TOXICOLOGY AND APPLIED PHARMACOLOGY 73, 42–47 (1984)

Increased Epidermal Transglutaminase Activity following 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: *In Vivo* and *in Vitro* Studies with Mouse Skin

S. M. PUHVEL,[1] D. C. ERTL, AND C. A. LYNBERG

Division of Dermatology, Department of Medicine, School of Medicine, Center for Health Sciences, University of California, Los Angeles, Los Angeles, California 90024

Received June 18, 1983; accepted October 25, 1983

Increased Epidermal Transglutaminase Activity following 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: *In Vivo* and *in Vitro* Studies with Mouse Skin. PUHVEL, S. M., ERTL, D. C., AND LYNBERG, C. A. (1984). *Toxicol. Appl. Pharmacol.* **73**, 42–47. In previous studies it has been shown that topical treatment of hairless mice with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) induces hyperproliferation and hyperkeratinization in the epidermis of hairless mice. The present investigation demonstrated that such TCDD-induced morphological changes in skin *in vivo* are accompanied by increased levels in activity of epidermal transglutaminase (ETG), the enzyme associated with terminal epidermal differentiation. Exposure of mouse epidermal cells in tissue culture to 10^{-9} M TCDD also resulted in a significant increase in ETG activity, despite the fact that morphologically these cultures (grown at 0.07 mM ionic calcium concentrations) exhibited no signs of terminal differentiation. Thus one mechanism of action of TCDD in inducing cutaneous changes appears to relate to the stimulation of increased ETG levels.

In humans one of the first signs of exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the development of a skin condition known as chloracne (Moore, 1978). This syndrome involves hyperkeratinization of ductal epithelium of cutaneous sebaceous follicles, resulting in comedo formation which may or may not proceed to pustular and cystic chloracne. Experimental chloracne has been induced in skin of hairless mice by topical application of TCDD (Puhvel *et al.*, 1982; Knutson and Poland, 1982). Even though follicular involvement in mouse skin is insignificant, the hyperproliferative, hyperkeratinizing changes induced in interfollicular epidermis are sufficiently well defined to permit the use of this animal model to monitor TCDD-induced enzymatic changes in skin.

In this report we investigated the effect of TCDD on epidermal transglutaminase (ETG) activity first in skin of hairless mice *in vivo* following topical application of TCDD. Then, to verify our observations in a more defined system, changes in ETG activity of mouse epidermal cells in tissue cultures were monitored following addition of TCDD to culture media.

METHODS

Chemicals. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was obtained from KOR, Incorporated (Boston, Mass.), and putrescine dihydrochloride, 2,3-^{3}H(N) (sp. act. 38.9 Ci/mmol) was obtained from New England Nuclear (Boston, Mass). Tissue culture Medium 199, calcium depleted Medium 199, fetal bovine serum (FBS), and antibiotic–antimycotic mixture were obtained from Grand Island Biological Co. (Grand Island, N.Y.); putrescine dihydrochloride, casein, dithiothreitol (DTT), and Trizma base were from Sigma Chemicals (St. Louis, Mo.); dimethylcasein was from Accurate Chemical and Scientific (Hicksville, N.Y.).

In Vivo Studies

Animals. HRS/J strain of hairless female mice were obtained from the Jackson Laboratories (Bar Harbor,

[1] To whom requests for reprints should be addressed.

0041-008X/84 $3.00

Maine). Animals were 10 to 11 weeks old at the beginning of the experiment. They were fed Wayne Lablox (Universal Feeds, Inc., Colton, Calif.) laboratory chow *ad libitum* and were housed four to a cage in disposable plastic cages inside a Biohazard safety containment box (Class III Glove box) for the duration of the experiments.

Treatment. Three times a week for 4 weeks animals were treated topically with 0.1 μg of TCDD dissolved in 0.1 ml of acetone. Control animals received 0.1 ml of acetone alone. Groups of four mice were killed by cervical dislocation at 7, 14, 21, and 28 days after the beginning of treatment. Skins were removed by dissection, and the epidermis was separated from dermis by first immersing the skins in a 55°C waterbath, followed by immersion in ice water. Following this treatment, the epidermis was scraped from the dermis with a scalpel, while maintaining the skins at 4°C. Epidermal samples were homogenized with a Polytron homogenizer, and the tissue cytosol was separated by centrifugation at 30,000*g* for 30 min. Supernatant fractions were used for the transglutaminase assays.

Transglutaminase assay of epidermal homogenates. The methods described by De Young and Ballaron (1982) were used. Briefly, 0.1 ml of tissue supernatant fractions was added to 0.5 ml of assay mixture containing 0.05 M Tris buffer (pH 8.1), 0.01 M $CaCl_2$, 0.05 M DTT, 0.6 mg dimethylcasein, and 0.37 μCi of [^{3}H]putrescine. Mixtures were incubated at 37°C for 60 min, and the reaction was stopped by adding 0.6 ml of 10% trichloroacetic acid (TCA) and 5 ml of 5% TCA containing 0.1% unlabeled putrescine. After 30 min at room temperature, incubation mixtures were filtered on 25-mm Whatman GF/A filter discs. Discs were washed once with 10 ml 5% TCA containing 0.1% unlabeled putrescine and twice with 10 ml 100% ethanol. After drying, the discs were placed in Aquasol II (New England Nuclear) and the radioactivity was counted. Soluble proteins in the supernatant fractions were quantified by the Bio-Rad protein assay with bovine serum albumin as standard (Bradford, 1976).

In Vitro Studies

Tissue culture. Basal keratinocyte cultures from neonatal BALB/c mice were established following the methods described by Marcelo *et al.* (1978). Basal keratinocytes were separated by discontinuous Ficoll gradient centrifugation, and 1.5×10^6 cells were inoculated per 30-mm-diameter culture dish. Cultures were grown in parallel in the following media: one-third of the cells were plated in Medium 199 supplemented with antibiotics and 10% FBS; the remainder were plated in calcium-depleted Medium 199 supplemented with antibiotics and 10% FBS (5% chelated, 5% normal). The final ionic concentrations of calcium in these media were 1.2 and 0.07 mM, respectively.

TCDD treatment. On the third day of culture, TCDD dissolved in dimethylsulfoxide (DMSO) was added in a final concentration of 10^{-9} M to half the low calcium cultures. The remainder of the low calcium cultures received DMSO alone in a final concentration of 0.1%. The medium was changed every other day and fresh TCDD and DMSO were added at every medium change.

Cells were harvested from four dishes of each of the three media on Days 3, 5, 7, 10, and 12 by scraping the plates with rubber spatula and collecting all cells (i.e., attached as well as free floating), by centrifugation. Cell pellets were washed twice in buffer and stored frozen at −60°C until used for the transglutaminase assay.

Transglutaminase assay of cell pellets. Cell lysates obtained by thawing and freezing cell pellets three to four times were used for the transglutaminase assay recommended by S. H. Yuspa and U. Lichti (personal communication). This procedure was an adaptation of the method described by Ogawa and Goldsmith (1976). Cells were pelleted in a buffer containing 50 mM Tris (pH 7.5), 2.5 mM DTT, 0.13 M NaCl, 0.83 mM EDTA, and 8.3 mM $CaCl_2$. The reaction mixture consisted of 100 μl of cell lysate, 20 μl of casein (20 mg/ml), 10 μl of [^{3}H]putrescine (5 μCi), and 20 μl of buffer. The mixture was incubated at 37°C for 10 min. Following incubation 50 μl of the mixtures was spotted on filter paper discs (Whatman 3MM) and immediately precipitated by immersion in 10% ice cold trichloroacetic acid (TCA) plus 0.1% putrescine. The precipitates were washed in four separate 30-min washes of 5% TCA plus 0.1% putrescine, then immersed in 100% ethanol, and dried. The radioactivity of the precipitates on the filter paper discs was counted in a Beckman LS-7500 liquid scintillation counter. Results were corrected for background radioactivity found in lysate-free parallel assays. Soluble proteins in the cell lysates were determined by the Bio-Rad protein assay.

To monitor the effect of 10^{-9} M TCDD on epidermal cell proliferation, cell counts were performed at regular intervals in cultures grown in low calcium medium, with or without the presence of TCDD in the medium. The effect of TCDD on terminal differentiation of epidermal cells in culture was investigated by transferring cultures grown in low calcium medium (with and without the presence of 10^{-9} M TCDD) to high calcium medium on the seventh day of culture, and monitoring the morphology of the cultures and the rate of cornified cell envelope formation at 24 hr to 5 days after the change to the 1.2 mM Ca^{2+} medium. Cornified envelope counts were performed according to the methods described by Yuspa *et al.* (1982) with insolubility in 2% SDS and 20 mM DTT at 90°C for 5 min as the criterion for defining cornified envelopes.

RESULTS

In Vivo Studies

Hairless HRS/J mice treated topically with TCDD developed the same pattern of well-defined cutaneous changes which have been described in detail elsewhere (Puhvel *et al.*,

1982). Essentially, epidermal hyperplasia accompanied by involution of sebaceous glands, hyperkeratinization of the epidermis, and keratinization of dermal cysts are the hallmarks of hairless mouse "chloracne."

These gross morphologic changes were accompanied by significant elevations in epidermal transglutaminase activity. By the end of the second week of treatment, the epidermal transglutaminase (ETG) levels were more than sixfold the baseline levels, or the levels in the skin of acetone-treated mice (Fig. 1). By the end of the third week of treatment, ETG levels had decreased from the initial peak but still remained at more than twice the baseline levels at the termination of the experiment at 4 weeks.

To test for the possibility that TCDD present in the treated mouse skins was carried into the ETG assay and exerted a direct activating effect on the enzyme in the epidermal cytosol preparation, 0.2 ng of TCDD was added directly to the ETG assay mixture and the effect monitored. No changes in radioactivity of the resulting precipitates was observed.

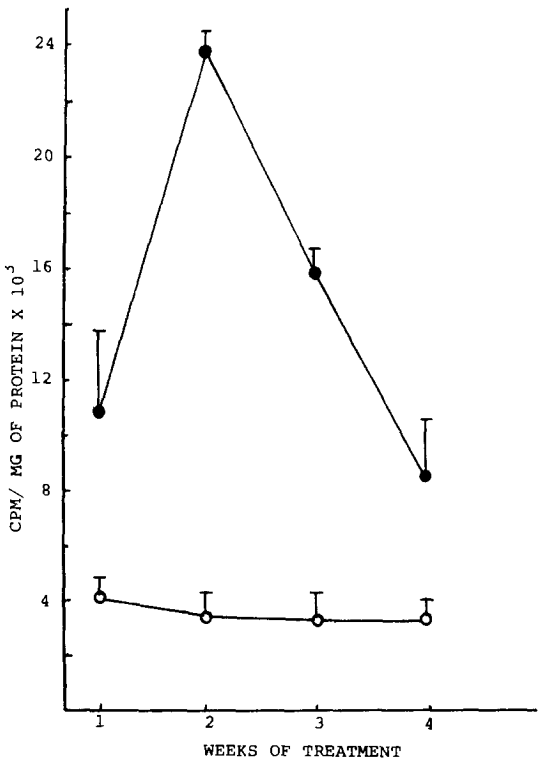

FIG. 1. Epidermal transglutaminase activity in skin of hairless mice treated with TCDD (● —— ●) and in controls (○ —— ○), expressed as cpm incorporated during 30 min of incubation/mg of epidermal cytosol protein. See Methods for details.

In Vitro Studies

The ionic concentration of calcium in the medium has been well established as a regulator of mouse epidermal cell terminal differentiation in *in vitro* tissues cultures (Hennings *et al.*, 1980) and our results were in line with this finding. In low calcium medium, mouse keratinocytes did not undergo terminal differentiation but continued to proliferate as basal cells in monolayers (Fig. 2A). Addition of 10^{-9} M TCDD did not change this pattern of growth at least in so far as could be determined by light microscopy (Fig. 2B). Nor did addition of TCDD have any effect on the rate of cell proliferation as measured by comparison of total cell counts of cultures, 24, 48, 72, and 96 hr after the addition of TCDD or DMSO to the low calcium medium. Total protein content (measured to the twelfth day) was similar in cultures grown with or without TCDD. Cells grown in high calcium medium started to stratify and differentiate 72 hr after plating (Fig. 2C).

ETG activity in the TCDD-treated cells in low calcium medium gradually increased over the 12-day period of growth, so that on the twelfth day ETG levels in the TCDD-treated, undifferentiated cells were almost identical to levels in the fully differentiating cells grown in high calcium medium (Fig. 3). ETG levels in the undifferentiating cells grown in low calcium medium without the addition of TCDD also increased slightly over the 12-day incubation period, but remained at about half the level of activity in the previously described cultures.

That this was a genuine increase of ETG production by the cells, rather than an activation of ETG activity already present in the cells, was shown by the fact that 10^{-9} M TCDD added directly to the transglutaminase assay did not have any effect on the results.

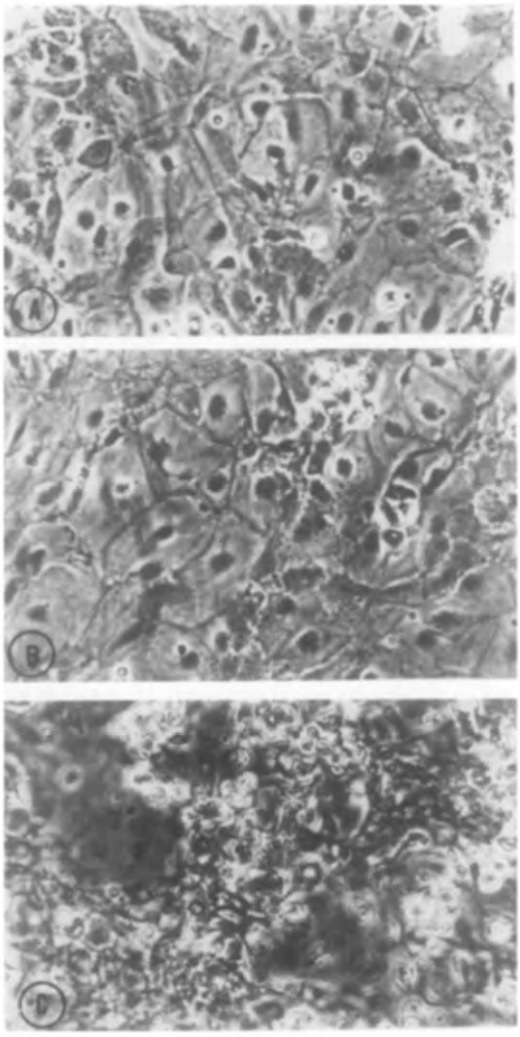

FIG. 2. Neonatal mouse epidermal cell cultures (A) in 0.07 mM Ca^{2+} medium; (B) in 0.07 mM Ca^{2+} and 10^{-9} M TCDD; and (C) in 1.2 mM Ca^{2+} medium at 7 days after plating. Note that TCDD did not affect cell culture morphology in the low calcium medium.

Changing keratinocyte cultures from low calcium to high calcium medium on Day 7, at a time when it had been established that ETG activity was high in the TCDD-treated, undifferentiated cells, indicated that terminal differentiation was not the same in TCDD-treated cells as in control cells grown in low calcium medium without the presence of TCDD. The cornified envelope count was consistently slightly higher in the TCDD-treated cells (0.7%) than in the control cells (0.3%), but it remained much lower than the counts of cornified envelopes in cells grown in high Ca^{2+} medium from the beginning (12 to 14%). Morphologically, terminal differentiation and cell death were delayed in the TCDD-treated cultures, in a manner similar to what has been reported for retinoic acid-treated cultures by Yuspa *et al.* (1981) (Fig. 4).

DISCUSSION

The changes induced in human skin by TCDD exposure are among the more observable and well-defined biological effects of this potent man-made toxin. In the hairless mouse model, topical application of minute amounts of TCDD induces a distinct hyperproliferative, hyperkeratinizing response (Puhvel *et al.* 1982; Knutson and Poland, 1982). Epidermal transglutaminase is considered a marker enzyme for terminal epidermal differentiation (Goldsmith and Martin, 1975; Buxman and Wuepper, 1976). This soluble, calcium-dependent enzyme which catalyses the formation of co-

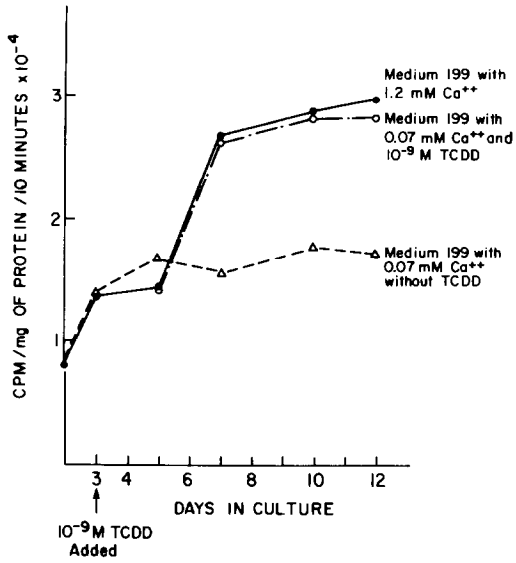

FIG. 3. Epidermal transglutaminase activity in cultures grown in 1.2 mM Ca^{2+} (● —— ●), in cultures grown in 0.07 mM Ca^{2+} plus 10^{-9} M TCDD (○ —— ○), and in 0.07 mM Ca^{2+} without TCDD (△ —— △).

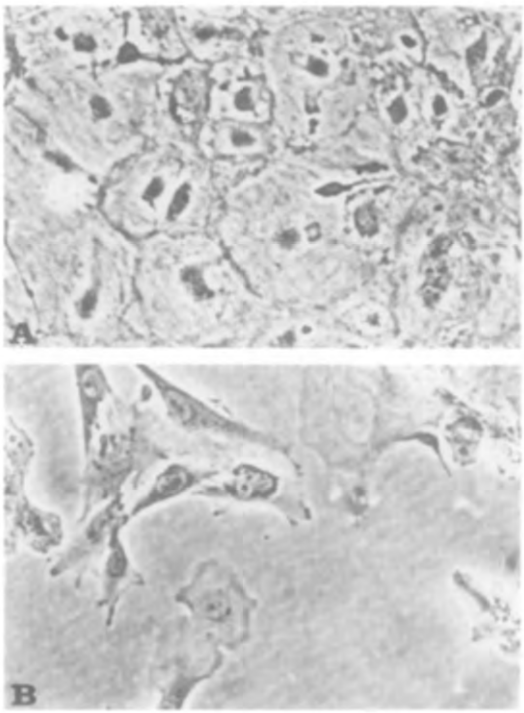

FIG. 4. Illustration of the delay in terminal differentiation of mouse epidermal cell cultures following treatment with 10^{-9} M TCDD. Parallel cultures were first grown in 0.07 mM Ca^{2+} for 7 days in the presence (A) and without the presence of 10^{-9} M TCDD (B). Cultures were then switched to 1.2 mM Ca^{2+} (without TCDD). Terminal differentiation and cell death were delayed in the TCDD-treated cultures (A) compared to controls (B). The photograph was taken 5 days after transfer to 1.2 mM Ca^{2+} medium.

valent crosslinks, involving ϵ (γ-gluytamyl-lysine) dipeptide bonds, is thought to be involved in the conversion of soluble structural protein beneath the plasma membranes of epidermal cells, to insoluble, high-molecular-weight proteins which form the cornified cell envelopes of differentiated keratinocytes.

Levels of transglutaminase activity *in vivo* in mouse ear epidermis are affected by agents which alter epidermal differentiation, such as anthralin, retinoic acid, and fluocinolone acetonide (DeYoung and Ballaron, 1982). In the present *in vivo* studies, a significant increase of this enzyme activity was demonstrated following changes in cutaneous differentiation after TCDD application. One possible complication in interpreting results of *in vivo* experiments which involve analyses of crude epidermal extracts is that during the dermal–epidermal separation procedure, dermal transglutaminases may theoretically contaminate the epidermal preparations. Substances applied topically to the skin, particularly those which induce an inflammatory cutaneous response, may affect the dermal as well as epidermal transglutaminase levels. Previous studies by DeYoung and Ballaron (1982) which investigated this problem suggested that their results were not affected by inflammatory changes. In the present paper, *in vitro* tissue culture studies were used to confirm the *in vivo* observations in a system which was free of dermal components.

The observation that mouse epidermal keratinocytes in low Ca^{2+} medium have lower levels of transglutaminase activity than differentiating keratinocytes in high Ca^{2+} medium has been established previously by Hennings *et al.* (1981). Our finding that TCDD increases unexpressed transglutaminase activity in the nondifferentiating cells in low Ca^{2+} medium confirms our *in vivo* observations about the increase of this enzyme by TCDD in epidermal cells.

Very similar effects on transglutaminase induction by epidermal basal cells *in vitro* have been demonstrated by the addition of retinoic acid (Yuspa *et al.*, 1981) or 12-*O*-tetradecanoylphorbol-13-acetate and other tumor promotors (Yuspa *et al.*, 1980) to low calcium mouse epidermal tissue cultures. In those studies as in the present experiments, it appeared that the effect was one of induction rather than activation of enzyme activity, since addition of the chemical (in this case 10^{-9} M TCDD) directly to the enzyme assay had no effect on the results. In the studies by Yuspa *et al.* (1980, 1981) retinoic acid had a different effect on terminal differentiation of the cell cultures after transfer to high calcium (1.2 mM) medium. Normally, when epidermal basal cells are transferred to high calcium medium after being cultured in low calcium medium for 5 to 7 days, terminal differentiation accompanied by cell death occurs within 2 to 5 days after transfer. In cells grown in the presence of retinoic acid (at 10^{-6} M concentration),

this pattern of terminal differentiation was significantly delayed. Phorbol esters did not affect the rate of terminal differentiation. In the present study, TCDD also delayed terminal differentiation of the cultures, despite the fact that insoluble cell envelope counts appeared to be higher in the TCDD-treated transferred cultures than in the untreated controls.

Knutson and Poland (1982) have suggested that cutaneous sensitivity to TCDD in mice is associated with the gene for hairlessness, and that hr/hr (hairless) animals have a more acute cutaneous response to TCDD than do the hr/+ (haired) counterparts. In the present studies, epidermal cell cultures were established with BALB/c hr/+ (haired) neonatal mice as tissue donors. Interestingly, such cells in culture responded to TCDD exposure. It could be that the effects of TCDD on epidermal cell cultures would be even more striking in epidermal cultures from hr/hr (hairless) skin donors.

ACKNOWLEDGMENTS

This research was supported in part by Grants AM 17425 and OH 1108 from the National Institute of Health, by Veterans Administration Medical Research funds, and funds from the Dermatologic Research Foundation of California, Inc.

REFERENCES

BRADFORD, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dying binding. *Anal. Biochem.* **72,** 248–254.

BUXMAN, M. M., AND WUEPPER, K. D. (1976). Isolation, purification and characterization of epidermal transglutaminase. *Biochim. Biophys. Acta* **452,** 356–369.

DEYOUNG, L., AND BALLARON, S. (1982). The effect of topical drugs on mouse ear transglutaminase activity. *J. Invest. Dermatol.* **79,** 189–193.

GOLDSMITH, L. A., AND MARTIN, C. M. (1975). Human epidermal transamidase. *J. Invest. Dermatol.* **64,** 316–321.

HENNINGS, H., MICHAEL, D., CHENG, C., STEINERT, P., HOLBROOK, K., AND YUSPA, S. H. (1980). Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell* **19,** 245–254.

HENNINGS, H., STEINERT, P., AND BUXMAN, M. M. (1981). Calcium induction of transglutaminase and the formation of ϵ (γ glutamyl) lysine cross-links in cultured mouse epidermal cells. *Biochem. Biophys. Res. Commun.* **102,** 739–745.

KNUTSON, J. C., AND POLAND, A. (1982). Response of murine epidermis to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Interaction of the *Ah* and *hr* loci. *Cell* **30,** 225–234.

MARCELO, C., KIM, Y. G., KLEINE, J. L., AND VORHEES, J. J. (1978). Stratification, specialization and proliferation of primary keratinocyte cultures. *J. Cell Biol.* **79,** 356–370.

MOORE, J. A. (1978). Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Ecol. Bull (Stockholm)* **27,** 134–144.

OGAWA, H., AND GOLDSMITH, L. A. (1976). Human epidermal transglutaminase. *J. Biol. Chem.* **251,** 7281–7288.

PUHVEL, S. M., SAKAMOTO, M., ERTL, D. C., AND REISNER, R. M. (1982). Hairless mice as models for chloracne: a study of cutaneous changes induced by topical application of established chloracnegens. *Toxicol. Appl. Pharmacol.* **64,** 492–503.

YUSPA, S. H., BEN, T., HENNINGS, H., AND LICHTI, U. (1980). Phorbol ester tumor promoters induce epidermal transglutaminase activity. *Biochem. Biophys. Res. Commun.* **97,** 700–708.

YUSPA, S. H., LICHTI, U., BEN, T., AND HENNINGS, H. (1981). Modulation of terminal differentiation and responses to tumor promoters by retinoids in mouse epidermal cell cultures. *Ann. N.Y. Acad. Sci.* **359,** 260–273.

YUSPA, S., BEN, T., AND STEINERT, P. (1982). Retinoic acid induces transglutaminase activity but inhibits cornification of cultured epidermal cells. *J. Biol. Chem.* **257,** 9906–9908.