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Reversible Inhibition of *in Vitro* Epithelial Cell Proliferation by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin[1]

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Reversible Inhibition of *in Vitro* Epithelial Cell Proliferation by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin. GIERTHY, J. F., AND CRANE, D. (1984). *Toxicol. Appl. Pharmacol.* **74**, 91–98. Subconfluent cultures of a mouse epithelial cell line, which after prolonged subculturing exhibited an elevated saturation density as compared to the original cell line, were treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Cultures of cells with or without TCDD grew at equal rates until confluency was reached. At confluency, cultures treated with as little as 10^{-11} M TCDD showed a decline in cell proliferation relative to controls as demonstrated by cell enumeration and supported by reduced [^{3}H]thymidine incorporation (both by liquid scintillation spectrometry of whole culture and autoradiography of individual cells). After 14 days of exposure, the saturation density of the treated culture was about 50% of the control culture. This TCDD-induced, increased sensitivity to density-dependent inhibition of replication (DDIR) was accompanied by a change from a fusiform morphology in the high-saturation-density control cells to a flat cobblestone appearance in the treated low-saturation-density cells. The nondividing cultures treated for 14 days with 10^{-11} M TCDD had the same viability as control cultures. Upon trypsin suspension and reseeding, these formerly quiescent cultures were again capable of growing to high cell density and of again showing susceptibility to TCDD-induced changes in cell growth and morphology. Evidence is presented to suggest that this reversible increase in sensitivity to DDIR and the morphological change are not a consequence of cell growth inhibition. This system may provide the basis for an *in vitro* model to study the effect of TCDD on the control of replication of these cells.

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic synthetic compound known (Poland and Kende, 1976; Poland and Knutson, 1982). Animal studies have demonstrated the pleotypic nature of this toxicity, which is characterized mainly by a prolonged wasting syndrome leading to death but also includes embryotoxicity and/or teratogenicity, thymic and lymphoid involution, hyperkeratoses, edema, hyperplasia of the epithelium of the stomach, intestine, urinary bladder, and bile duct, and hepatocellular damage (Kimbrough, 1974; Huff *et al.*, 1980). The development of acneform lesions is one of the most common toxic manifestations of TCDD exposure seen in, but not confined to, humans (Kimbrough, 1974; Huff *et al.*, 1980). The development of this chloracne appears to result from hyperkeratoses of the sebaceous glands leading to a cystic response (Kimbrough, 1974; Knutson and Poland, 1980b). The specific pathology and sensitivity to TCDD vary greatly among animal species. The LD50 for the species tested ranges from 1 μg/kg body weight in the guinea pig to 5000 μg/kg in the hamster (Schwetz *et al.*, 1973; Henck *et al.*, 1981). The specific cause of death due to TCDD intoxication is unknown.

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Extensive studies of TCDD in animal and in *in vitro* systems over the past decade have suggested that the toxic response elicited by TCDD isomers and congeners is associated with their binding to a specific cytosolic receptor (Poland and Glover, 1975; Kende *et al.*, 1974). The affinity of these compounds for this receptor corresponds in rank order to their *in vivo* toxicity, as well as to the induction of a battery of enzymes which include the cytochrome P_1-450-mediated microsomal monooxygenase aryl hydrocarbon hydroxylase (AHH) (Poland and Glover, 1977; Poland and Kende, 1976; Kende *et al.*, 1974). Additional evidence suggests that the induction of AHH is dependent upon the translocation of the TCDD–receptor complex from the cytoplasm into the nucleus (Greenlee and Poland, 1979; Okey *et al.*, 1979, 1980), where evidence suggests it induces genetic activation resulting in the production of cytochrome P_1-450 mRNA (Tukey *et al.*, 1982).

Many cultured cell lines respond to TCDD exposure by inducing AHH. Despite this enzyme induction and the toxicity of TCDD in animals, extensive studies have not demonstrated significant *in vitro* toxicity in cultured cell lines exposed to TCDD. No change in growth or morphology was seen in 23 cell lines exposed to TCDD (Knutson and Poland, 1980a). It was reported, however, that *in vitro* keratinization could be induced in the cloned mouse-teratoma cell line, XB, by exposure to very low levels of TCDD (Knutson and Poland, 1980b). These results suggested the use of this system as an *in vitro* model for the *in vivo* keratinization response. These experiments also showed that this phenotype could change under conditions of cell passage. We have also observed a change in phenotype and determined that, under our conditions of cell culture, late-passage XB cells no longer keratinize upon exposure to TCDD although they continue to proliferate and to grow to a higher density than the early-passage XB cells. The late-passage XB cells, which we designate XBF, have been found to respond to very low levels of TCDD by an increased sensitivity to density-dependent inhibition of replication (DDIR) and the appearance of a flat epithelial morphology, as compared to the fusiform morphology of high-density nonexposed replicate cultures. We also present evidence that this TCDD-induced decrease in saturation density observed in these cells does not result from a general toxic response and is reversible. The TCDD-induced DDIR seen in this cell line may provide the basis for an *in vitro* system to study the effect of this compound on the control of cell replication in these cells.

METHODS

TCDD was obtained from Dow Chemical (Midland, Mich.). Purity was determined by mass spectrometry to be >99%. The XB teratoma-derived mouse epithelial cells and the 3T3 fibroblast feeder cells were the generous gift of Dr. Howard Green (Harvard University). Analytical grade dimethyl sulfoxide (DMSO) was obtained from the Aldrich Chemical Co. (Milwaukee, Wis.).

Cell culture. XB cell stocks were grown in Costar plastic tissue culture flasks (75 cm^2) in Dulbeccos Modified Eagle Medium (DMEM; Gibco) supplemented with 20% fetal bovine serum (Flow, Rockville, Md.), 100 U of penicillin/ml, and 100 μg of streptomycin/ml. This medium had been conditioned by exposure to a confluent culture of 3T3 feeder cells for 24 hr (25 ml of medium/75 cm^2 flask) and sterilized by filtration. The 3T3 feeder cell stocks were grown in DMEM supplemented with 10% calf serum (Flow).

The XB cells were routinely propagated every 1 to 2 weeks, when confluency was reached, by trypsinization (0.25%) and replating at a concentration of 2×10^4 cells per cm^2. The culture medium was renewed once a week between passages. The confluent density of these early XB cultures was about 4×10^4 cells per cm^2 under these conditions. These cells, when seeded with lethally irradiated 3T3 cells, were found to exhibit the TCDD-induced keratinization response described by Knutson and Poland (1980b). The magnitude of this keratinization response to TCDD exposure gradually declined after passage seven, and by passage 15 this response was greatly reduced compared to the early passage XB cells, although the sensitivity of this reduced response to TCDD was similar to that of the early passage cells. At this time the XB stock cultures, which were originally flat and polygonal, now had become more fusiform in morphology and reached confluency earlier than the low passage XB cultures. This alteration in growth and morphology at high density progressed dur-

ing the next five to seven passages as the keratinization response to TCDD exposure disappeared. The confluent density of the cells at this time was about 15×10^4 cells per cm^2. At this time it was discovered that medium conditioned by exposure to 3T3 cells was no longer required for the growth of these cells, which were now designated XBF to indicate their divergence from the XB line. Subsequently, stock cultures of XBF cells were routinely grown in DMEM supplemented with 20% fetal bovine serum (Flow), 100 U of penicillin/ml, and 100 μg of streptomycin/ml. The cultures were propagated by suspension in trypsin (0.25%), replated at a density of 3×10^4 cells per cm^2 once or twice a week as confluency was reached, and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Exposure of cells to TCDD. XBF cells were suspended by trypsinization and seeded into 24-well plates (16-mm-diameter wells, 5×10^4 cells per ml per well) in conditioned medium with 3T3 cells (5×10^5 per ml per well) which had been irradiated with 6000 rads from a cesium source. The cultures were incubated overnight and refed with a series comprising 10-fold dilutions of a stock solution of TCDD in DMSO or with DMSO alone in DMEM supplemented with 20% fetal bovine serum. This refeeding was repeated every 3 or 4 days for the duration of the experiment. The highest cumulative DMSO concentration was 0.1%.

At the end of incubation the cultures were washed with phosphate-buffered saline (PBS), fixed with formalin in PBS, and stained with Giemsa. Alternatively, the cultures were suspended by trypsinization at varying times after the initial exposure to TCDD. After thorough disaggregation of the cells by trituration, the number of cells per culture was determined with a Coulter Particle Counter (Coulter Instrument, Hialeah, Fla.). Complete disaggregation was confirmed by microscopic examination of the cell suspension immediately before counting. [^{3}H]Thymidine (New England Nuclear; specific activity = 0.67 μCi/mmol) incorporation at various times after exposure to TCDD was determined by pulse-labeling the cultures for the indicated times and counting the acid-precipitable radioactivity in a Packard Tricarb liquid scintillation spectrometer (Ellem and Gierthy, 1977). Autoradiography was performed as described elsewhere (Ellem and Gierthy, 1977).

Cell viability was determined by measuring the percentage of trypsin-suspended cells capable of excluding 0.1% trypan blue dye. Cloning efficiency was determined by seeding 200 cells in 5 ml of medium into a 60-mm plastic tissue-culture Petri dish (Falcon) and incubating for 2 weeks. Cultures were then washed in PBS, fixed in 10% formalin, and stained with Giemsa, and colonies were counted. Absolute cloning efficiency was defined as the percentage of the cells seeded capable of forming colonies.

Safety considerations. Safe handling, containment, and disposal of the substances used in this study conformed to the general principles and standards of biologic safety proposed by the Office of Biohazards and Environmental Control, National Cancer Institute, for class I hazards and to the guidelines of the Laboratory Chemical Carcinogen Safety Standards Subcommittee of the Department of Health and Human Services Committee to coordinate toxicology and related programs.

RESULTS

Induction of Morphological Change in XBF Cells by Exposure to TCDD

The XBF cells, cocultured with irradiated 3T3 cells for 14 days, grew to very dense cultures characterized by apparent multilayering and the appearance of parallel growth patterns indicative of a spindlelike morphology. Treatment with 0.1% DMSO had no effect on this morphology (Fig. 1a). Cultures grown in the presence of 10^{-9} M TCDD grew to confluency, and after 7 to 10 days began to develop into a cobblestonelike monolayer comprised of flat, evenly distributed cells very apparent by Day 14 (Fig. 1b). Cultures of irradiated 3T3 feeder-layer cells, seeded from the same stock used for these experiments, did not show any indication of growth or TCDD-induced morphological change. By Day 14 these cultures had essentially disintegrated.

This change in growth and morphology was induced by TCDD concentrations ranging from 10^{-11} to 10^{-8} M, the highest concentration tested. This dose–response effect was apparent in unmagnified, stained preparations (Fig. 2), as well as in the number of cells in each culture after 14 days of exposure (Fig. 3). Solvent control cultures treated with DMSO at concentrations corresponding to those in the dilutions did not show this effect. The presence of 0.1% DMSO did not affect the TCDD dose–response. The ability of TCDD to induce these changes in morphology and growth in XBF cells has remained stable for over a year of cell passage as described under Methods. Representative experiments are presented in the figures.

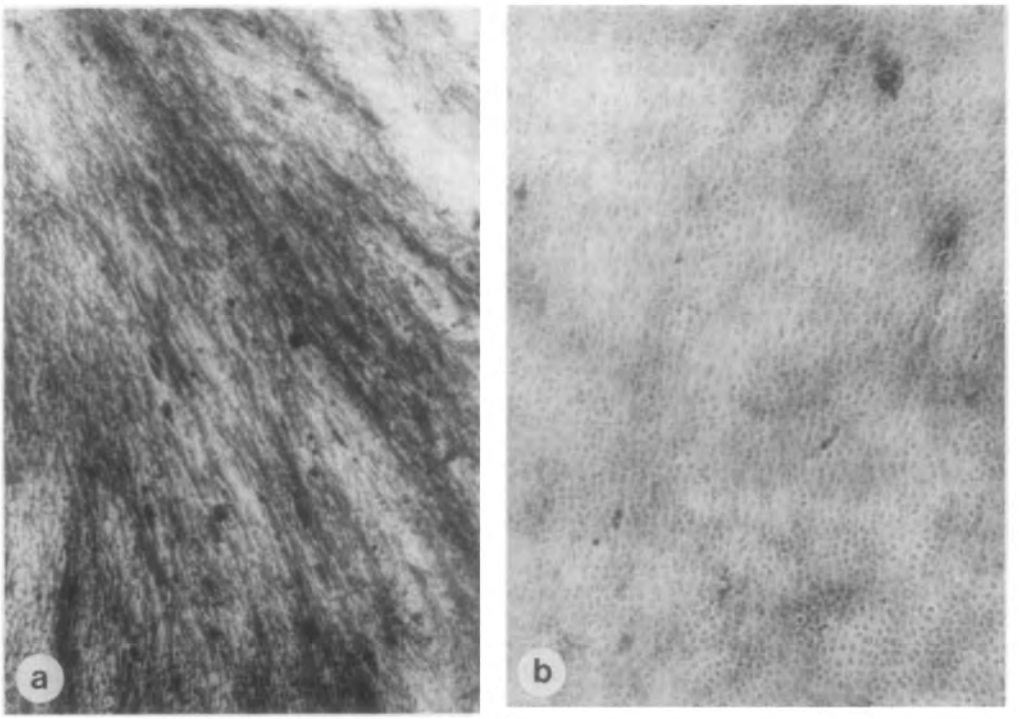

FIG. 1. Induction of the flat-cell response by TCDD. (a) The dense packing and multilayering of control XBF/3T3 cultures. (b) The apparent induction of density-dependent inhibition of cell proliferation and flat-cell morphology characteristic of XBF/3T3 cultures after 14 days of exposure to 10^{-9} M TCDD. Giemsa stain and methods, as described in text. 100× magnification.

Reduction in Cell Growth during Exposure to 2,3,7,8-TCDD

Investigation of the kinetics of cell growth in exposed cultures revealed that the decline in cell proliferation after TCDD exposure was not immediate. Cultures were seeded below confluency, as described under Methods, and after 24 hr were treated with 10^{-9} M TCDD and DMSO (0.1%) or with DMSO alone. Figure 4 shows that both treated and untreated cultures accumulated cells at equal rates until Day 7, at about which time confluency was reached. After this time the solvent control cultures continued to increase in cell number, while the 2,3,7,8-TCDD cultures did not. This cessation of cell proliferation is apparently TCDD-induced, as reflected in the TCDD-concentration-dependent decline in [^{3}H]thymidine incorporation into the acid-insoluble fraction of treated cultures (Fig. 5). This experiment was done after 7 days of exposure to TCDD, at which time the cultures had reached confluency, and the cell numbers were the same per culture. The range of TCDD concentrations capable of diminishing [^{3}H]thymidine incorporation corresponds to that which caused the morphological change and is supportive of the decline in DNA synthesis which would be expected to occur with the observed inhibition of cell growth. Further evidence that the lack of increase in cell number was due to a cessation of cell division, rather than to the TCDD-treated cells simply sloughing off resulting in a steady-state, was a decrease in the number of cells incorporating [^{3}H]thymidine into DNA, as shown by autoradiography. A 6-hr pulse with [^{3}H]thymidine at Day 6, when the treated and untreated cultures showed equal growth rates,

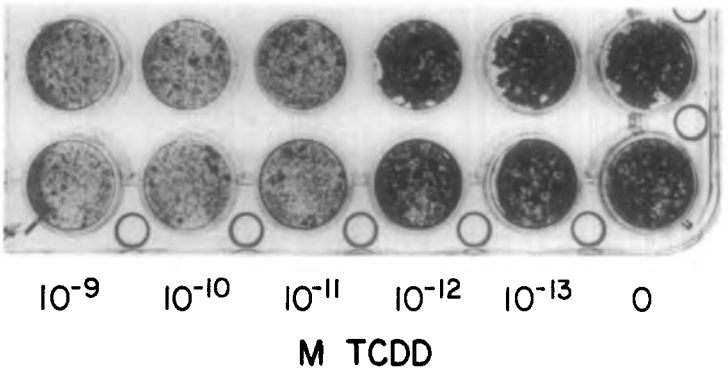

FIG. 2. Dose–response relation of flat-cell induction by TCDD. XBF/3T3 cultures were exposed to various concentrations of TCDD as described in text. After 14 days the cultures were fixed and stained with Giemsa stain. The less intensely stained low-density cultures (10^{-9} to 10^{-11} M) are similar to that shown in Fig. 1b, while the intensely staining high-density cultures (10^{-12}, 10^{-13}, and 0 M) are similar to that seen in Fig. 1a. The flat-cell induction is first evident with 10^{-11} M TCDD.

resulted in 45 ± 4.4% of the cells being labeled. By Day 13 of treatment the cultures exposed to 10^{-9} M TCDD showed a reduction in labeling to 1.6 ± 1.2%.

The viability of cells from these treated cultures was tested by trypan blue exclusion, which indicated a high viability of 87 ± 2.5% in the 10^{-8} M TCDD-treated cultures, 85 ± 4.2% in control cultures (0.1% DMSO), and 88 ± 4.1% in untreated cultures. Cells from these cultures gave plating efficiencies of 33 ± 3.6% for 10^{-8} M TCDD-treated cultures, 37 ± 6.3% for control cultures, and 34 ± 4.5% for untreated cultures.

When cells treated with 10^{-9} M TCDD for 14 days were suspended with trypsin, reseeded, and allowed to grow with or without added TCDD, again no difference in proliferation was seen until confluency was reached. At

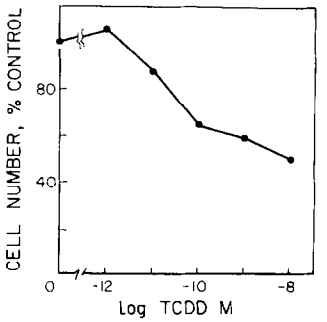

FIG. 3. Effect of TCDD on cell growth in XBF cultures. XBF cells were seeded with irradiated 3T3 feeder cells and incubated as described under Methods. Medium was replaced 24 hr after seeding and every 3 or 4 days thereafter with medium containing the indicated concentrations of TCDD in DMSO or DMSO alone. The cultures were trypsinized after 14 days, and cell number per culture well was determined. The data points represent the percentage of cells in the TCDD-treated cultures compared to control cultures and are the mean of four replicate cultures.

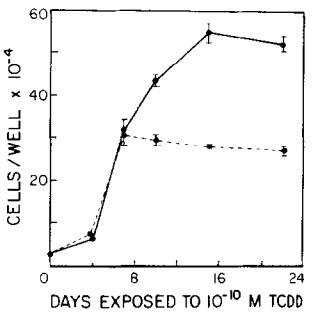

FIG. 4. Effect of TCDD on XBF cell proliferation kinetics. XBF cells were seeded and incubated with irradiated 3T3 feeder cells as described under Methods. Medium was replaced after 24 hr and every 3 or 4 days thereafter with medium containing TCDD with DMSO or DMSO alone. At the indicated times of exposure, the cultures were trypsinized, and cell number per culture well was determined. ●——●, XBF/3T3, 0.01% DMSO; ●- - -●, XBF/3T3, 10^{-10} M TCDD, 0.01% DMSO; data points represent the mean of four replicates ± SE.

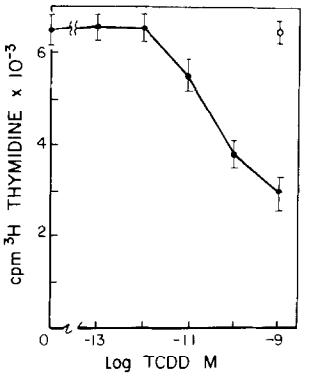

FIG. 5. Effect of TCDD on incorporation of [^{3}H]thymidine into XBF cells. XBF cells were seeded with irradiated 3T3 cells and incubated as described under Methods. Medium was replaced after 24 hr and after 4 days with medium containing the indicated concentration of TCDD. On Day 7 all cultures had reached confluency and had the same number of cells. At this time the cultures were pulse-labeled with [^{3}H]thymidine (0.5 μCi/ml, 6.7 Ci/mmol) for 1 hr, fixed, and counted for radioactivity as described under Methods. A DMSO concentration of 0.1% was the highest used in this experiment (O). Data points represent counts per minute $\times$ 10^{-3} per 16 mm culture well and are the mean of four replicates $\pm$ SE.

about this time the TCDD-treated cells showed a decline in growth, while the untreated cells continued to proliferate to a higher saturation density (Fig. 6). The reduction in cell growth of these cultures of pretreated cells had the same sensitivity to TCDD (10^{-11} M) as cells which were not pretreated as seen by microscopic flat-cell evaluation and in the gross observation of fixed and stained cultures (Fig. 2).

Inhibition of Cell Division and the Morphological Change

Subconfluent XBF cells were treated with other inhibitors of proliferation, which have various mechanisms, to determine if the flat-cell morphology was a consequence of a general toxic effect related to inhibition of specific macromolecular synthesis. Inhibitors of DNA (hydroxyurea), RNA (actinomycin D), and protein (cycloheximide) synthesis, as well as the mitotic inhibitor cholchicine, were tested in 11 10-fold dilutions starting with a concentration of 100 μg/ml. The effects ranged from obvious toxicity, characterized by cell disintegration at the high concentrations, through inhibition of cell division, to no observable effects at the low ranges. The induction of a flat-cell morphology similar to that induced by TCDD in replicate XBF/3T3 cultures was not seen at any concentration of these agents.

DISCUSSION

The XBF cells used in this study evolved, after serial passage, from the cloned XB epithelial cell line derived from a mouse teratoma. The XBF cells no longer have the requirement for a fibroblast feeder layer or for medium conditioned by a fibroblast culture for growth, as did the original XB line. The loss of the ability of the XBF line to be induced

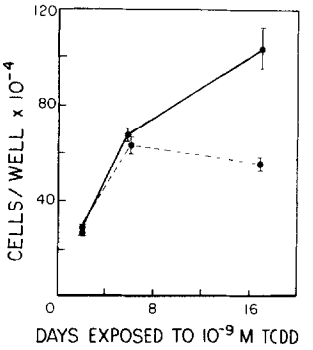

FIG. 6. Effect of TCDD on cell growth in XBF cultures of cells previously treated with TCDD. XBF cells, which had been exposed in the presence of irradiated 3T3 cells to 10^{-9} M TCDD for 14 days and exhibited growth inhibition and morphological changes, were seeded with fresh, non-TCDD-treated irradiated 3T3 feeder cells and incubated as described under Methods. Medium was replaced 24 hr after seeding and every 3 or 4 days thereafter with medium containing either 10^{-9} M TCDD in 0.01% DMSO or 0.01% DMSO. The cultures were trypsinized at the indicated times and the cell number per culture well was determined. ● —— ●, XBF/3T3, 0.01% DMSO; ● - - - ●, XBF/3T3, 10^{-9} M TCDD, 0.01% DMSO. Data points represent the mean of four replicates $\pm$ SE.

by TCDD to keratinize is consistent with reports of others describing a change in this phenotypic expression in some XB cells after serial culture (Knutson and Poland, 1980b; Rheinwald and Green, 1975). We have found that continued culturing of XBF cells resulted in an increased cell density at saturation and acquisition of a fusiform morphology at high density, in contrast to the cobblestonelike appearance of the original XB parent cell line. This change in saturation density and morphology may be the result of a spontaneous *in vitro* transformation and has been stable for over a year of twice weekly subculturing.

The XBF cells exposed to TCDD have stopped replicating based on the absence of [^{3}H]thymidine-labeled cells and the lack of increase in cell number. This is supported by the decline in [^{3}H]thymidine incorporation into acid-insoluble products. These effects were not immediate, as would have been expected if a toxic response of the type seen with previously discussed inhibitors of macromolecular synthesis had occurred. The XBF cells were seeded at low density and divided in the presence of TCDD at a rate no different from that of control cultures, until confluency was reached. The cessation of cell division at that time, unlike that seen in the keratinized stratum corneum of the epithelium was probably not a consequence of terminal differentiation which results in cell death. This conclusion is based on the observation that the quiescent XBF cells, when trypsinized and reseeded at low density, grew to high density unless treated with TCDD, in which case they again grew to a lower saturation density than the untreated cultures. The population of cells that regrew was probably representative of the entire culture rather than of a selected resistant population, since trypan blue exclusion and cloning efficiency indicate the same degree of viability in high-density control cultures, low-density TCDD-treated cultures, and untreated stock cultures of XBF cells. These results demonstrate the reversibility of this effect in response to TCDD.

The fusiform nature of epithelial cells at high density was probably a consequence of the high saturation density of the XBF cells. The change from the morphology and growth characteristics of the XB to the XBF cells may reflect a progression to a less-differentiated progeny, with loss of regulatory control of growth or a decreased sensitivity to contact inhibition of proliferation, as seen in other cell cultures as a spontaneous *in vitro* transformation. Exposure to TCDD may reestablish this regulation, resulting in a return to the parent-cell morphology and saturation density characteristics.

The effect of TCDD on other cells in culture has been described previously and, to a lesser extent, in this report. In one of these studies (Knutson and Poland, 1980a) involving suspension cultures and anchorage-dependent cells grown on a substrate, no toxic effects (altered morphology, decreased viability, or diminished growth rate) were seen in 23 cultured cell types. Sparse cultures of the 16 anchorage-dependent cell types grown on a substrate were unaffected by exposure to 10^{-7} M TCDD in regard to the previously mentioned end points from the time of seeding to confluency, at which time they were trypsinized and evaluated for viability and density. Our results for preconfluence growth with the XBF line agree with these findings. However, the observed change in cell growth rates and morphology in TCDD-treated XBF cells was apparent only after confluency was reached, as the untreated cultures continued to proliferate resulting in the observed high density. This effect was not due simply to inhibition of cell growth since inhibitors of macromolecular synthesis and mitosis did not induce this effect. TCDD is extremely potent for inducing certain gene products and keratinization. Further studies may indicate the usefulness of TCDD for inducing a specific gene product associated with the regulation of cell proliferation in the XBF system.

The abilities of TCDD to induce keratinization in the parent XB cell line and mor-

phological and proliferative change in the XBF cells may be related. The existence of a high-affinity cytosolic receptor for TCDD in the XB cells has been demonstrated by others (Knutson and Poland, 1980b), and its interaction with TCDD has been postulated to be obligatory for a number of TCDD-induced responses (AHH induction, keratinization, etc.) (Poland and Knutson, 1982). Further studies of the XBF cell response to TCDD may determine if this receptor is part of the mechanism of the induction of the flat-cell effect. The reduction of saturation density and morphological change seen *in vitro* may be analogous to the *in vivo* epithelial change from proliferating basal cells to nondividing cells of the stratum spinosum. Studies using ultrastructural and biochemical analyses for specific markers of this differentiation may answer this question. The sensitivity of this effect suggests the use of this system as an assay for TCDD if specificity for this compound can be demonstrated.

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