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Application of an *in Vitro* Keratinization Assay to Extracts of Soot from a Fire in a Polychlorinated Biphenyl-Containing Transformer[1]

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Application of an *in Vitro* Keratinization Assay to Extracts of Soot from a Fire in a Polychlorinated Biphenyl-Containing Transformer. GIERTHY, J. F., CRANE, D., AND FRENKEL, G. D. (1984). *Fundam. Appl. Toxicol.* **4**, 1036–1041. A fire in the State Office Building in Binghamton, New York, involving a polychlorinated biphenyl-containing electrical transformer, resulted in contamination of the structure with soot containing 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 2,3,7,8-tetrachlorodibenzofuran. Benzene extracts of soot collected from various areas of the building were tested for *in vitro* keratinization-inducing activity by the method of J. C. Knutson and A. Poland (*Cell* **22**, 27–36, 1980). The results, in terms of relative keratinization-inducing activity, are compared to a high-resolution gas chromatographic/mass spectrometric analysis for total polychlorinated dibenzofurans in the same samples. This comparison showed a good correlation and suggests that the *in vitro* keratinization model has potential for use as a semiquantitative assay for dioxinlike activity.

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is detected and quantitated in environmental samples by high-resolution gas chromatography and mass spectrometry. While these methods are highly sensitive, accurate, and isomer specific, they are also costly and time consuming and therefore of limited application to routine large-scale screening of environmental samples (Hutzinger *et al.*, 1981). A rapid, inexpensive screening assay for semiquantitative determination of halogenated dioxin congener and isomer concentrations would allow priority ranking of large numbers of samples for more rigorous chemical analysis.

Animal studies as well as incidents of human exposure have demonstrated the pleomorphic nature of TCDD toxicity. In animals this includes prolonged wasting syndrome prior to death; lymphoid involution; embryotoxicity and/or teratogenicity; hyperkeratosis; edema; hyperplasia of the epithelium of the stomach, intestines, and urinary bladder; hepatocellular damage; and thymic involution. In humans, acneform lesions (chloracne) are the most common toxic manifestation of TCDD exposure (Kimbrough, 1974; Huff *et al.*, 1980). This acnegenic response is thought to be caused by a thickening of the epidermis (acanthosis), hyperkeratosis, and a metaplastic change in the sebaceous glands to a squamous epithelium. Chloracne results as the keratinaceous material plugs hair follicles and the sebaceous glands become cystic (Poland and Knutson, 1982).

Knutson and Poland (1980a) have developed an *in vitro* system which has been suggested as a model for the hyperkeratinization response to TCDD exposure. XB epithelial cells, derived as a cloned cell line from a mouse teratoma (Rheinwald and Green, 1975), exhibit a keratinization response to TCDD exposure when grown at high cell density in co-culture with irradiated

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3T3 cells. Rheinwald and Green (1975), the developers of the XB/3T3 culture system, had shown previously that high-density cultures would not keratinize spontaneously.

The involvement of an electrical transformer containing a mixture of polychlorinated biphenyls (PCBs) (Aroclor 1254) and chlorinated benzenes in a fire at the Binghamton New York State Office Building (BSOB) led to contamination of the structure with polychlorinated dibenzofuran (PCDF)- and polychlorinated dioxin (PCDD)-laden soot (Smith *et al.*, 1982b). Pyrolysis converts PCBs and chlorinated benzenes to PCDFs and PCDDs respectively (Buser *et al.*, 1978a,b). Benzene extracts of soot samples from above the ceiling tiles of various floors were therefore analyzed for PCDD and PCDF by mass spectrometry (Smith *et al.*, 1982a) to determine the extent and variability of this contamination. Portions of these extracts were made available for preliminary evaluation and verification of *in vitro* keratinization as an assay for dioxinlike activity, so designated because 2,3,7,8-TCDD is the most potent inducer of this response. The results of these studies are the subject of this report.

METHODS

Sample preparation. A soxhlet extract of the soot sample in benzene (Smith *et al.*, 1982a) at a volume of 1.0 ml was mixed with 100 μl of dimethyl sulfoxide (DMSO, Aldrich, Milwaukee, Wisc.), and evaporated at room temperature for 24 hr in darkness to allow for solvent exchange from benzene to DMSO (final volume of 100 μl). A solvent exchange was performed with positive controls and solvent controls (DMSO). Subsequently, in a modified procedure, the solvent exchange was performed in 20 min under a flow of dry nitrogen in a closed vessel.

The extract in DMSO was diluted 1:1000 in tissue culture medium (Dulbecco's modified Eagle's medium, supplemented with 20% fetal calf serum), and four more 10-fold dilutions were made in this medium for application to the cell cultures. This dilution series was freshly made for each of the twice-weekly refeedings.

2,3,7,8-TCDD, used as a positive control, was obtained from Dow Chemical Company (Midland, Mich.).

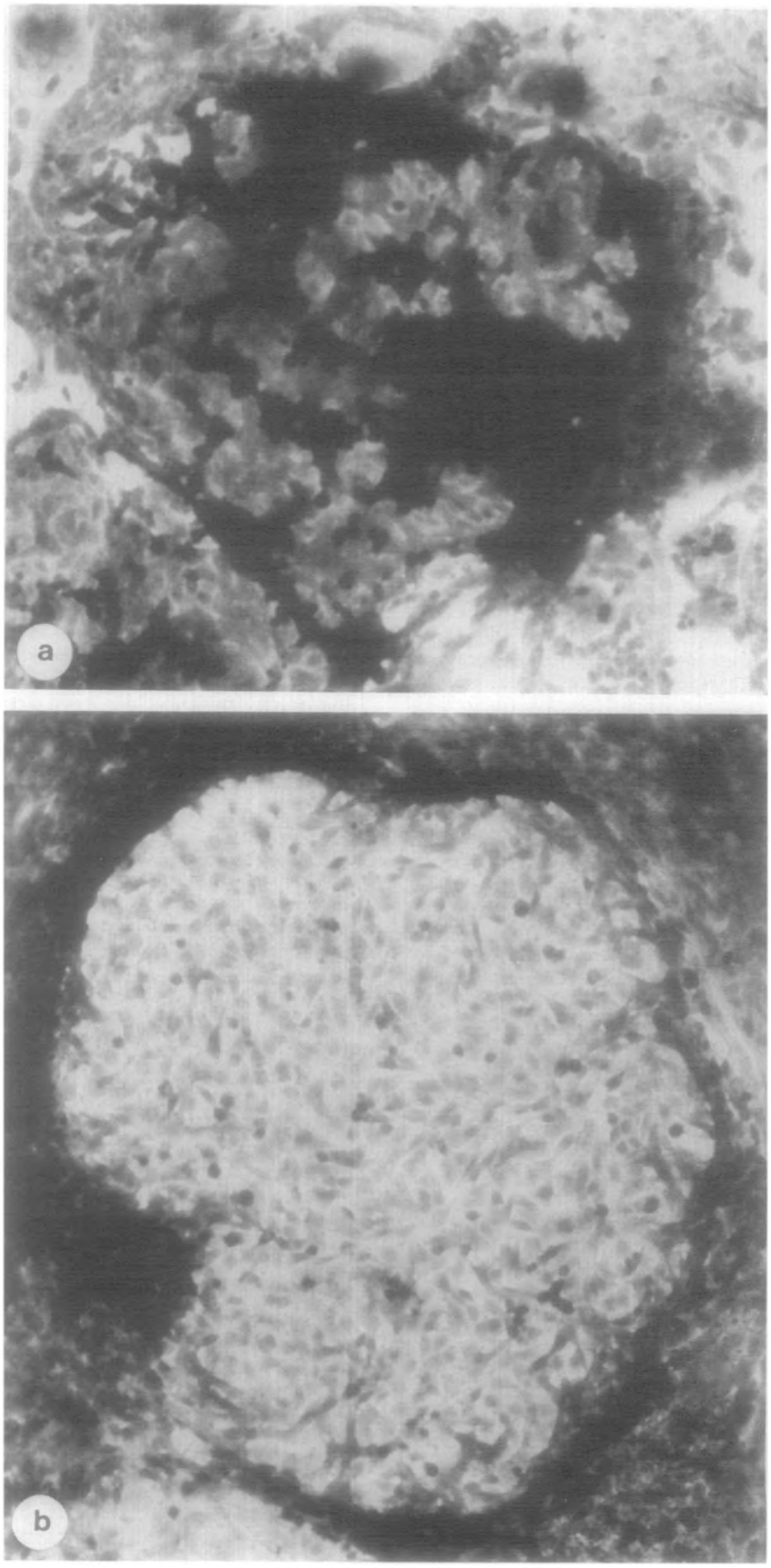
Cells. The XB cell line and the 3T3 feeder cells were obtained from H. Green, Harvard University. The cultures were grown in Dulbecco's modified Eagle's medium (Flow, Rockville, Md.) with 100 U of penicillin and 100 μg of streptomycin/ml. The XB line was cultured in medium conditioned by a 24-hr exposure to confluent 3T3 cells (25 ml/75 cm^2). The medium was supplemented with 20% fetal calf serum (Flow) for the XB cells and 10% calf serum (Flow) for the 3T3 cells. The cells were cultivated at 37°C and 5% CO_2 in air in a humidified incubator. Cells were suspended in 0.25% trypsin for passage. No contamination with mycoplasma was detected by the Hoechst fluorescence staining method (Chen, 1974).

Keratinization assay. Testing for dioxinlike activity was done essentially as described by Knutson and Poland (1980a). Confluent cultures of 3T3 cells were lethally irradiated with 4000 rads by a cesium source. Costar Multiwell dishes (2.1 cm^2) were seeded with 5×10^4 XB epithelial cells and 5×10^4 irradiated 3T3 cells in one ml of 3T3 conditioned medium per well. Incubation was at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium (Dulbecco's modified Eagle's medium with 20% fetal calf serum) was replaced twice weekly. The 10-fold dilution series of the sample was added to the assay cultures 24 hr after seeding and again with each medium change. The solvent controls and the 2,3,7,8-TCDD reference dilution series were processed identically.

After 21 days the cultures were rinsed with phosphate-buffered saline (PBS), fixed for 30 min in 10% formalin–PBS, and stained with 1% Rhodamine B, which stains keratinized tissue red (MacConaill and Gurr, 1964). When the cultures were evaluated microscopically, colonies of keratinizing cells, similar to those described by Rheinwald and Green (1975), could be seen on a background of nonkeratinizing cells (Fig. 1). Microscopic evaluation was carried out on each of three replicates for each sample by two observers using a double blind technique. The highest dilution of sample capable of inducing keratinization greater than background was taken as the endpoint. The keratinization response was evident after 14 days of exposure, as reported by others (Knutson and Poland, 1980a), and this shorter incubation time has been used in subsequent experiments.

A decline in the magnitude, but not the sensitivity, of the keratinization response to 2,3,7,8-TCDD was seen in these cultures under our conditions of cell passage. Changes in phenotype during passage of XB cells had also been noted by others (Knutson and Poland, 1980a; Rheinwald and Green, 1975). To overcome this problem standard XB cells were stored in liquid nitrogen, and were placed in culture as necessary.

Safety considerations. Safe handling, containment, and disposal of the substances used in this study were essential. Every attempt was made to conform 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 Car-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cinogen Safety Standards Subcommittee of the DHEW Committee to coordinate toxicology and related programs.

RESULTS

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 keratinization. The results (Table 1) show that keratinization inducing activity was detected in all 10 samples. However, the minimum amount of soot equivalent of the extract needed to induce keratinization greater than background varied from 0.4 to 114.2 μg. The relative activity of each sample was calculated in relation to the activity of the first floor sample (Table 1).

In order to determine if these results reflected actual variation between samples, these values were compared to the total PCDF analysis for these samples (Smith *et al.*, 1982a). Total PCDF values were used in this comparison, since, as predicted by pyrolysis studies of polychlorinated biphenyls (Buser *et al.*, 1978a,b), PCDF concentrations in the soot were found to be up to 100 times greater than PCDDs (Smith *et al.*, 1982a,b). As with the keratinization activity, the total PCDF concentrations varied greatly from floor to floor (Smith *et al.*, 1982a). Comparison of the relative keratinization activity (Table 1) with relative total PCDF concentrations of the various soot extracts demonstrated a good correlation between the results of the two methods (correlation coefficient equals 0.89) (Fig. 2).

TABLE 1

KERATINIZATION ACTIVITY OF BINGHAMTON STATE OFFICE BUILDING (BSOB) SOOT SAMPLES

Floor No.	Endpoint (μg soot/ml)[a]	Relative activity[b]
1	114.2	1.0
4	14.0	8.2
6	15.9	7.2
7	0.3	380.5
8	1.0	114.2
9	1.5	76.1
10	0.5	228.3
14	12.9	8.9
15	0.4	285.4
17	0.4	285.4

[a] Lowest concentration of a series of 10-fold dilutions of soot extract capable of inducing keratinization greater than background. Each endpoint is an average of two evaluations of three replicates.

[b] Relative to activity of first floor sample.

DISCUSSION

Exposure to 2,3,7,8-TCDD causes chloracne in humans, probably by an induced differentiation of the squamous epithelium resulting in hyperkeratinization. This effect is considered to be produced by a sustained stimulation of the normal keratinization response (Poland and Knutson, 1982). Knutson and Poland (1980a) have used the *in vitro* XB/3T3 cell keratinization system (Rheinwald and Green, 1975) to study this effect of chlorinated dioxin isomers and congeners. We have now shown the feasibility of the use of this system as an assay for dioxinlike activity in environmental samples.

Keratinization-inducing activity was found in extracts of 10 soot samples taken from a building contaminated with soot from a PCB-containing transformer fire. The level of activity in these samples varied over about three orders of magnitude. This variation in keratinization activity correlated well with the variation seen in the total PCDF concentrations in the same samples as determined by mass spectrometric analysis (Smith *et al.*, 1982a). This demonstrates the ability of the *in vitro* keratinization assay to discriminate between samples having relatively high and low levels of PCDFs.

FIG. 1. 2,3,7,8-TCDD induction of keratinized colonies in XB/3T3 cultures. (a) Notice darkly stained keratinized cells induced by 10^{-9} M 2,3,7,8-TCDD. (b) Solvent control colony grown in the presence of 0.1% DMSO with no evidence of keratinization. Rhodamine B stain, ×100.

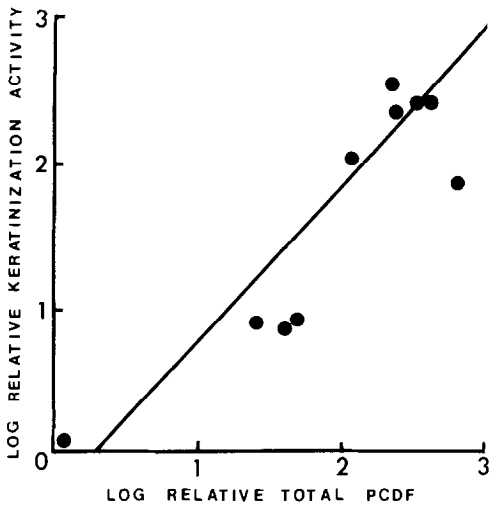

FIG. 2. Comparison of data for relative keratinization induction (from Table 1) and relative mass spectrometric analysis for PCDFs (Smith *et al.*, 1982a) for the various soot extracts from the Binghamton State Office building. The relative keratinization activity and relative total PCDF in each sample is plotted as a ratio to the values on floor 1. The correlation coefficient for these two sets of data is 0.89.

Exact quantitative correlation between the two methods was not possible in this experiment because the exact amount of any specific isomer or congener in these samples is unknown. It has been demonstrated that as for arylhydrocarbon hydroxylase induction and cytosolic receptor binding, the potency of a compound to induce keratinization in the *in vitro* XB/3T3 system is dependent on its structure. 2,3,7,8-TCDF, for example, has about 1/20th of the keratinization potential of 2,3,7,8-TCDD. Other PCDF isomers and congeners have even lower activity (Knutson and Poland, 1980a).

The relative specificity of the induction of *in vitro* keratinization for the most toxic PCDD and PCDF isomers and congeners has been demonstrated by Knutson and Poland (1980a). Here it was found that other halogenated aromatic hydrocarbon congeners, including chlorinated dibenzo(*p*)dioxins, dibenzofurans, biphenyls, and azo(xy)benzenes, also produce keratinization in the XB/3T3 system, but to a lesser degree than 2,3,7,8-TCDD. They also demonstrated that unrelated toxins, including direct-acting alkylating agents, inhibitors of nucleic acid synthesis, and a spindle microtubule inhibitor, all failed to produce keratinization in the XB/3T3 culture system. The *in vitro* keratinization response seems specific for the XB/3T3 culture system since 2,3,7,8-TCDD did not produce changes in cell growth, viability, and morphology in 23 other cell lines (Knutson and Poland, 1980b). The XB/3T3 system is extremely sensitive: a concentration of 2,3,7,8-TCDD as low as 3.2 pg/ml induced keratinization in these cultures (Knutson and Poland, 1980a).

Other *in vitro* cell culture models have been investigated and proven to be useful in the detection of planar polychlorinated organic compounds. The *in vitro* induction of aryl hydrocarbon hydroxylase (AHH) in rat hepatoma cell cultures (Bradlaw and Casterline, 1979) has been one of the most extensively studied. AHH is part of the P_1-450 monooxygenase system, which is a general detoxification mechanism, and which is inducible in a relatively short time by a wide variety of polynuclear aromatic hydrocarbons. While 2,3,7,8-TCDD is the most potent known inducer of AHH activity, other unrelated compounds such as 3-methylcholanthrene and benzo[*a*]pyrene also induce this enzyme. These compounds have been shown to be inactive in the induction of keratinization under routine culture conditions (Knutson and Poland, 1980a). This may allow the keratinization system to have a greater specificity for the detection of polychlorinated dioxin isomers and congeners.

Thus, an important advantage of the XB/3T3 *in vitro* keratinization system for detection of dioxinlike activity is its apparent sensitivity to the most toxic members of the PCDDs and PCDFs. Therefore this *in vitro* system detects relevant biological activity associated with a sample rather than depending on quantitation of specific isomers whose toxic potential may be unknown.

The relative specificity and sensitivity of the *in vitro* keratinization system for 2,3,7,8-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 a keratinization assay would be most useful in screening many samples, reserving subsequent high resolution chemical analysis for the identification of specific isomers and congeners in the most relevant samples. Application of this *in vitro* system to the detection of dioxinlike activity in other types of environmental samples and pollutants is under investigation.

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