analogues in SENCAR mice. Carcinogenesis 7:1755–1760, 1986
25. Kruszewski FH, Chenicek KJ, DiGiovanni J: Effect of application frequency on epidermal ornithine decarboxylase induction by chrysarobin in SENCAR mice. Cancer Lett 32:263–269, 1986
26. Argyris TS: Epidermal growth following a single application of 12-O-tetradecanoylphorbol-13-acetate in mice. Amer J Pathol 98:639–646, 1980
27. Naito M, Naito Y, DiGiovanni J: Comparison of the histological changes in the skin of DBA/2 and C57BL/6 mice following exposure to various promoting agents. Carcinogenesis 8:1807–1815, 1987
28. DiGiovanni J, Kruszewski FH, Chenicek KJ: Studies on the skin tumor promoting actions of chrysarobin (1,8-dihydroxy-3-methyl-9-anthrone). In: Butterworth B, Slaga TJ (eds.) Banbury Report 25: Nongenotoxic Mechanisms in Carcinogenesis. Cold Spring Harbor Laboratory, 1987, pp 25–39
29. Boutwell RK: The function and mechanisms of promoters of carcinogenesis. Crit Rev Toxicol 2:419–443, 1974
30. Scribner JD, Suss T: Tumor initiation and promotion. Int Rev Exp Path 18:137–198, 1978
31. Argyris TS: An analysis of the epidermal hyperplasia produced by acetic acid, a poor promoter, in the skin of female mice initiated with dimethylbenz-anthracene J Invest Dermatol 80:430–435, 1983
32. Argyris TS: Nature of epidermal hyperplasia produced by mezerein, a weak tumor promoter, in initiated skin of mice. Cancer Res 43:1768–1773, 1983
33. Argyris TS: The regulation of epidermal hyperplastic growth. CRC Crit Rev Toxicol 9:151–200, 1981
34. Klein-Szanto AJP, Chiba M, Lee S-H, Conti CJ, Thetford D: Keratinocyte damage produced by 12-O-tetradecanoylphorbol-13-acetate in rodent epidermis. Carcinogenesis, 5:1459–1465, 1986
35. Raick AN, Brudzy K: Ultrastructural and biochemical changes induced in mouse epidermis by a hyperplastic agent, ethylphenylpropriolate. Cancer Res 33:2221–2230, 1973
36. Raick AN: Cell differentiation and tumor-promoting action in skin carcinogenesis. Cancer Res 34:2915–2925, 1974
37. Chiba M, Slaga TJ, Klein-Szanto AJP: A morphometric study of dedifferentiated and involutional dark keratinocytes in 12-O-tetradecanoylphorbol-13-acetate-treated epidermis. Cancer Res 44:2711–2717, 1984
38. Murakami Y, Hibino T, Arai M, Kuroki T: Appearance of dark keratinocytes following intracutaneous injection of cholera toxin in mouse skin. J Invest Dermatol 85:115–117, 1985
39. Glaso M, Ree K, Iversen OH, Hovig T: The influence of different fixatives and a tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), on the induction of so-called dark cells in mouse epidermis. Virchows Arch [Cell Pathol] 50:355–372, 1986
40. Slaga TJ: Mechanisms involved in multistage skin tumorigenesis. In: Huberman E, Barr SH (eds.). Carcinogenesis, Vol. 10: The Role of Chemicals and Radiation in the Etiology of Cancer. Raven Press, New York, 1985, pp 189–199