Direct Detection of Dioxins and Related Chemicals in Small Quantities of Human Serum Using a Recombinant Cell Bioassay

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Introduction

Halogenated and non-halogenated aromatic hydrocarbons, such as polychlorinated dibenzo-p-dioxins, biphenyls, benzo(a)pyrene and related chemicals have been recognized as significant and widespread pollutants in the environment. In mammalian systems, exposure to such xenobiotics have been observed to produce a variety of species- and tissue-specific toxic and biologic effects, including tumor production, lethality, birth defects, organ toxicity and induction of many metabolic enzymes, including the cytochrome P450 (CYP) system[1-3]. Induction of these enzymes is thought to be mediated by the Ah receptor (AhR), a soluble intracellular protein which strongly binds these chemicals and, through translocation and DNA binding, causes increased transcription of CYP1A1 enzymes[4-7]. This induction, consequently, has been one response that has been used not only as a bioassay to evaluate the relative biological/toxicological potency of HAHs and complex mixtures containing these chemicals, but, through implication, as a biomarker of exposure to xenobiotics[3].

While the quantification of CYP enzymes may act as an excellent "bioassay of effect" in some species, because of differing intra- and interspecies response to various concentrations of xenobiotics[4] and inhibition of CYP enzymatic activity by many AhR inducers , it is difficult to assess exposure using the resultant data. Additionally, analytic methods that are routinely employed to determine pollutant exposures often require both prior knowledge of exposure chemical class as well as significant amounts of biologic tissue for analysis. In order to accurately assess such exposure using easily obtainable samples, we have modified a specific "bioassay of exposure" for environmental samples in order to detect these substances in the serum of vertebrates. As described previously, this assay uses the AhR-dependent mechanism of action (CYP1A1 induction) in cell culture combined with a recombinant luciferase gene vector as an indicator to quantify TCDD-like ligand present in samples through measurement of chemically-induced luminescence[8,9].

While this assay works well for samples that contain high levels of AhR ligands and has been optimized for this use, at levels normally associated with chronic or sub-acute exposures (as well as exposure to those chemicals not associated with extremely strong induction), direct analysis of serum may not be adequate to separate true xenobiotic exposure from "background" (i.e. non ligand-

dependent) induction of cells. In order to improve both precision and validity of the assay, two additional optimization steps have recently been incorporated into the protocol. First, by first extracting serum in a simple liquid-phase manner, the sensitivity of the assay improves dramatically, into the picomolar range for TCDD. Secondly, through the parallel analysis of samples with a potent Ah receptor antagonist (4-amino-3-methoxyflavone or AMF), ligand independent luciferase activity can be easily corrected for.

Here, we describe the methods we employ to analyze samples for AhR ligands and present preliminary data on samples from a human population.

Experimental Methods

Serum. Serum samples were collected from a variety of human subjects with HAH exposure as part of a larger baseline exposure trial.

Cell Line. Mouse hepatoma (hepa1c1c7) cells were stably transfected with the HAH-inducible luciferase expression vector pGudLuc1.1 (H1L1.1c2) as described[9]. This vector contains the luciferase gene under HAH-inducible control of four dioxin responsive elements such that, when exposed to AhR ligands, induction of luciferase occurs in a time-, dose- and AhR-dependent manner.

Sample preparation. Serum samples were mixed twice with an equal volume of acetone (to precipitate serum proteins), centrifuged and the resultant supernatants were collected. The extractant was blown to dryness under N_2 and then resuspended in DMSO.

Induction Protocol. Plates of stable cell clones were trypsinized and resuspended in 10 ml alpha-MEM. One-hundred µl of this cell suspension was aliquoted into all wells of a sterile 96-well microtiter plate. Plates were incubated for 24 hours prior to ligand exposure, allowing cells in all wells to return to confluence. Cells were then induced by the addition of culture media containing extractant (50:1), as well as addition culture media containing extractant as well as the AhR antagonist AMF (50:1). All inductions were done in triplicate. After a 4 hour induction period, cells were washed with PBS, then lysed. Subsequent luciferase activity was determined using the Promega luciferase assay system in a Dynatech ML300 automated microplate luminometer.

Protein Correction. In order to correct for cell number per well, protein concentrations were determined in triplicate using a modification of a previously-developed method[10]. Briefly, fluorescamine in acetone was added to all wells, including a standard curve of 0 to 75 µg bovine serum albumin assayed in triplicate. Subsequent fluorescence was quantified using a multiplate fluorometer at 400 nm excitation and 460 nm emission wavelengths.

Data Analysis. All samples were expressed as relative light units (RLUs) per mg protein.

Results and Discussion

We have previously shown that this luciferase-based bioassay works in a dose-, time- and AhR-dependent manner for the screening of chemicals, natural and manufactured products[9]. Also, we

have shown the utility of this bioassay in analysis of HAHs in sediment, milk and whole serum[11-13], however, at the physiologic levels that HAHs are seen *in vivo* in most samples, the lower detection limits of the assay may not be sufficient. Although, when this assay is coupled with a simple liquid-phase sample extraction technique and the elimination of non-ligand dependent induction through the use of an AhR antagonist, sufficient sensitivity for lower level exposures may be seen.

As an example of these techniques, serum samples collected from HAH-exposed humans were extracted with acetone and analyzed using the bioassay. As is seen in Figure 1, when results are compared with TCDD as a positive control, most are seen to be positive and, in fact, some have fairly high levels of activity. Using this screening information, further analyses, including the analyses of serial dilutions of positive extracts in order to calculate relative TEQs in the serum, as well as incorporating additional analytic measures for specific classes of compounds (via GC/MS), can be conducted in a time- and cost-effective manner.

Although this assay, as described, does not differentiate between HAHs, PAHs and other inducing chemicals, the coupling of this extraction procedure with our recently-optimized acid denaturation, which inactivates non-HAH chemicals[13] will allow direct estimation of HAH-equivalents in these samples. These data presented represent a fraction of the total number of blood samples that we have begun to analyze. Since GC/MS analysis has already been carried out on each of these samples, determination of HAH TEQs in each of these using the bioassay will provide direct TEQ comparison of data from this biologically-based system with that from instrumental analysis.

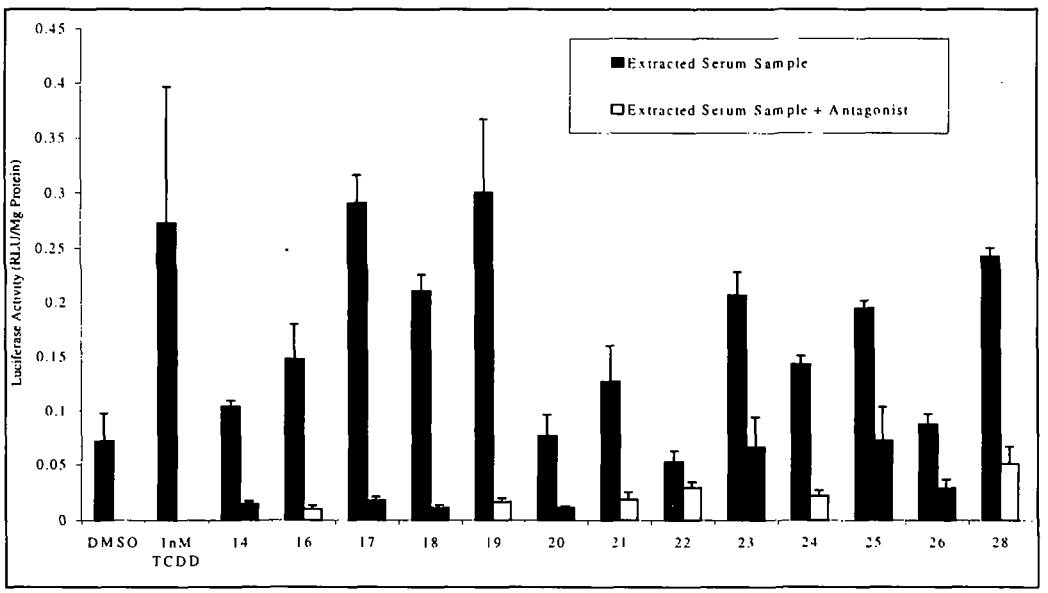

Figure 1: Induction of luciferase activity by DMSO extracts of human serum samples. H1L1.1c2 cells grown in 96-well microtiter plates were incubated with an aliquot of acetone-extracted serum both in and not in the presence of an Ah receptor antagonist, 4-amino-3-methoxyflavone for 4 hours, followed by measurement of luciferase activity. Values represent the mean ± SD of triplicate determinations.

While these additional techniques increase both our analysis time and cost, when compared with the extensive and expensive clean-up procedures necessary (i.e. GC/MS), to the lower sensitivity (i.e. fluorescent/spectrophotometric methods) or to the requirement of difficultly-acquired tissues (i.e. EROD) of other methods, this bioassay provides us with a sensitive and relatively rapid procedure for large-scale screening of samples from human populations for HAHs. As such, this bioassay complements conventional chemical analytical procedures by low cost screening of samples in order to identify those to be further characterized by GC/MS.

Acknowledgments

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