

Effect of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin on Murine Skin

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The effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on skin of congenic haired and hairless newborn and adult HRS/J mice was studied. In all adult animals topical application of TCDD caused an involution of sebaceous glands. Epidermal/epithelial hyperplasia and hyperkeratinization was induced in the hairless, but not the haired mice. Transglutaminase (TG) activity was stimulated in both haired and hairless animals. A single application of 1 μ g of TCDD did not stimulate significant ornithine decarboxylase activity in the skin in either strain. Other than a reduction in the density

of the inflammatory cell infiltrate in the dermis, topical treatment with antiinflammatory agents fluocinolone acetonide and indomethacin did not affect the cutaneous response to TCDD. Skin of newborn mice treated topically with TCDD over a 2-wk period reacted much the same as adult skin in that sebaceous glands were reduced in size and TG activity was stimulated in both haired and hairless neonates; but epidermal hyperplasia occurred only in the hairless, not the haired newborns. *J Invest Dermatol* 90:354-358, 1988

In humans the skin is a primary target tissue for manifesting the toxic effects of the unique group of polyhalogenated aromatic hydrocarbons of which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most potent known prototype [1]. Exposure to these chemicals leads to the development of an acneform eruption in humans, which involves metaplasia and involution of sebaceous glands, and hyperplasia/hyperkeratinization of the epithelium lining ducts of cutaneous sebaceous follicles [2,3]. This syndrome, termed chloracne, has led to the use of the term chloracne to describe the chemicals that induce this effect.

The skin of laboratory animals generally does not exhibit a similar sensitivity, and in most species cutaneous changes are seen as late manifestations of systemic toxic effects following chloracne exposure [4]. Known exceptions to this are skin of the inner surface of the rabbit ear, facial skin of monkeys, and skin of hairless mice. This latter animal model has been used in recent years to analyse the mechanisms of TCDD toxicity in skin [5-8].

As little as 0.6 μ g of TCDD applied topically in 0.1 μ g aliquots to the dorsal skin of hairless mice over a 2-wk period induces a well-

defined pattern of cutaneous changes that involve involution and rapid disappearance of sebaceous glands, hyperplasia/hyperkeratinization of the epidermis, and hyperkeratinization of epithelial cysts (which are vestigial remnants of hair follicles characteristically present in the dermis of these animals). In many ways the changes in mouse skin are similar to the changes seen in human skin in TCDD induced chloracne.

Poland and Knutson [5,8] observed that skin of congenic haired mice that were genetically identical to the hairless animals whose response to TCDD was described above, except for a difference at the hr locus on chromosome 14, were totally unaffected after similar exposure to TCDD. They postulated that in murine skin, expression of TCDD-induced toxicity was dependent on the coordinate gene expression at the Ah and hr genetic loci. The Ah locus is considered the putative structural gene for the Ah receptor and is thought to mediate ligand recognition, while the hr locus that controls for hairlessness in mice appeared to determine the extent of the responses under the control of the TCDD receptor.

Recently we observed that keratinocytes derived from both haired as well as hairless newborn mice, when grown in vitro in tissue cultures, responded to TCDD stimulation by an increase in cell proliferation and terminal differentiation [9,10]. Thus the differences described by Poland and Knutson at the in vivo level, were not manifested by keratinocytes in vitro.

In the present study we have re-examined the comparative cutaneous response of congenic newborn and adult haired and hairless mice to TCDD exposure in vivo in an effort to define this in greater detail. Histologic changes in TCDD-treated skin, as well as changes in transglutaminase (TG) and ornithine decarboxylase (ODC) activity of skin after TCDD treatment, and the effect of fluocinolone acetonide and indomethacin on the parameters listed above, were monitored.

MATERIALS AND METHODS

Chemicals Dithiothreitol (DTT), pyridoxal-5-phosphate, L-ornithine HCl dimethyl casein, indomethacin, fluocinolone acetonide, putrescine, Tris base, ethylenediaminoacetic acid (EDTA), trichloroacetic acid (TCA), and 12-O-tetradecanoylphorbol

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Abbreviations:

- DTT: dithiothreitol
- EDTA: ethylenediaminoacetic acid
- ETG: epidermal transglutaminase
- FA: fluocinolone acetonide
- I: indomethacin
- NCS: tissue solubilizing solution
- OCS: nonaqueous liquid scintillation solution
- ODC: ornithine decarboxylase
- TCA: trichloroacetic acid
- TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
- TG: transglutaminase
- TPA: 12-O-tetradecanoylphorbol 13-acetate

13-acetate (TPA), were purchased from Sigma Chemicals (St. Louis, Missouri). [^3H]Putrescine (sp act 28 Ci/mmol) was obtained from New England Nuclear (Cambridge, Massachusetts) and L-[1- ^{14}C]-ornithine HCl (sp act 56 mCi/mmol), NCS tissue solubilizing and OCS scintillation solutions from Amersham (Arlington Heights, Illinois).

Six- to eight-week old female congenic HRS/J (hr/hr) hairless, and HRS/J (hr/+ or +/+) haired mice were obtained from the Jackson Laboratories, Bar Harbor, Maine. Age-matched animals were divided into groups of 3 to 4 animals per group. Animals in each group were housed together in plastic cages. The TCDD-treated animals were kept inside biohazard safety containment boxes (Class III Gloveboxes) for the duration of the experiments.

The effect of TCDD on newborn mouse skin was studied in haired and hairless newborn mice obtained from genotypically segregated breeding colonies that had been established at UCLA. Because hairless HRS/J females routinely fail to care for their pups (possibly due to a reduction in mammary gland tissues, which is an anomaly that has been associated with these mutants), newborns from hairless mothers were kept with surrogate haired mothers for the duration of TCDD treatment.

Treatment

Depilation The day before treatment was initiated, the backs of haired animals were shaved and depilated by a 7-min exposure to a mixture of sodium and calcium thioglycolate and calcium hydroxide in a lotion base (Nair, Carter Products, New York). To standardize the treatment, hairless mice were also treated with depilatory lotion in a similar manner.

2,3,7,8-Tetrachlorodibenzo-p-Dioxin One-tenth microgram of TCDD in 0.1 ml of acetone was applied topically to the backs of adult animals three times a week for 2 wk. Newborn mice were treated with 0.01 μg of TCDD dissolved in 10 μl of acetone, three times a week for 2 wk. Similar numbers of animals in parallel groups were treated with acetone alone.

Antiinflammatory Agents The effect of topical application of two antiinflammatory agents, fluocinolone acetonide (FA) and indomethacin (I) on TCDD-induced changes in cutaneous morphology and TG activity was compared in haired and hairless mice. Animals were treated with TCDD in acetone as described previously. Additional treatment groups received 10 μg of FA in 0.1 ml of acetone topically three times a week for 2 wk, or 50 μg of I in 0.1 ml of acetone once daily for 2 wk, in addition to the treatment with TCDD or with acetone, as already described. Animals receiving more than one treatment were treated with one agent in the morning and the other in the afternoon. Additional control groups received FA and I alone.

Histologic Studies At the end of treatment, the mice were killed by cervical dislocation, and biopsy specimens were obtained from treated areas of skin for histologic studies. The specimens were fixed in 10% formalin and paraffin embedded. Tissue sections were stained with hematoxylin and eosin, Masson's trichrome stain (for collagen), colloidal iron (for mucin) and Verhoeff's stain (for elastic fibers) [11].

Transglutaminase Assays Back skins were removed immediately after mice had been killed by cervical dislocation. Epidermis was separated from the dermis by immersing skins in a 55°C waterbath for 30 s followed by immersion in ice-cold water. Skins were blotted dry and the epidermis scraped off the dermis with a scalpel while maintaining the skin flat on a cooled glass plate. Each epidermis was added to 0.75 ml of ice-cold homogenizing buffer containing 0.25 M sucrose, 0.05 M Tris (pH 7.5), and 1 mM EDTA, and homogenized with a Polytron tissue homogenizer. After centrifugation the supernatants were removed and the pellet resuspended in 0.5 ml of ice-cold homogenizing buffer. Transglutaminase activity was measured in supernatants and pellets separately by using the methods outlined by de Young and Ballaron [12]. A 0.1-ml sample was tested in 0.5 ml of assay solution containing 0.05 M Tris (pH 8.1), 0.01 M

CaCl_2 , 5 mM DTT, 0.6 mg of dimethyl casein, 0.4 μCi [^3H]putrescine (dissolved in 1 mM cold putrescine). Tubes were incubated for 60 min at 37°C with shaking. Activity was terminated by adding 0.6 ml of 10% TCA and 5 ml of 5% TCA containing 0.1% cold putrescine. After 30 min at room temperature, the tube contents were filtered on 25-mm Whatman GF/A filter discs. The discs were washed once with 10 ml of 5% TCA containing 0.1% cold putrescine, and then washed twice with 10 ml of 100% ethanol. After drying, the discs were placed in Aquasol and the radioactivity counted. Background counts were obtained by using boiled enzyme solution. Soluble proteins were quantitated by the Bradford assay for proteins [13]. Specific activity was calculated by dividing total transglutaminase activity by total soluble protein.

Ornithine Decarboxylase Assays The skin for ODC assays was obtained from depilated haired and hairless HRS/J mice 4, 8, and 24 h after treatment with a single application of 1 μg (3.1 nmole) of TCDD. As positive controls, the epidermises of mice that had been treated once with 17 nmole of 12-0-tetradecanoylphorbol 13-acetate (TPA) and killed 4 h later were processed in parallel to epidermal samples from TCDD-treated animals and from acetone treated negative controls. Epidermis was separated from the dermis by heat treatment at 58°C for 30 s followed by immersion in ice water and scraped off with a scalpel. A Polytron tissue homogenizer was used to homogenize epidermal samples individually in 1 ml of buffer containing 50 mM sodium phosphate buffer, pH 7.2, 0.4 mM pyridoxal-5-phosphate, and 5 mM DTT. Homogenates were stored frozen at -60°C. The ODC assays were performed according to the methods previously published by Connor and Lowe [14] by measuring the release of $^{14}\text{CO}_2$ from L-[1- ^{14}C]ornithine HCl substrate. Duplicate samples of 0.25 ml of homogenate were incubated in tubes fitted with side arms. The $^{14}\text{CO}_2$ released was collected directly into scintillation vials containing 0.15 ml of NCS, which were attached to the incubation tubes via the side arms. Fifty microliters of ornithine substrate was added to each tube and the mixture incubated at 37°C in a metabolic shaker for 1 h. The reaction was terminated by adding 0.5 ml of 2 M citric acid to each tube through a sleeve and continuing the incubations for an additional 45–60 min to insure absorption of all released $^{14}\text{CO}_2$ by the NCS. At the end of the second incubation, 10 ml of OCS counting solution was added to the vials and the radioactivity counted. Controls consisted of duplicate blanks containing all components except tissue homogenates, and duplicate standards containing 50 μl of ornithine substrate in 0.15 ml of NCS. The ornithine substrate contained 0.5 μCi of L-[1- ^{14}C]ornithine HCl per 0.05 ml of a solution containing 6 mM L-ornithine in the homogenizing buffer. Proteins were measured using the Bradford protein assay [13].

RESULTS

TCDD Treatment In skin of hairless mice (Figs 1A and 1B), treatment with a total of 0.6 μg of TCDD over a 2-wk period induced a doubling in the thickness of the epidermis, from 2–4 cell layers to 4–8 cell layers. Focal areas of epidermal parakeratosis indicated a rapid turnover of epidermal cells. Sebaceous glands had disappeared entirely. Most of the epithelial cysts normally present in the dermal layer of hairless mouse skin had been transformed into large keratinized cysts, and in many of these the cyst wall was disrupted. Granuloma formations were evident in areas around disrupted cysts, presumably as a result of the intrusion of keratinous material from the cysts into the dermis. Verhoeff's stain indicated some loss of elastic fibers in the dermis, possibly a secondary effect of the marked inflammatory response. Other stains for specialized dermal components were unchanged in TCDD-treated skin, compared to acetone treated controls.

In skin of haired mice (Figs 1C and 1D), sebaceous glands are connected to hair follicles and do not open directly to the epidermal surface (as they do in hairless mice). The epithelial cysts present in hairless mouse skin are absent in skin of haired mice. The TCDD treatment induced an involution of sebaceous glands in skin of haired mice, just as had occurred in hairless animals. However,

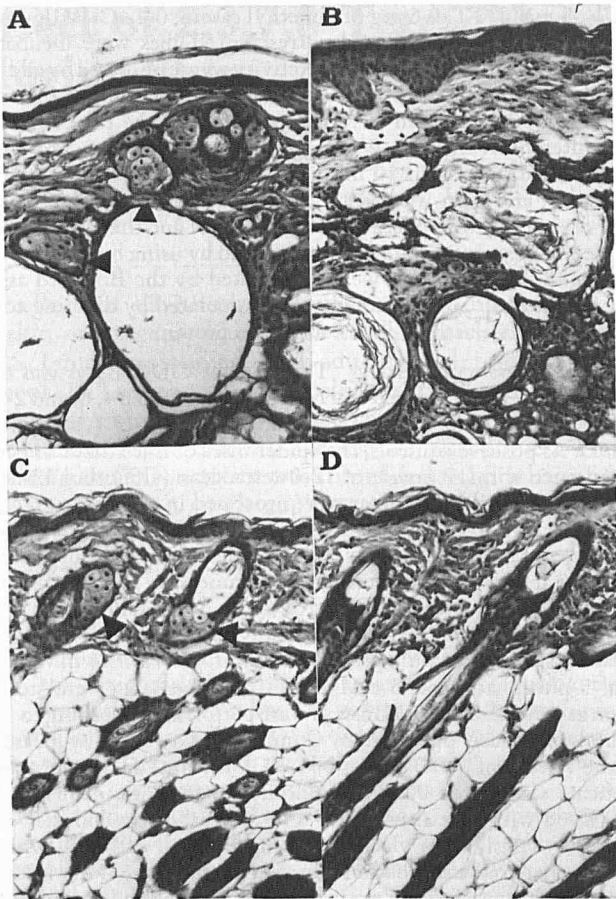


Figure 1. Light microscopic findings in (A,B) adult hairless (hr/hr) and (C,D) haired (hr/+ or +/+) HRS/J mouse skin after topical application of acetone (A,C) and TCDD (B,D) over a 2-wk period (see Methods). Note the presence of sebaceous glands (black triangles) in acetone-treated skin, and absence of such glands in both hr/hr and hr/+ skin after TCDD treatment. Also note the epidermal hyperplasia, hyperkeratinization of epithelial cysts, and inflammatory cell infiltrate in the dermis of hairless mice (B) and the absence of these changes in the haired mice (D).

epidermal thickness remained unchanged and no inflammatory cell infiltrate was seen. Stains for specific dermal components did not reveal any further changes following TCDD treatment.

Indomethacin and Fluocinolone Acetonide Treatment In the skin of hairless mice, topical application of FA and I in conjunction with TCDD treatment reduced the intensity of the inflammatory cell response in the dermis, but it had no other visible modifying effects on the epidermal hyperplasia and hyperkeratinization of epithelial cysts seen in hairless mouse skin in response to TCDD treatment.

Treatment with FA alone induced a thinning of the epidermis. In haired mice, treatment with I had no detectable effect on cutaneous histology. Treatment with FA induced epidermal thinning and involution of the hair follicles. This occurred in mice treated with FA alone, as well as in mice treated with both TCDD and FA.

Newborn Mouse Skin In newborn mouse skin, sebaceous glands are small and associated with the developing hair follicles. After TCDD treatment, sebaceous glands had involuted in both haired and hairless mouse skin. Epidermal hyperplasia was evident in skin of the hairless, but not in the haired genotypes (Fig 2). To confirm the histologic observations relative to epidermal hyperplasia, the soluble protein content of defined areas (1.65 cm²) of epidermis was measured in newborn mouse skin. The TCDD treatment induced a doubling of total soluble protein in epidermis of hairless mice, but it had no effect on soluble epidermal protein content in skin of haired newborns.

Transglutaminase Activity Topical treatment with TCDD over a 2-wk period resulted in a two- to threefold increase in cutaneous TG activity in both haired and hairless mice (See Table I). The basal TG activity in haired mice was significantly higher than in hairless animals. Thus, although final levels of TG activity in haired animals treated with TCDD were higher than in TCDD-treated hairless mice, the magnitude of the increase in activity (compared with acetone-treated controls) was consistently more pronounced in the hairless compared to the haired animals. Although TG activity increased in both the soluble and the insoluble fraction of epidermal homogenates, this increase was always higher in the soluble fraction suggesting that the cytosolic TG was primarily affected by treatment of skin with TCDD.

Treatment with I had no significant effect on the levels of TG activity in any of the treated or control groups. Fluocinolone ace-

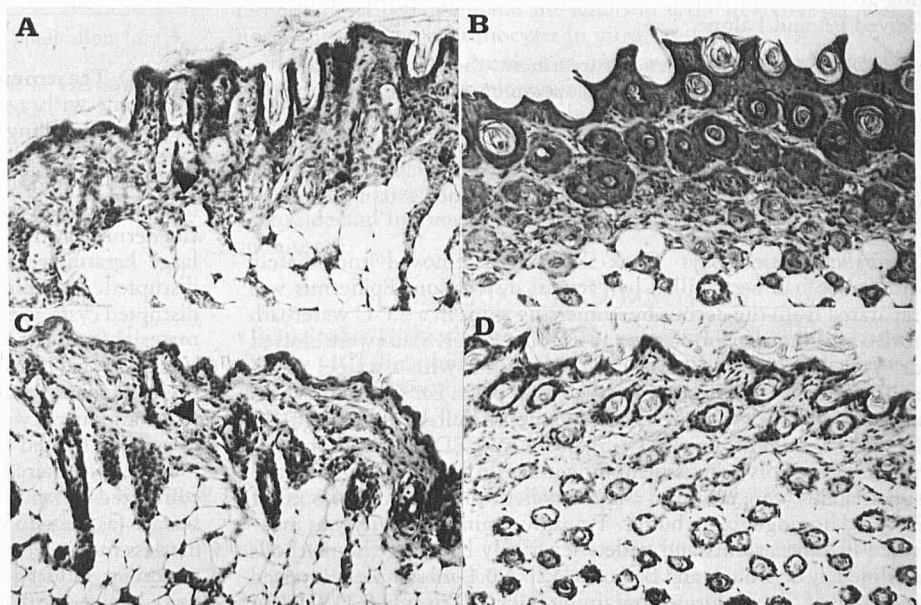


Figure 2. Light microscopic findings of (A,B) newborn hairless (hr/hr) and (C,D) haired (+/+) HRS/J mouse skin treated with acetone (A,C) or with TCDD (B,D) over a 2-wk period. Note presence of hair follicles in all sections. Sebaceous glands are present in acetone treated skin (A,C, black triangle), but have disappeared in TCDD treated skin (B,D). Epidermal hyperplasia is present in skin of hr/hr newborn skin treated with TCDD (B) but not in skin of +/+ newborns treated with TCDD (D).

Table I Transglutaminase Activity^a in Epidermis of HRS/J Adult Mice

Treatment ^b	Haired (+/+)		Hairless (hr/hr)	
	Supernatant	Pellet	Supernatant	Pellet
TCDD	17.5 ± 6.7	13.3 ± 5.8	13.3 ± 5.8	4.1 ± 0.8
Acetone	8.3 ± 3.8	4.1 ± 0.8	4.1 ± 0.8	4.1 ± 0.8

^a Expressed as nanomoles putrescine incorporated per hour per milligram of protein at 37°C by combined soluble and insoluble fractions of epidermal homogenates (measured separately), mean values of three separate experiments ± SDs. Total number of mice in each treatment group was 10. The increase in TG activity in TCDD-treated mice (with TG values of acetone-treated animals = 100%) was supernatant 270%, pellet 180% for haired mice and supernatant 370%, pellet 270% for hairless mice.

^b Mice were treated three times a week for 2 wk with 0.1 µg TCDD in 0.1 ml of acetone, or with acetone alone.

tonide treatment by itself increased TG activity in skin of hairless but not haired mice. However, FA treatment had no effect on TG levels in any of the other treatment groups.

Transglutaminase activity in the skin of both haired and hairless newborn mice increased following TCDD treatment in approximately the same proportions as in skin of adult animals (Table II).

Ornithine Decarboxylase Activity The ODC response induced in murine epidermis after topical application of 1 µg of TCDD was minimal compared to the response induced by application of 17 nmoles of TPA (Table III). Time-course studies indicated that the time of maximal ODC response to TCDD (during a 24-h period) was at 4 h.

DISCUSSION

Knutson and Poland, and Poland et al [5,8] established that there are fundamental differences in the cutaneous response of congenic haired and hairless mice to TCDD exposure *in vivo*. According to their work, the skin of haired mice was entirely unaffected by TCDD exposure, whereas in skin of congenic hairless littermates, sebaceous glands involuted and the epidermis and epithelial cysts developed a characteristic hyperplastic/hyperkeratotic response. The present studies have attempted to delineate the TCDD-induced changes in murine skin in greater detail and suggest that although clear differences exist, haired mouse skin is not unaffected by TCDD exposure.

In the present study one of the first effects of TCDD in mouse skin was the involution of sebaceous glands. This was seen in skin from haired as well as hairless animals, and thus appears to be unrelated to the presence or absence of a mutation at the *hr* locus. It is known that topical application of other polycyclic aromatic hydrocarbons, particularly those containing the benzenanthracene structure, induces suppression of sebaceous gland activity in mouse skin [15-18]. Within this class of compounds, the sebaceous gland "suppression index" has been found to correlate with tumor-producing capacity in mice and a mouse skin sebaceous-gland suppression test has been described as one of several short-term tests for screening organic chemicals for carcinogenic potential [19]. Whether the sebaceous suppressive action of TCDD is effected by the same mechanisms as that of the polycyclic tumor promoters is unknown.

Table II Transglutaminase Activity^a in Epidermis of HRS/J Newborn Mice

Treatment ^b	Haired (+/+)		Hairless (hr/hr)	
	Supernatant	Pellet	Supernatant	Pellet
TCDD	7.5	11.0	7.7	12.3
Acetone	2.8	6.3	2.6	5.5

^a Expressed as nanomoles putrescine incorporated per hour per milligram of protein at 37°C. Total number of mice in each group was 5.

^b Mice were treated three times a week for 2 wk with 0.01 µg of TCDD in 10 µl of acetone, or with acetone alone.

Table III Ornithine Decarboxylase Activity^a HRS/J adult mice

Treatment ^b	N	hr/hr	N	+/+
Acetone	6	0.28 ± 0.11	8	0.26 ± 0.18
TPA	6	10.21 ± 1.39	8	20.48 ± 6.84
TCDD	5	0.40 ± 0.18	12	0.31 ± 0.18

^a Expressed as nanomoles of CO₂ released per hour per milligram of protein, taken from the mean values of two to three experiments ± SDs.

^b Animals treated with 0.1 ml acetone (control), 17 nmoles TPA, or 1 µg of TCDD, 4 h prior to being killed by cervical dislocation.

N = Total number of animals tested in each group.

Two other effects of TCDD that typically occur in skin of hairless mice (hyperkeratinization of epithelial cysts and development of an intense inflammatory cell response in the dermis) are absent in skin of haired animals. The dermal epithelial cysts in skin of hairless mice are vestiges of involuted hair follicles that develop after the shedding of the first coat of hair between the 12th and 24th days of age [20]. In haired animals, the hair follicles are not lost, the epithelial cysts are therefore absent, and consequently not affected by exposure to TCDD. The inflammatory cell infiltrate seen in skin of hairless mice after treatment with TCDD appears to be a secondary response to the rupture of the contents of the keratinized cysts into the dermis, as evidenced histologically by the presence of multinucleolated giant cells in close proximity to the disrupted cyst walls.

Thus, in effect, the main difference between skin of haired and hairless congenic mice to TCDD exposure that remains unexplainable is the seemingly total absence of epidermal hyperplasia in haired animals, and the induction of marked epidermal hyperplasia/hyperkeratinization in the hairless mutants. The fact that this is expressed already in skin of newborn mice in which the genotypical differences are masked (until the 3rd week of life), suggests that the difference in response to TCDD is under genetic control and not a secondary effect of phenotypic differences (such as variations in epidermal thickness, presence or absence of hair follicles) that occur in the skin of congenic adult haired and hairless HRS/J mice.

Despite the fact that the intensity of the inflammatory cell infiltrate in the dermis appeared slightly reduced after the topical administration of high concentrations of either the potent steroidal antiinflammatory agent fluocinolone acetonide or the nonsteroidal prostaglandin inhibitor indomethacin, neither compound was effective in inhibiting TCDD-induced epidermal hyperplasia and epithelial cyst keratinization in hairless mouse skin. The fact that indomethacin treatment did not interfere with the effect of TCDD confirms Knutson and Poland's finding that inhibitors of arachidonic acid metabolism, including indomethacin, failed to inhibit TCDD-induced keratinization of XB/3T3 cells *in vitro* [21]. Lack of effect of fluocinolone acetonide treatment in this regard, however, was more surprising. Others have found FA to markedly inhibit TPA induced epidermal hyperplasia in mouse skin [22]. The fact that fluocinolone acetonide failed to interfere with TCDD-induced epidermal hyperplasia and epithelial keratinization strongly suggests that the mechanisms of action of TPA and TCDD follow different pathways. This contention is supported by the results of the ODC assays. Induction of ODC activity has been regarded as a marker for cell proliferation, particularly after exposure of cells to tumor promoters such as phorbol esters [23,24]. In the present studies, TCDD did not stimulate significant epidermal ODC activity in either haired or hairless mice. This contrasts with the findings of Knutson and Poland [5] who reported that a single application of TCDD stimulated an ODC response in skin of hairless, but not haired, mice. The present results suggest that TCDD-stimulated ODC activity in mouse skin is minimal compared with the activity induced by application of potent ODC stimulators such as TPA. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin has been shown to be an effective tumor promoter in the two-step assay for carcinogenesis in hairless mouse skin [7]; however, the mechanisms involved in its activity may be different than that of other chemical tumor pro-

motors for which stimulation of epidermal ODC activity is regarded as an initial step.

The particulate form of epidermal transglutaminase is thought to be the marker enzyme for terminal epidermal differentiation [25]. In a previous study [26] in which we demonstrated that TCDD treatment of hairless mouse skin stimulated an increase in TG activity, we suggested that this response was related to the epidermal hyperkeratinization such exposure induced. In *in vitro* studies [9,10], we have also shown that TCDD induces a two- to four-fold increase in TG activity of murine keratinocyte cultures that can be correlated with increased competency to form cornified cell envelopes. This is seen with cultures derived from haired as well as hairless HRS/J mice [9,10], and it suggests that murine epidermal keratinocytes have the capacity to express TCDD-induced increased terminal differentiation regardless of whether or not they are derived from animals carrying a mutation at the *hr* locus. The finding in the present study that TCDD stimulated TG activity in skin of haired mice even in the absence of cell proliferation/hyperkeratinization suggests that for reasons unknown, the TCDD-stimulated terminal differentiation is only partially expressed in these animals *in vivo*. Further studies are in progress to identify the exact nature of the transglutaminase activity stimulated by TCDD in murine skin.

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