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Application of the CALUX bioassay system for the detection of dioxin-like chemicals (Ah receptor ligands) in whole serum samples and in extracts from commercial and consumer products.

M.S. Denison, W.J. Rogers and M.Fair
Department of Environmental Toxicology, University of California, Davis, CA, 95616, USA .

M.Ziccardi
Wildlife Health Center, Institute of Toxicology and Environmental Health, University of California, Davis, CA, 95616, USA .

G. Clark
Xenobiotic Detection Systems, Inc., 310 Alcona Ave., Durham, NC, 27703, USA.

A.J. Murk and A. Brouwer
Department of Toxicology, Agricultural University, PO Box 8000, 6700 EA Wageningen, The Netherlands

1. Abstract

Halogenated aromatic hydrocarbons (HAHs), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are a diverse group of widespread and persistent environmental contaminants which produce a wide variety of species- and tissue-specific toxic and biological effects, many of which are mediated by the Ah receptor (AhR). Using a recently developed recombinant cell line which responds to TCDD and related chemicals with the AhR-dependent induction of firefly luciferase, we have developed a chemical-activated luciferase gene expression (CALUX) cell bioassay system for the detection of TCDD-like chemicals. We have utilized this bioassay as a screening tool not only to determine the widespread nature of these chemicals in industrial, commercial, consumer and environmental samples, but also for the detection of TCDD-like chemicals in whole serum samples. Here we report the identification of CALUX-positive compounds present in DMSO extracts from a wide variety of commercial and consumer plastic, rubber and paper products and we have now optimized the bioassay system or detection of TCDD in small (25-50 µl) aliquots of whole serum. Although the identity of the specific chemical(s) responsible for the positive CALUX signals currently remain to be determined as well as their toxicological and biological impact, our data demonstrate the widespread distribution of chemicals in everyday products that can specifically activate gene expression in an AhR-dependent manner. In addition, the availablility of a rapid and small volume serum bioassay for TCDD-like chemicals provides an avenue for screening of samples were limited blood volumes are available (e.g. endangered species) as well as large scale screening of populations for epidemiological studies.

2. Introduction

Polychlorinated diaromatic hydrocarbons (PCDHs) are a diverse group of widespread environmental contaminants, which include polychlorinated biphenyls (PCBs), dibenzofurans (PCDFs), dibenzo-*p*-dioxins (PCDDs) as well as many other subclasses of PCDHs and polycyclic aromatic hydrocarbons (PAHs).[1-3] Some PCDHs are not only lipophilic and persistent in the environment but they are known to bioaccumulate and biomagnify in the food chain.[1,2] Exposure

to TCDD-like chemicals produces a wide variety of species- and tissue-specific toxic and biological effects, many of which are mediated by the Ah receptor (AhR). The AhR is a ligand DNA regulatory protein which activates gene expression in a ligand (TCDD/PCDH)-dependent manner. Following ligand binding, the AhR complex is transformed into its DNA binding form, AhR complexes accumulate within the nucleus[2-5] and the binding of the PCDH:AhR complex to its specific DNA recognition site (the dioxin responsive element) stimulates expression of the adjacent gene.[4,5] We have recently developed a novel recombinant cell line which responds to TCDD and related chemicals (PCDHs and PAHs) with the induction of firefly luciferase and this bioassay, like the induction of EROD activity in rat hepatoma (H4IIE) cells, has been used has been used for the detection of TCDD-like chemicals in complex mixtures of chemicals from a variety of matrices.[1,6,10] Given the broad applicability of this assay and the documented widespread distribution of PCDHs and PAHs in a wide variety of products, we have begun screening for the presence of PCDH-like chemicals in industrial, commercial, consumer and environmental samples as well as optimization of the bioassay system for direct detection of these chemicals in biological materials (e.g., whole serum).

3. Materials and Methods

Sample Preparation: The indicated samples (Figure 1) were finely chopped up with scissors and extracted overnight (~16 hours) in glass tubes with DMSO (1.5 µλ of DMSO/mg of material for all samples except paper products which were extracted with 10 µl/mg of DMSO). DMSO was collected from each sample and stored in a teflon-capped vial until use.

Luciferase Expression Assay: A mouse hepatoma (Hepa1c1c7) cell line stably transfected with the HAH-inducible reporter plasmid pGudLuc1.1 [10] (H1L1.1c2) were used in these studies. The pGudLuc1.1 vector contains the firefly luciferase gene under PCDH-inducible control of four dioxin responsive elements and exposure of these cells to TCDD and related AhR agonists results in induction of firefly luciferase activity in a time-, dose- and AhR-dependent manner.[10] Cells, grown in 96 well microplates, were incubated with 2.5 µl of extract for 4 hours followed by lysis in the wells and automatic measurement of luciferase activity (using the Promega luciferase assay system) in a Dynatech ML-3000 luminescence plate reader. Activity was corrected for the amount of luminescence present in the DMSO-treated sample wells.

4. Results

We have previously demonstrated that the recombinant mouse hepatoma cell line, H1L1.1c2, responds to dioxin-like chemicals in a dose-, time- and AhR-dependent manner with the induction of firefly luciferase. The induction was specific for PCDHs and PAHs which are known to exert their action on gene expression via the AhR. Given the rapidity, inexpensive and sensitive nature of this assay (minimal detection limits of ~10 ppb), as well as its insensitivity to substrate inhibition (as observed with P4501A1[8]) this bioassay provides an avenue for the large scale screening of samples.

Commercial/Consumer Samples: Analysis of DMSO extracts from a variety of plastic, rubber and paper products using the CALUX assay system has revealed the presence of AhR ligands in many of these samples. Specifically, DMSO extracts from every paper product examined (Figure 1) induced luciferase activity (with maximal induction to ~25% of that observed with TCDD). Although the highest level of induction from these sample extracts was observed using printed newspaper, it was unclear whether the activity we observed was due to the paper and or the ink used in the printing process. Consequently, we carried out additional experiments utilizing DMSO extracts of ink-free newspaper and compared the results to that obtained using extracts from inked paper. These results (data not shown) clearly demonstrate that the majority of the activity observed with the newspaper extracts results from the ink used in the printing process, although low levels of activity were observed with a DMSO extract of ink-free paper itself. To date, all

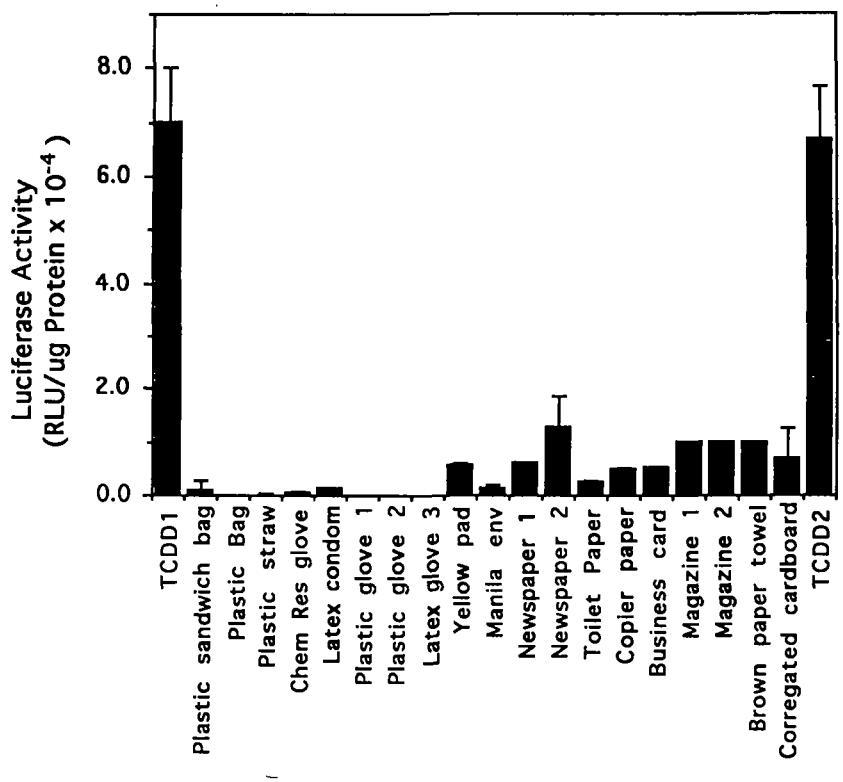

Figure 1. Induction of luciferase activity by DMSO extracts of the indicated products. H1L1.1c2 cells grown in 96 well microplates were incubated with an aliquot of a DMSO extract of the indicated products for 4 hours followed by measurement of luciferase activity. Values represent the mean ± SD of at least three determinations and were corrected for background luminescence observed with DMSO alone.

DMSO extracts of paper products we have examined have resulted in a positive CALUX response, although the magnitude of the signal was varied. We have also screened and observed the presence of CALUX-positive chemicals in a variety of rubber and plastic products, including:. rubber stoppers and O-rings, sneaker soles, a variety of rubber/silicone tubing, PVC pipe, rubber bands, baby pacifiers and nipples and other products. It should be noted that although this assay can be used to detect TCDD and related PCDHs it also can detect a variety of less/non-toxic PAHs as well. Thus, the identification of a positive CALUX response from a given sample does not necessarily indicate the presence of TCDD or the more toxic PCDHs. In fact, several lines of evidence suggest that the majority of the CALUX positive samples actually are not due to dioxins themselves. Measurement of the dioxin-like activity of these extracts using an in vitro AhR ligand or DNA binding assay would indicate that these extracts should be signficantly more active in the cell bioassay than they are. This decreased CALUX response is very likely the result of metabolism of

the inducing chemicals by the degradation enzymes present in these cells, something that is more characteristic of PAHs inducers rather than most PCDHs. In addition, preliminary elisa assays using an antibody against TCDD (and which cross-reacts with other dioxins) would support these conclusions. Although the identity of the specific chemical(s) responsible for the positive CALUX signals currently remain to be determined as well as their toxicological and biological impact, our data demonstrate the widespread distribution of chemicals in everyday products that can specifically activate gene expression in an AhR-dependent manner. Experiments are in progress to identify the CLAUX positive chemicals in the most active samples.

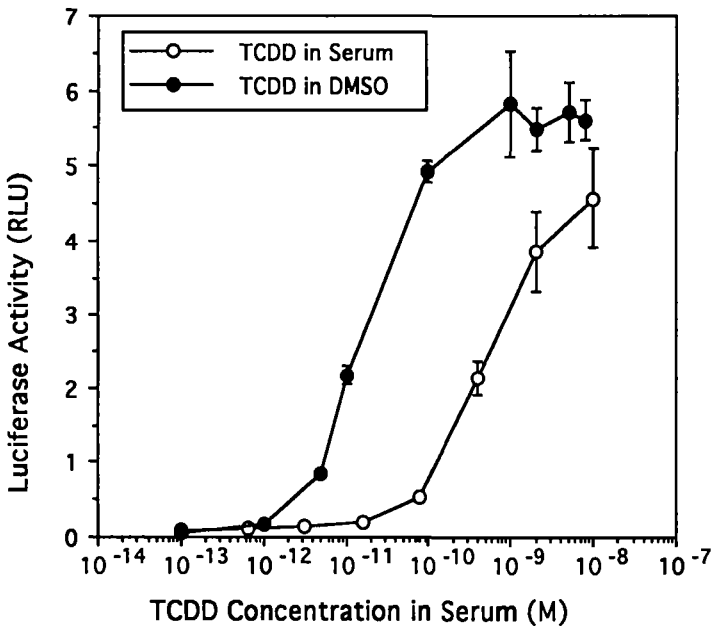

Figure 3. Dose-dependent induction of luciferase activity in H1L1.1c2 cells by TCDD added to each well in DMSO or in whole serum. H1L1.1c2 cells were grown overnight in 96 well microplates followed by the addition of TCDD in DMSO (added to 100 µl of media present in each well) or by the addition of 50 µl of whole serum (containing the indictated TCDD concentration) to 50 µl of media present in each well. Cells were incubated for 4 h at 37°C followed by analysis of luciferase activity as described in Figure 1. Values represent the mean ± SD of at least three determinations.

Serum Bioassay: Measurement of TCDD-like chemicals in biological materials (particularly blood and serum) has required solvent extraction of the material, followed by concentration and subsequent analysis by biological/analytical methodologies. The availability of a bioassay which can directly detect and provide a relative measurement of TCDD-like chemicals in whole serum would be a significant advance relative to large scale screening of blood samples. The sensitivity of the CALUX bioassay, combined with our modification of the bioassay system which allows direct growth of cells and subsequent analysis of activity in 96 well microplates, provides an avenue to develop a rapid screening assay for small volumes of serum. Utilizing this system, we have now optimized the CALUX bioassay for direct measurement of TCDD-like chemicals present in small

volumes of samples. Serum was spiked with TCDD at varying concentrations and added to 96 well microplates containing H1L1.1c2 cells and after 4 hours luciferase activity was measured (Figure 2). For comparison, induction was also carried out by directly adding TCDD, in DMSO, to the sample wells. As depicted in Figure 2, dose dependent induction of luciferase activity was observed by both methods with the dose-response curve for induction by TCDD in serum requiring approximately a 10-fold greater concentration for a similar induction response. This is likely due to the decreased availability of TCDD to the cells because of serum protein binding. These results demonstrate that the CALUX system can be used to directly detect TCDD present in whole serum samples and can be used to provide a relatively accurate estimation of TCDD-like activity in a sample. In addition, we have also determined that the optimal whole serum volume for these analysis is only 50 µl (mixed with 50 µl of culture media) and the results present in Figure 2 were obtained using 50 µl of fetal calf serum containing the indicated TCDD concentration. Overall, the rapidity and sensitivity of the CALUX assay, combined with the low volume of sample needed for analysis provides us with a unique assay system for the direct analysis of very small volumes of serum samples for the presence of TCDD-like chemicals. This modification of the CALUX assay not only has applications for the screening of samples where only small volumes of blood are available (e.g., endangered and/or small species), but it will allow for large scale screening of populations for epidemiology studies.

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