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In Vitro Bioassay for Dioxinlike Activity Based on Alterations in Epithelial Cell Proliferation and Morphology

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In Vitro Bioassay for Dioxinlike Activity Based on Alterations in Epithelial Cell Proliferation and Morphology. GIERTHY, J. F., AND CRANE, D. (1985). *Fundam. Appl. Toxicol.* **5**, 754–759. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to induce changes in morphology and proliferation characteristics of a nonkeratinizing derivative (XBF) of a keratinizing epithelial cell line (XB), cloned from a mouse teratoma, when cocultured with irradiated feeder cells. Polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), polynuclear aromatic hydrocarbons (PAHs), and pesticides (24 compounds in total) were tested for their ability to induce these effects. The results indicated that, for the representative compounds tested, these changes are relatively specific for—and that the XBF cells are extremely sensitive to—PCDDs and PCDFs. TCDD was the most potent congener tested, capable of inducing the effects at a concentration as low as 10^{-11} M. The activities of other tested PCDDs and PCDFs ranged from 10^{-1} to 10^{-3} of TCDD activity. The PCBs, PAHs, and pesticides had lower activities ranging from 10^{-3} to 10^{-6} that of TCDD. This assay system using XBF cells cocultured with irradiated 3T3 fibroblast feeder cells was examined as a possible *in vitro* screening assay for dioxinlike activity by testing benzene extracts of soot from a fire involving a PCB-containing transformer. The results were compared to a high-resolution gas chromatographic/mass spectrometric analysis for total PCDFs in the same samples. This comparison showed a good correlation, suggesting that the XBF-3T3 system has potential for use as a semiquantitative assay for dioxinlike activity.

Because of the possible human health hazards associated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) exposure, great emphasis has been placed on its detection in the environment (Kimbrough *et al.*, 1984). The polychlorinated dibenzofurans (PCDFs), which have been formed in heating fluids from polychlorinated biphenyl (PCB) pyrolysis (Buser *et al.*, 1978a,b), in soot produced by a fire associated with a PCB-containing transformer (Smith *et al.*, 1982a,b), and in incinerator flyash (Hutzinger *et al.*, 1981) are also of concern. In some cases PCDF exposure has induced acute toxicity in humans, including chloracne, similar to that of TCDD (Kimbrough, 1974; Huff *et al.*, 1980).

Polychlorinated dibenzodioxins (PCDDs) and PCDFs are currently detected and quantitated in environmental samples by high-resolution gas chromatography and mass spectrometry (GC/MS). While these methods are highly sensitive, accurate, and isomer-specific, they are also costly and time-consuming. Current capabilities may not be adequate to handle the large numbers of samples generated by testing programs (Hutzinger *et al.*, 1981). A rapid, inexpensive screening assay for semiquantitative determination of dioxinlike activity (i.e., specific biological activity induced by TCDD, the most toxic member of this group), would allow priority ranking of large numbers of environmental samples for subsequent isomer-specific quantitation by high-resolution chemical analysis. Perhaps more importantly, such an assay would also identify samples which have no

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dioxinlike activity at a predetermined level and for which the more rigorous and costly chemical analysis is not needed.

There are 75 possible PCDD congeners and 135 possible PCDF congeners. A correlation has been demonstrated between the structures of these compounds and their biologic potency with regard to toxicity, enzyme induction, aryl hydrocarbon (Ah) receptor binding, and hyperkeratinization (Poland and Knutson, 1982). The potency of these compounds is modulated by the positions chlorinated. The occurrence of many isomers and congeners in a sample necessitates an even more complex chemical analysis in order to make an assessment of the aggregate toxicity associated with such a sample.

Chloracne is one of the most common human clinical manifestations resulting from exposure to these compounds. Hyperkeratinization of the epithelium is considered to be responsible for the occurrence of chloracne in humans exposed to PCDDs and PCDFs. This effect is thought to be caused by an induced differentiation of the squamous epithelium (Poland and Knutson, 1982). In this regard TCDD is considered to produce a sustained stimulation of a normal physiologic response which leads to chloracne.

Knutson and Poland (1980, 1984) have developed an *in vitro* model to study the induction of cell keratinization by PCDD isomers and congeners. We have demonstrated the utility and deficiencies of the keratinization system to detect these compounds in soot extracts from a fire which involved a PCB-containing electrical transformer (Gierthy *et al.*, 1984b). For that investigation the keratinizing epithelial cell line (XB) cloned by Rheinwald and Green (1975) from a mouse teratoma was cocultured with lethally irradiated mouse fibroblasts (3T3) as described by Knutson and Poland (1980).

Subsequent experiments in our laboratory led to the identification of a nonkeratinizing derivative of the XB line, which we designated XBF. This variant, when cocultured with lethally irradiated 3T3 cells, grew to higher saturation density than the original XB line grown under similar conditions. TCDD, at a minimal concentration of 10^{-11} M, induced both a morphologic change and reversible inhibition of postconfluent cell proliferation in the XBF/3T3 system (Gierthy and Crane, 1984a). The morphologic change induced by TCDD in these cultures is characterized by the appearance of flat, cobblestonelike cells, as compared to the fusiform, high-density control cultures. This phenotype is more stable than the induction of keratinization during extended serial culture and therefore may be more useful as an indicator of dioxinlike activity. We refer to this TCDD-induced morphologic change as the flat-cell effect.

In the present study we have investigated the chemical specificity and sensitivity of the TCDD-induced flat-cell effect in the XBF/3T3 culture system and have demonstrated the feasibility of its use as a bioassay to detect dioxinlike activity in soot extracts.

METHODS

Chemicals. TCDD was obtained from Dow Chemical (Midland, Mich.); its purity was determined by mass spectroscopy to be >99%. Other PCDDs and PCDFs (purity > 99%) were obtained from the National Institute of Environmental Health Sciences, the Illinois Institute of Technology, and Analabs (North Haven, Conn.). Single PCB isomers were obtained from Analabs at 99% purity. Commercial Aroclor 1254 was from Monsanto (St. Louis, Mo.). Polynuclear aromatic hydrocarbons (PAHs) were obtained from Aldrich Chemical (Milwaukee, Wisc.), Eastman Organic (Rochester, N.Y.), and K and K Laboratories (Plainview, N.Y.) and recrystallized by B. Bush as described by Choudhury and Bush, (1981). Pesticides were obtained as reference standards from the Environmental Protection Agency at >99% purity. Analytical-grade dimethyl sulfoxide (Me_2SO) was obtained from Aldrich Chemical Company.

Samples of soot were collected from the upper surfaces of ceiling panels on various floors of the Binghamton State Office Building (BSOB) in Binghamton, New York, after it was involved in a PCB-containing transformer fire.

Cell culture. The XBF cells were derived from the cloned XB mouse epithelial cell line, which, along with the 3T3 feeder cells, was a gift from H. Green, Harvard University (Rheinwald and Green, 1975). The derivation

of the XBF line from the XB line has been described in detail elsewhere (Gierthy and Crane, 1984a). XBF cells were routinely propagated once or twice a week, when confluency was reached, by trypsinization (0.25%) and replating at a concentration of 3×10^4 cells/cm^2. These cells were grown in Dulbecco's 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 in a humidified atmosphere of 5% CO_2. The 3T3 feeder cell stocks were grown under the same incubation conditions in DMEM supplemented with 10% calf serum (Flow).

Assay procedure. XBF cells were suspended by trypsinization and seeded into 24-well plates (16-mm-diameter wells, 5×10^4 cells/ml per well) with irradiated 3T3 cells (5×10^5/ml per well) in propagation medium conditioned by 24-hr exposure to confluent cultures of 3T3 cells (25 ml of medium per 75-cm^2 flask). After overnight incubation (37°C, 5% CO_2, humidified) the cultures were refed with a series of 10-fold dilutions of a stock solution of test chemical or soot extract in Me_2SO, or with Me_2SO alone, in nonconditioned DMEM supplemented with 20% fetal bovine serum. Soxhlet benzene extracts of soot samples from the BSOB were produced and solvent-exchanged to Me_2SO as described elsewhere (Gierthy *et al.*, 1984b). These extracts were diluted 1:1000 in the culture medium before the 10-fold dilutions were made. The medium containing the sample extract or test chemical was replaced every 3 or 4 days with freshly prepared dilutions. The highest cumulative Me_2SO concentration was 0.1%. After 14 days the culture was assessed for flat-cell induction, washed with phosphate-buffered saline (PBS), fixed with Formalin in PBS, and stained with either Giemsa or 1% rhodamine B in water.

The cultures were evaluated for the flat-cell effect by phase-microscopic assessment and confirmation that the tested cultures had grown to form a confluent monolayer of morphologically flat cells as compared to the high density, control cultures comprised of multilayered fusiform cells as described in detail previously (Gierthy and Crane, 1984a). Microscopic evaluation of staining intensity, i.e., an indication of cell culture density, was then made on the fixed and stained cultures as shown previously for TCDD (Gierthy and Crane, 1984a) and in Fig. 1 for TCDF as compared to the TCDD calibration standard.

RESULTS

Validation of flat-cell assay. The effects of a series of 10-fold dilutions of the TCDD standard and 2,3,7,8-tetrachlorodibenzofuran (TCDF) on XBF/3T3 cultures exposed and stained with Giemsa stain are shown in Fig. 1. The lightly stained wells correspond to the flat-cell, low-saturation density appearance as described previously (Gierthy and Crane, 1984a). The intensity of the staining can be used as an indicator of the flat-cell effect. Here, the TCDF exposure has an endpoint of 10^{-10} M, while that of TCDD is 10^{-11} M, indicating that TCDD is approximately 10-fold more potent.

For a series of 24 chemicals, including PCDDs, PCDFs, PCBs, polycyclic aromatic hydrocarbons (PAHs), and pesticides, there was at least a 63-million-fold range in the potential for inducing the flat-cell effect (Table 1). These data show that the flat-cell effect is most sensitive to the more toxic PCDDs and PCDFs, with TCDD the most potent. Specifically 1,2,4,7,8-penta-CDD, which lacks chlorination in a lateral position of one of the benzene rings, shows 100-fold less activity than TCDD. TCDF was the most potent PCDF congener tested with flat-cell-inducing activity an order of magnitude lower than that of TCDD. Other PCDFs tested gave a range of activity, with hexa-CDF more potent than octa- or di-CDF. The range of potency observed for the PCDDs and PCDFs tested,

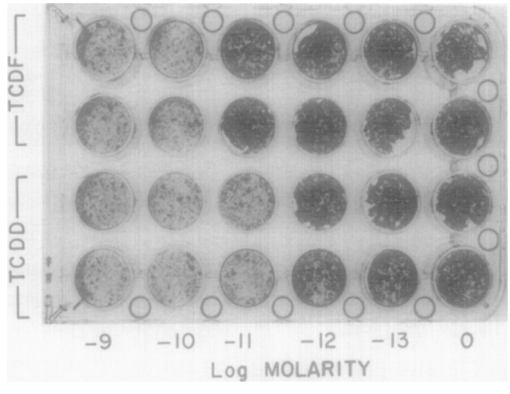

FIG. 1. Dose–response relation of flat-cell induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF). XBF/3T3 cultures were exposed to indicated concentrations of TCDD and TCDF for 14 days, fixed, and stained with Giemsa stain. The less intensely stained, low-density cultures (10^{-9} to 10^{-11} M for TCDD and 10^{-9} to 10^{-10} M for TCDF) exhibit the flat-cell morphology. Flat-cell induction is first evident with 10^{-11} M TCDD and 10^{-10} M TCDF.

TABLE 1

INDUCTION OF THE FLAT-CELL EFFECT BY VARIOUS CHEMICALS

Compound	Minimum detectable concentration (ppb)
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	0.0032
1,2,4,7,8-Pentachlorodibenzo-*p*-dioxin	0.359
2,3,7,8-Tetrachlorodibenzofuran	0.032
2,3,4,6,7,8-Hexachlorodibenzofuran	0.378
Octachlorodibenzofuran	4.48
2,6-Dichlorodibenzofuran	>2.38
3,4,3',4'-Tetrachlorobiphenyl	100
2,4,5,2',4',5'-Hexachlorobiphenyl	1,000
2,5,2',5'-Tetrachlorobiphenyl	>10,000
2,3,4,2',4',5'-Hexachlorobiphenyl	>10,000
2,3,4,2',3',4'-Hexachlorobiphenyl	>10,000
Aroclor 1254	10,000
Dibenzo[*a,h*]anthracene	10
Benz[*a*]anthracene	100
3-Methylcholanthrene	>100[a]
Benzo[*a*]pyrene	>100[a]
β-Naphthoflavone	1,000
Pyrene	>10,000
Mirex	10,000
Dieldrin	>10,000
Aldrin	>10,000
*o,p'*DDT	>10,000
Lindane	>10,000
α–BHC	>200,000

[a] Toxic concentration was 1000 ppb.

relative to TCDD, was about 1000-fold. In contrast the various PCBs tested were 10^4–10^6 times less potent than TCDD.

The PAHs varied in activity relative to TCDD. Dibenzo[*a,h*]anthracene and benz[*a*]anthracene were about 10^3 and 10^4 times less potent than TCDD, respectively. 3-Methylcholanthrene and benzo[*a*]pyrene were both toxic at concentrations which were insufficient to induce a flat-cell effect. Of the pesticides only Mirex induced the flat-cell response at the concentrations tested, and was 10^6 times less active than TCDD.

Application to Binghamton State Office Building soot samples. Benzene extracts of 10 soot samples taken from above the ceiling tiles of different floors of the BSOB were tested for their ability to induce the flat-cell effect. Although flat-cell-inducing activity was detected in all 10 samples (Table 2), the minimum amount of soot-equivalent of the extract needed to induce this effect to greater than background levels varied from 0.3 to 114.2 μg.

These activities were compared to the total PCDF concentrations determined by GC/MS analysis for these samples (Smith *et al.*, 1982a) in order to determine if these differences reflected actual variation in flat-cell-inducing potential between samples. Total PCDF concentrations were used in this correlation since, as predicted by pyrolysis studies of PCBs (Buser *et al.*, 1978a,b), PCDF concentrations in the soot were up to 100 times greater than those of the PCDDs (Smith *et al.*, 1982a,b). The relative flat-cell-inducing activities (Table 2) correlated well with the relative total PCDF concentrations of the various soot extracts (correlation coefficient, 0.82; Fig. 2).

TABLE 2

FLAT-CELL-INDUCING ACTIVITY OF BINGHAMTOM STATE OFFICE BUILDING SOOT EXTRACTS

Floor	Endpoint (μg soot/ml)[a]	Relative activity[b]
1	114.2	1
4	1.4	82
6	15.9	7
7	0.3	381
8	10.0	11
9	1.5	76
10	0.5	228
14	12.9	9
15	0.4	284
17	0.4	284

[a] Lowest concentration of a series of 10-fold dilutions of extracts of soot from various floors of the Binghamtom State Office Building capable of inducing a flat-cell effect greater than background. Each endpoint is an average of two evaluations of three replicates.

[b] Relative to activity of 1st-floor sample.

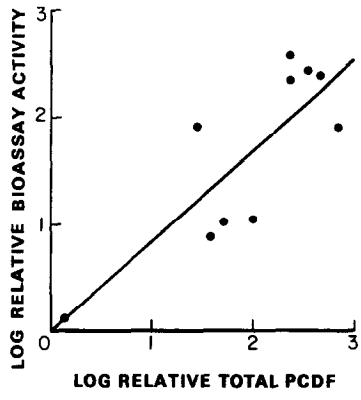

FIG. 2. Correlation of the data for relative flat-cell induction (Table 2) and relative mass spectrometric analysis for polychlorinated dibenzofurans (PCDFs) (Smith *et al.*, 1982a) for extracts of 10 soot samples from various floors of the Binghamton State Office Building. Both the relative flat-cell-inducing activity and the relative total PCDF in each sample are plotted as a ratio to the value on floor 1. The correlation coefficient is 0.82.

DISCUSSION

The potential health hazards associated with human exposure to TCDD have become of major public concern. Other polychlorinated dioxin isomers and congeners, as well as other dioxinlike compounds such as PCDFs and PCBs, also exhibit toxic effects associated with TCDD (dioxinlike activity). Detection of these less potent compounds is as important as detection of TCDD, since at sufficiently high concentrations their overall hazard may equal or exceed that of TCDD in a particular sample. Thus an analytical approach is needed which will provide an overall indication of the aggregate toxicity of an environmental sample. GC/MS analysis, while determining the concentrations of specific components of the environmental sample, does not indicate the overall toxicity.

We have previously demonstrated the feasibility of the keratinization system developed by Knutson and Poland (1980) as an assay for dioxinlike compounds, e.g., correspondence of relative keratinization inducing potential with relative PCDF concentration as determined by GC/MS analysis of extracts from soot produced by a fire which involved a PCB-containing electrical transformer (Gierthy *et al.*, 1984b). However, we also noted, as had others, a change in phenotype which was apparently associated with extended serial culture of the target cell line. In our experience, this change was characterized by a decline in the magnitude of the keratinization response to TCDD exposure, so that low-passage, frozen cell stocks were necessary for extensive use of this system as a screening assay (Gierthy *et al.*, 1984b).

The XBF line, when cocultured with irradiated 3T3 cells, exhibits a dioxin-induced, reversible, postconfluent inhibition of cell proliferation (Gierthy and Crane, 1984a). This XBF/3T3 phenotype has been stable for over a year of routine culturing, unlike the XB/3T3 culture system, which lost its dioxin-induced keratinization characteristic under similar conditions.

The sensitivity and stability of the XBF/3T3 flat-cell effect for TCDD suggested its application as an assay for dioxin. Previous studies using inhibition of macromolecular synthesis and mitosis indicated that the morphologic change seen in postconfluent cultures was not simply a consequence of inhibition of cell division (Gierthy and Crane, 1984a). The experiments described in the present report indicate that, for the representative chemicals tested, this effect occurs most sensitively with TCDD and TCDF. Other compounds, such as PCBs and certain PAHs, demonstrate relatively little potential for inducing this activity. Ranking of the flat-cell-inducing potentials of these chemicals also suggests a structure–function relationship which is consistent with those of the keratinization system, TCDD receptor binding, AHH induction, and animal toxicity (Knutson and Poland, 1981). Our results also demonstrate the ability of the flat-cell assay to detect flat-cell-inducing activity in the tested soot extracts and to discriminate between soot samples having relatively high and low levels of PCDFs as determined by GC/MS analysis.

The potency of a compound for induction of aryl hydrocarbon hydroxylase and TCDD receptor binding is dependent on its structure (Knutson and Poland, 1981). We have shown that TCDF is about 10 times more potent in the flat-cell assay than 1,2,4,7,8-pentachlordibenzofuran and 2,3,4,6,7,8-hexachlorodibenzofuran. Other PCDF congeners tested are even less potent. Thus an important advantage of the XBF/3T3 system is its apparent sensitivity to the most toxic PCDDs and PCDFs. Data resulting from this *in vitro* system reflect the total dioxinlike activity associated with a sample, rather than quantitation of specific isomers, as in GC/MS, whose toxic potential may be unknown.

The relative specificity and sensitivity of the XBF/3T3 system for TCDD—and the agreement of the results obtained by the biologic and chemical methods reported here for the soot extracts contaminated with PCDFs and PCDDs—suggest its use as an alternative or supplement to chemical analysis. Such an assay would be most useful for screening many samples, reserving the subsequent high-resolution chemical analysis for samples which demonstrate dioxinlike activity. Application of this *in vitro* system to detection of dioxinlike activity in other types of environmental samples and pollutants is under investigation.

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