Effects of Peroxides on Rodent Skin: Epidermal Hyperplasia and Tumor Promotion

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Free radical generating peroxides are potent skin irritants. After a single topical application of either 10, 20, or 40 mg of lauroyl peroxide or benzoyl peroxide on the dorsal skin of Sencar mice, the epidermal thickness increased markedly. No major inflammatory or vascular alterations were noted. On the other hand, 15 or 30% hydrogen peroxide produced an extensive epidermolysis, as well as inflammation and vascular injury, followed by quick regeneration and epidermal hyperplasia.

Both lauroyl peroxide- and benzoyl peroxide-induced hyperplasias were characterized by a sustained production of dark basal keratinocytes, which constituted approximately 10% of the basal cell population during the first week after single topical application. Hydrogen peroxide-induced epidermal hyperplasias also exhibited numerous dark cells, but their presence was less sustained.

Although all these peroxides were inactive either as initiators or as complete carcinogens, lauroyl peroxide was as effective as benzoyl peroxide when used as a skin tumor promoter in a two-stage carcinogenesis protocol. In a similar experimental protocol, hydrogen peroxide proved to be a very weak skin tumor promoter.

Hydrogen peroxide (H_2O_2), lauroyl peroxide (LP), and benzoyl peroxide (BP) are free radical generating compounds widely used in the chemical industry, especially BP in pharmaceutical preparations. Although earlier studies showed that peroxides are not carcinogenic when applied topically on the mouse skin [1], if these compounds are used undiluted or in high concentrations they are potent skin irritants [2,3], Recently we have confirmed that BP is not a complete carcinogen, but it is a very effective tumor promoter if repetitively applied after a subcarcinogenic dose of dimethylbenzanthracene (DMBA) in a two-stage carcinogenesis protocol [4]. In addition, as has been seen with many other skin tumor promoters, BP induces a large number of dark basal keratinocytes, i.e., intensely basophilic or electron dense cells [4]. In other published experiments we have shown that after topical application of several promoters and hyperplasiogenic agents the number of induced dark basal keratinocytes correlates well with the respective tumor-promoting efficiencies of these agents [5–7]. The purpose of this study is to show the morphological changes induced by peroxides in skin, especially epidermal hyperplasia and induction of dark keratinocytes, and to describe the skin tumor-promoting abilities of these free radical generating compounds.

MATERIALS AND METHODS

Animals

Sencar mice originally obtained from Dr. R. K. Boutwell, Madison, Wis., are presently raised in Oak Ridge, Tenn, as previously described [8]. Female animals 7 to 9 weeks of age, were shaved with surgical clippers 2 days before treatment, and only those in the resting phase of the hair cycle were used.

Morphological Studies

A total of 132 animals were treated with BP or LP in acetone or with H_2O_2. Using 16 to 21 animals per group, BP and LP were used in 3 different doses, i.e., 10, 20, and 40 mg in 0.2 ml acetone. H_2O_2 was applied either as a concentrated solution (30%) or diluted 1:1 and 1:5 in acetone. The 0.2-ml solution containing the peroxides was applied topically on the dorsal skin. Except for the LP treatments, in which due to the low solubility of the compound 10 mg were applied in 0.2 ml 2 or 4 times to give a total dosage of 20 and 40 mg, respectively, in all other treatments a single topical application of 0.2 ml of solution containing the peroxide dose was applied topically on the dorsal skin. A group of 12 control animals were treated with acetone alone.

Following a single application, 2–4 animals were sacrificed 1, 2, 4, 6, 8, and 10 days after treatment. After excision of 1 cm^2 of treated skin, 1-mm thick blocks were fixed in 3% glutaraldehyde in 0.05 M sym-collidine buffer (pH 7.4) for 2 hr at 4°C. After washing the tissues in 0.1 M sodium cacodylate solution, the tissues were postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated, and embedded in Epon 812. One micron-thick sections were prepared and stained with toluidine blue as previously described [5]. The number of darkly stained basal cells and the total number of basal cells were counted in 1–2 blocks for each treated animal. A total of 10,212 cells were counted. In the same blocks, epidermal thickness measured randomly at 10 different sites of the interfollicular epidermis per block was determined. Ultrathin sections were also prepared as previously described [5], stained with uranyl acetate and lead citrate, and observed in a Hitachi HU11B electron microscope.

Carcinogenesis Studies

The tumor-promoting, tumor-initiating, and complete carcinogenic activities of H_2O_2 and LP were investigated in the dorsal skin of 780 animals. Thirty (LP) or 60 (H_2O_2) Sencar mice were used in each experimental group. In order to test the tumor-promoting activity of the compound, the animals received 10 nmoles of DMBA in 0.2 ml acetone or acetone only as control, followed 1 week later by twice-weekly topical applications of various dose levels of either H_2O_2 or LP in 0.2 ml acetone for 25 weeks. The tumor-initiating abilities of the peroxides were determined by applying either H_2O_2 (diluted 1:1 in acetone) or 20 mg LP in 0.2 ml acetone or acetone only as control, and promoted 1 week later with twice-weekly applications of 2 µg 12-0-tetradecanoyl-phorbol-13-acetate (TPA) for 25 weeks. The complete carcinogenic activity of the peroxides was studied by performing twice-weekly topical applications of H_2O_2 (diluted in acetone 1:1) or 20 mg LP in 0.2 ml acetone, or acetone only as a control, for 25 weeks.

RESULTS

Early Effects of Single Topical Applications of Peroxides

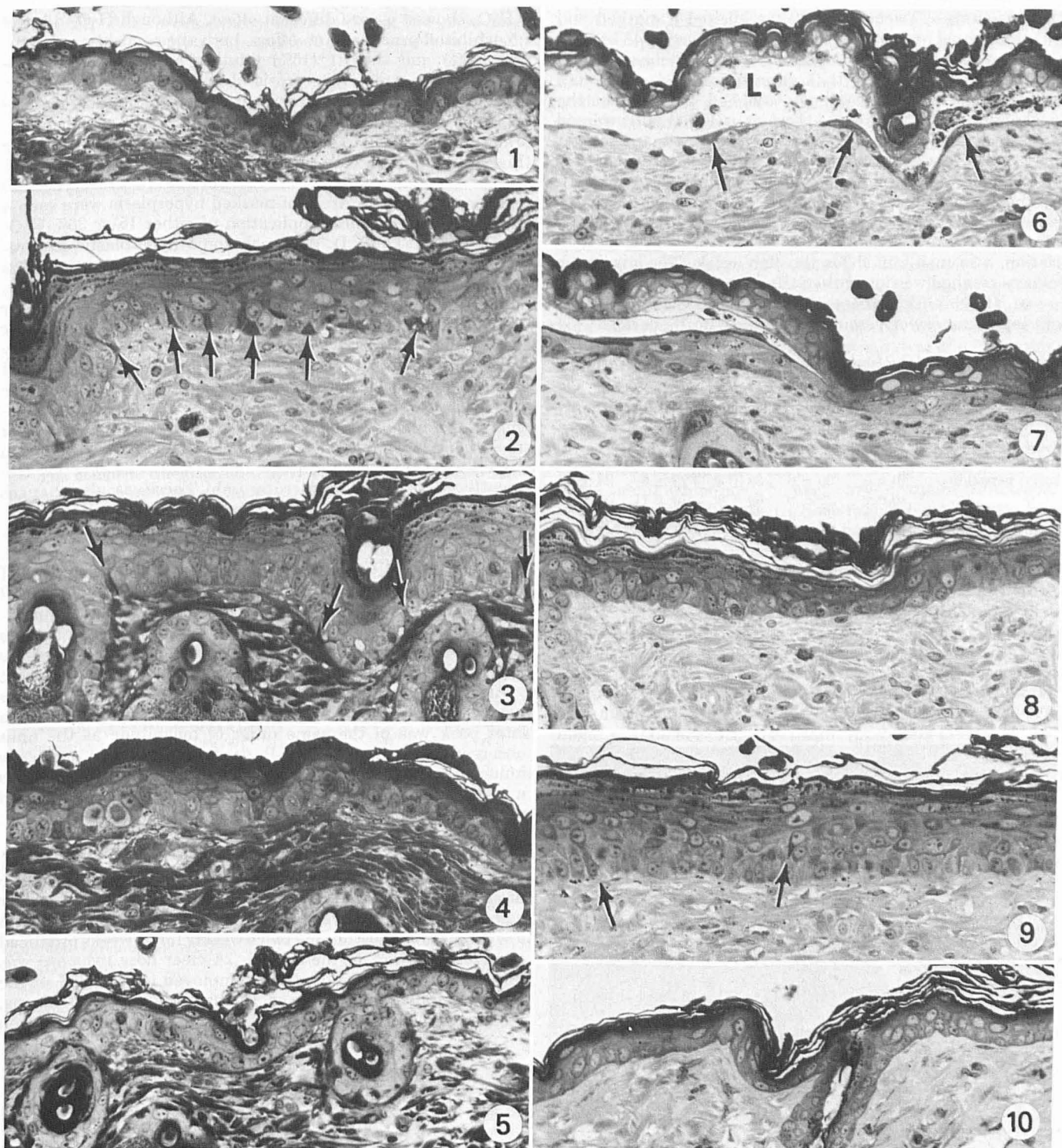BP and LP proved to be epidermal hyperplasiogenic agents. BP (10 mg) produced very slight or no response (Fig 1) in the

FIG 1 to 10. Epon-embedded material stained with toluidine blue (× 250).

FIG 1. No major alterations are seen in the epidermis 48 hr after topical application of 10 mg benzoyl peroxide.

FIG 2. Epidermal hyperplasia with acanthosis, hypergranulosis, and hyperorthokeratinization. Note numerous dark basal keratinocytes (*arrows*). Four days after topical application of 20 mg benzoyl peroxide.

FIG 3. Similar epidermal hyperplasia as in Fig 2, 4 days after topical application of 40 mg benzoyl peroxide. Dark cells are indicated by *arrows*.

FIG 4. Moderate epidermal hyperplasia 8 days after topical application of 20 mg benzoyl peroxide. Note hyperorthokeratinization and decrease of dark basal keratinocytes.

FIG 5. The epidermis looks almost normal 10 days after topical application of 20 mg benzoyl peroxide.

FIG 6. The epidermis is completely separated from the dermis. Lacunae or bullae (*L*) are formed subepidermally; they often contain polymorphonuclear leukocytes and cell debris. Epithelial cells usually originating in the infundibular area migrate and cover the deepithelialized areas (*arrows*). Twenty-four hr after hydrogen peroxide (30%) treatment.

FIG 7. Almost complete regeneration of the epidermis is accomplished 2 days after topical application of hydrogen peroxide 15%.

FIG 8. Epidermal hyperplasia 4 days after topical application of 15% hydrogen peroxide.

FIG 9. Marked epidermal hyperplasia 6 days after topical application of 30% hydrogen peroxide. Dark cells are indicated by *arrows*.

FIG 10. Normal epidermis 10 days after topical application of 15% hydrogen peroxide.

cutaneous tissues. Twenty and 40 mg elicited a marked and similar epidermal hyperplasia (Figs 2 and 3), starting 48 hr after topical application of the compounds. This was characterized by acanthosis with hyperorthokeratinization. The maximum epidermal thickness (Table I) was reached 4 days after the topical application; it decreased thereafter (Fig 4) and attained almost normal values after 10 days (Fig 5). LP induced similar, although less marked, alterations, i.e. practically no changes were observed with 10 mg, whereas a mild hyperplasia was seen with the higher doses. The pattern of epidermal thickness variations offered some differences (Table I), i.e., the maximum epidermal hyperplasia was reached at 2 days, and, with slight variation, was maintained for the first week. The maximum thickness reached was lower than the one attained with similar doses of BP. Neither of these compounds produced more than slight inflammatory or vascular alterations in the dermis.

TABLE I. *Epidermal thickness changes after a single application of peroxides*

Treatment	Dose	Epidermal thickness (μm)[a]			
		1 day	2 days	4 days	6 days
Acetone	0.2 ml	28 ± 4	27 ± 3	28 ± 5	26 ± 4
Benzoyl peroxide	10 mg	30 ± 6	38 ± 4	29 ± 6	28 ± 4
	20 mg	28 ± 5	63 ± 8	70 ± 11	55 ± 6
	40 mg	32 ± 4	71 ± 9	73 ± 20	61 ± 8
Lauroyl peroxide	10 mg	31 ± 4	36 ± 7	38 ± 10	36 ± 4
	20 mg	32 ± 7	38 ± 8	23 ± 9	38 ± 10
	40 mg	38 ± 5	41 ± 7	31 ± 8	40 ± 8
Hydrogen peroxide	30%	10 ± 5[b]	28 ± 6	50 ± 18	37 ± 6
	15%	11 ± 6[b]	18 ± 11	44 ± 15	40 ± 5

[a] Epidermal thickness from basal to granular layer excluding horny layer. Each number represents mean ± standard deviation obtained from 2 to 3 animals (20–30 measurements per animal).

[b] Areas of erosion and ulceration were seen in these specimens and were avoided in this quantitative study.
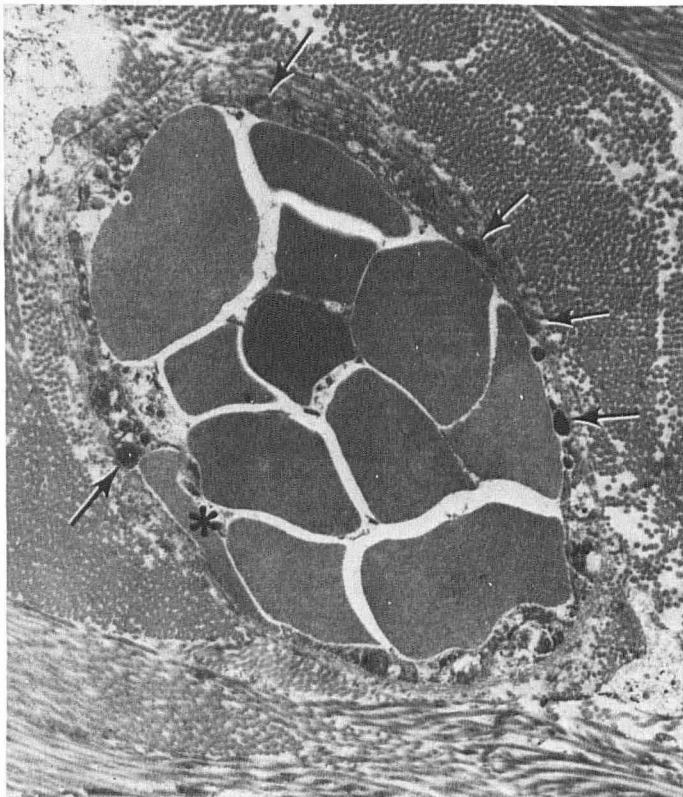

FIG 11. Dilated capillary vessel. The endothelial cells are markedly damaged, few normal organelles are seen, numerous dense bodies (*arrows*) can be noted. In one area the endothelial lining is not visible (*asterisk*) (× 5,800).

H_2O_2 showed a very different effect. Although H_2O_2 diluted 1:5 exhibited practically no effect, both the concentrated solution (30%) and the 1:1 (15%) dilution produced an intense reaction with extensive necrosis of the epidermis which was seen sloughing off *in toto* as early as 24 hr after application (Fig 6). Some basal cells remained attached to the basement membrane and were seen to proliferate and regenerate the epidermis (Fig 7). In addition, cells migrating from the infundibular portion of the hair follicles also contributed to the epidermal regeneration (Fig 6). Areas of marked hyperplasia were seen 4 and 6 days after topical application of either 15 or 30% H_2O_2 (Fig 8 and 9, Table I). Although some hyperplastic foci remained at 8 and 10 days, overall the epidermis reverted to its normal thickness (Fig 10). During the first 4 days after H_2O_2 application and coinciding with the extensive erosion and/or ulceration of the epidermis, an intense leukocytic infiltration of the dermis was observed. Many hair follicles were lethally damaged, exhibiting necrotic cells and several involutional changes (absence of mitosis, atrophy, shortening of the follicle and disappearance with granulation tissue replacement). Although the blood vessels were apparently normal at the light microscopy level, marked involutional changes were seen with the electron microscope in the endothelial cells (Fig 11) frequently accompanied by microthrombi. These vascular changes were not seen in the BP- and LP-treated skins.

Of particular interest was the presence of dark basal keratinocytes in the 1-μm thick sections stained with toluidine blue. Both LP- and BP-treated epidermis exhibited an increase of dark cells which constituted approximately 10% (a five-fold increase from the normal value of 2%) of the basal cell population 2 to 4 days after treatment. At 6 and 8 days the percentage decreased to approximately 7%, returning to control values by day 10. No dose response could be seen (Fig 12).

After H_2O_2 treatment the percentage of dark keratinocytes reached a maximum somewhat later (6 days). Although this later peak was of the same order of magnitude as the ones observed with either BP or LP, the total amount of dark cells induced by H_2O_2 during the entire experimental period was markedly lower than the total number of dark cells induced with either of the other peroxides.

Carcinogenesis Studies

The skin tumor induction studies carried out with LP are described in Table II. LP was an efficient skin tumor promoter when applied repetitively after initiation with DMBA. Even low promoting doses of 1 mg twice weekly for 25 weeks produced papillomas in 25% of the animals. A clear dose response was observed when other doses were employed (43 and 66% of the experimental animals showed tumors, using 10 and 20 mg, respectively). LP was not effective as a complete carcinogen
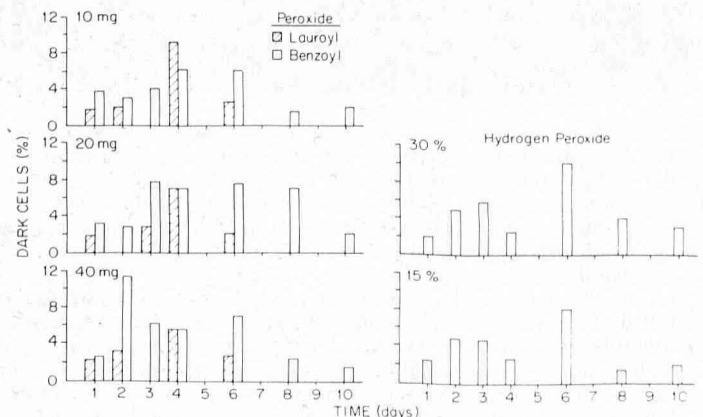

FIG 12. Dark cell percentage in the basal layer of peroxide-treated epidermis.

TABLE II. *Skin carcinogenicity, tumor-initiating and -promoting activities of lauroyl peroxide (LP) in Sencar mice*

Treatment protocol (LP, amount/application)	No. of mice alive at 25 weeks	No. of papillomas per mouse at 25 weeks	Percentage of mice with papillomas at 25 weeks
Tumor-promoting activity (DMBA initiation followed by twice-weekly application of LP)			
1 mg	28	0.3	25
10 mg	30	1.3	43
20 mg	29	3.1	66
acetone (0.2 ml)	30	0	0
Tumor-initiating activity (LP one application followed by TPA promotion)			
20 mg	28	0.2	15
acetone (012 ml)	29	0.2	15
Complete carcinogenic activity (LP 2×/wk)[a]			
20 mg	28	0.03	3
acetone (0.2 ml)	29	0	0

[a] The complete carcinogenic activity of LP was investigated for 50 weeks. No squamous cell carcinomas developed during this period.

TABLE III. *Skin carcinogenicity, tumor-initiating and -promoting activities of hydrogen peroxide (H_2O_2) in Sencar mice*

Treatment protocol (H_2O_2, amount/application)	No. of mice alive at 25 weeks	No. of papillomas per mouse at 25 weeks	Percentage of mice with papillomas at 25 weeks
Tumor-promoting activity, (DMBA initiation followed by either once or twice-weekly applications of H_2O_2)			
Concentrated solution (2×/wk)	58	0.06	6
H_2O_2:acetone 1:1 (1×/wk)	59	0.10	5
H_2O_2:acetone 1:1 (2×/wk)	59	0.08	8
H_2O_2:acetone 1:2 (2×/wk)	59	0.10	10
H_2O_2:acetone 1:5 (2×/wk)	60	0.15	10
Acetone 0.2 ml (2×/wk)	60	0	0
Tumor-initiating activity (H_2O_2 one application followed by TPA promotion)			
H_2O_2:acetone 1:1	58	0.1	10
Acetone 0.2 ml	57	0.06	6
Complete carcinogenic activity[a] (H_2O_2 2×/wk)			
H_2O_2:acetone 1:1	57	0.06	6

[a] The complete carcinogenic activity of H_2O_2 was investigated for 50 weeks. No squamous cell carcinomas developed during this period.

and was not able to induce tumor formation when used as an initiator followed by repetitive application of TPA.

H_2O_2 was also ineffective as an initiator or as a complete carcinogen but, in contrast to LP, was an extremely weak promoter (Table III).

DISCUSSION

Although the importance of free radicals in radiation and chemical carcinogenesis has been suggested [9–12], both LP and BP failed to effectively produce skin tumors in a series of experiments carried out almost two decades ago [1]. Recently we have demonstrated that although BP is ineffective either as an initiator or as a complete carcinogen, it is a potent tumor promoter [4].

In the present report we have described analagous properties of LP. On the other hand, H_2O_2 did not show strong tumor-promoting capacity.

Free radical producing peroxides have been described as strong skin irritants [2,3]. H_2O_2, especially in concentrated solutions, is extremely toxic and we were able to produce massive epidermal necrosis accompanied by extensive endothelial damage of the dermal blood vessels. Neither BP nor LP has shown similar properties at the dosages used. Although the toxicity of H_2O_2 could account for the lack of promoting efficiency of undiluted or highly concentrated H_2O_2 solutions, because of a destruction of initiated epidermal cells, this does not adequately explain the lack of promoting ability of the 1:5 diluted solution which does not produce extensive epidermal necrosis. On the other hand, the diluted solutions of H_2O_2 neither produce epidermal hyperplasia nor induce the formation of dark basal cells. The more concentrated solutions do produce hyperplasia and induce dark keratinocytes, but these phenomena are part of a regenerative effort following extensive epidermal necrosis. It is possible that repetitive treatments with concentrated H_2O_2 solutions, although having tumor-promoting capacity, are too toxic to permit the survival of a large population of initiated cells, whereas the low concentration solutions are too weak to unfold the necessary proliferative reactions which accompany and probably are necessary for tumor promotion. In addition, cellular catalase and glutathione peroxidase, which can detoxify intracellular H_2O_2 and other hydroperoxides [13], may prevent some of the intracellular effects of these agents which may be necessary for tumor promotion. In preliminary experiments, we have found that tert-butyl hydroperoxide, like H_2O_2, is a very weak tumor promoter and a potent epidermal hyperplasiogenic agent. Glutathione peroxidase can effectively use H_2O_2 and other hydroperoxides as a substrate but not LP (dilauroyl peroxide) and other dialkyl peroxides [14]. Free radicals generated by hydroperoxides and dialkyl peroxides may cause lipid peroxidation in the plasma membrane which may cause the epidermal hyperplasia. Once these peroxides enter the cell the hydroperoxides are rapidly detoxified, whereas dialkyl peroxides are not; thus the free radicals generated may have additional effects, such as DNA damage, which might also be necessary for tumor promotion [14].

Raick [15–16] has shown that potent tumor promoters induce dark basal keratinocytes in the epidermis. In recent reports we have described a good correlation between the percentage of dark basal keratinocytes and the promoting efficiency of hyperplasiogenic compounds [5–7]. In the present report we describe a five-fold increase (10%) in the percentage of dark basal cells induced by both LP and BP 2 to 4 days after topical application, as well as a sustained three- to four-fold increase which is seen throughout the first 6 days after treatment. This is accompanied by a very early epidermal hyperplasia. On the other hand, H_2O_2, although also inducing a large number of dark cells, exhibits a less sustained effect with a later peak of 10% at day 6 and markedly lower values before and after this maximum. It has to be noted that H_2O_2 produces a marked destruction of the epidermis, followed by a relatively late (6 days) regenerative hyperplasia, which also peaks 6 days after topical application.

In conclusion, the early changes induced by single application of LP and BP, i.e., early epidermal hyperplasia and especially the sustained induction of dark keratinocytes, tend to correlate well with their respective relatively potent tumor-promoting abilities.

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Epidermal Cell Production of Thymocyte Activating Factor (ETAF)

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Since epidermal Langerhans cells share many of the surface marker characteristics and functions of cells of the monocyte-macrophage series, we sought to determine whether Langerhans cells, like macrophages, produce Interleukin 1 (IL-1), formerly called lymphocyte activating factor. Heterogenous suspensions of murine epidermal cells were cultured for 3 days with or without various stimulants and their cell-free supernatants were tested for IL-1 activity in a thymocyte proliferation assay. Significant augmentation of lectin induced thymocyte proliferation was produced by the supernatants of these cultures. However, when epidermal cells were depleted of Langerhans cells by treating with anti-Ia antiserum and complement, the supernatants still produced significant augmentation. As the augmenting activity was due to a factor found in the supernatants of epidermal cell suspensions devoid of Langerhans cells the factor is called Epidermal Cell Thymocyte Activating Factor (ETAF). ETAF was produced by unstimulated epidermal cells, but significantly more was produced by epidermal cells that were incubated with phorbol myristic acetate, a low molecular weight tumor promoting agent or muramyl dipeptide, the synthetic analog of the cell wall of mycobacterium smegmatis. ETAF production was reduced by X-irradiation and was completely abolished when protein synthesis was inhibited. The molecular weight of this factor was approximately 15,000 dalton, which is similar to the molecular weight of macrophage derived IL-1; its heat stability and pH stability were similar to that of IL-1. ETAF, like IL-1 also enhanced lymphocyte production of Interleukin 2 (IL-2). The data indicate that epidermal cells, devoid of Langerhans cells, are capable of producing factors with IL-1 like activity and may thereby modulate immune responses locally in the skin and perhaps systemically.

The role of the macrophage in T-cell activation is mediated in part by a 12,000 to 16,000 molecular weight factor called lymphocyte activating factor or Interleukin 1 (IL-1) [1–8]. IL-1 is capable of enhancing lectin-induced DNA synthesis in thymocytes [5,9] and appears to be involved in peripheral helper T cell activation [1–3]. Macrophages produce IL-1 in response to a wide variety of stimuli including endotoxin, phorbol esters, lectin-activated T cells and lymphokines [9–12]. In view of the relationship between certain macrophages and epidermal Langerhans cells (LC) (e.g., both bear receptors for the Fc portion of IgG and for C3b, synthesize and express Ia antigens, present