Ah RECEPTOR-BASED BIOASSAYS FOR DIOXINS AND RELATED CHEMICALS: APPLICATIONS AND LIMITATIONS.

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Introduction

Halogenated aromatic hydrocarbons (HAHs), such as polychlorinated dibenzo-p-dioxins (PCDDs), biphenyls (PCBs) and dibenzofurans (PCDFs), represent a large group of compounds which have been identified worldwide in a variety of wildlife and human tissues as well as in food, water, and soil samples. Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the prototypical and most potent member of this class, produces a wide variety of species and tissue-specific toxic and biological effects. Epidemiological and risk assessment analysis of HAHs/TCDD in humans and animals requires that an accurate measurement of the internal level of exposure be made. Although sophisticated cleanup and instrumental analysis (GC/MS) procedures can separate, identify and quantitate individual PCDD, PCB and PCDF congeners, these procedures require sophisticated equipment and large amounts of tissue for analysis and are also very costly and time-consuming, particularly when samples may contain large numbers of different HAH isomers and congeners. Thus, inexpensive and rapid screening bioassays capable of detecting and estimating the relative biological/toxic potency of complex mixtures of HAHs would have utility. Several bioassays meeting these criteria have been developed for HAH/TCDD detection and they are amenable to large scale screening analysis necessary for these types of studies. These assays are based on the ability of the compounds to be recognized and bound by antibodies (immunoassays) as well as their ability to induce a biological response in cells or whole organisms (bioassays). Current bioassays are based on the biochemical mechanism of action of TCDD and related HAHs and involves the Ah receptor (AhR), a ligand-dependent factor which mediates both the toxicity and the activation of expression of target genes specifically in response to these chemicals. We and others have developed in vitro and cell-based AhR-dependent screening bioassay systems for use in detection of TCDD and related chemicals. Given the potential utilization of these AhR-based bioassays for large scale chemical and environmental screening, further characterization of the in vitro Gel Retardation of AhR Binding (GRAB) and the cell-based Chemically-Activated LUciferase eXpression (CALUX) bioassays is necessary. Here we describe several applications for these screening systems and their current limitations.

Materials and Methods

GRAB Analysis. Male Hartley guinea pigs cytosol was used as the source of AhR for the GRAB assay. Cytosol was incubated with DMSO (20 ul/ml), TCDD (20 nM) or the indicated chemicals or extracts for 2 h at 20°C followed by electrophoretic separation of protein complexes and autoradiography of the dried gels as we have described in detail (1). Quantitation of the amount of protein-DNA complex formed was carried out using a Molecular Dynamics Phosphorimager SI.

<u>CALUX</u>. The CALUX assay utilizes recombinant mouse hepatoma (H1L1.1c2) cells which we previously generated and which were grown and maintained as described in detail (2). These cells, derived from the Hepa1c1c7 line, were engineered to contain a stably integrated DRE-driven firefly luciferase reporter gene plasmid whose transcriptional activation occurs in a time-, dose-, ligand- and AhR-dependent manner (2). For chemical treatment, H1L1.1c2 cells were grown in 24 to 96 well culture plates and incubated with DMSO, TCDD, 25-50 ul of whole serum or pure chemical or extracts for 4 hours at $37^{o}C$. After incubation, the cells were lysed and luciferase activity determined using a Dynatech ML3000 Microplate Luminometer with automatic injection of Promega stabilized luciferase reagent as we have described in detail (2). Luciferase activity, normalized to sample protein concentration determined using the fluorescamine protein assay (3) and bovine serum albumin as the standard, was calculated as relative light units (RLU) per mg of protein and expressed relative to that induced by TCDD.

<u>Preparation of Crude Newspaper Extracts</u>. DMSO extracts of commercial newspapers, virgin unprinted paper and newpaper ink were prepared for CALUX/GRAB analysis. Approximately 0.1 g of minced newsprint was deposited into individual Teflon-lined screw cap tubes followed by the addition of 10 volumes of DMSO; ink was extracted with an equivalent volume of isooctane. The capped sample was allowed to stand overnight (16 h) followed by transfer of the crude solvent "extract" into a clean Teflon-capped vial and an aliquot (20 µl/ml) analyzed by GRAB and CALUX.

Results and Discussion

The CALUX and GRAB bioassays provide us with inexpensive and relatively rapid systems for the detection TCDD and related chemicals. With TCDD, these systems are rapid (~5h) and sensitive (with detection limits below 1 ppt for TCDD in extracts and ~5 ppt for TCDD in whole serum) and it has been adapted to a 96 well microplate format for increase sample analysis. Validation studies have also revealed a good correlation between CALUX induction and TCDD/HAH concentrations (2,4-6). We have also used the CALUX assay for direct detection of TCDD/HAHs in small volumes (25-50 ul) of whole serum and extracts from tissue and other matrices and these results will be presented. The inclusion of a potent Ah receptor antagonist (to correct for non-ligand dependent induction) has also increased the sensitivity and selectivity of the assay system, thus improving its diagnostic value.

However, a major limitation of these bioassays is that they are not specific for TCDD alone, but they will respond to any chemical which can bind to and activate the AhR. We commonly observe that the GRAB assay not only responds to a much greater spectrum of chemicals than the CALUX bioassay, but the magnitude of response to a given crude extract is generally far greater as well, likely due to its ability to be activated by other chemicals in the mixture. For example, we observe positive signals using a DMSO extract of a commercial newspaper (Print) with both assays (Figure 1), although the response with the GRAB assay is much greater (equivalent to 1 nM TCDD). The minimal response observed using an extract of virgin unprinted paper suggests that the positive activity results from the ink used in the printing process. In fact, an isooctane extract of the printers ink used to print the specific newspaper we analyzed, contained significantly greater activity than the printed paper alone. Thus the activity likely results from the printers ink. Although both assays could be used to determine this, the GRAB assay responded to a greater degree. Additional studies (7) have not only revealed that the GRAB assay responds positively to

a greater number of chemicals than detected by the CALUX bioassay, but the GRAB assay can be activated by a large number of chemicals which fail to induce in the CALUX bioassay. These results reveal a significant discrepancy between the ability of these two bioassays to detect AhR agonists.

Analysis using only the GRAB assay can provide information as to the ability of a chemical to stimulate transformation of the AhR into its DNA binding form, but not whether it will be able actually to induce AhR-dependent gene expression in intact cells. GRAB is a useful bioassay to identify and characterize chemicals which can bind to the AhR (i.e. induce AhR transformation and DNA binding) and can be used to examine the ligand-specificity of the AhR. However, the ability of chemicals and chemical extracts to activate the AhR *in vitro* but not in intact cells, as shown in our results, is clearly a major limitation of GRAB or AhR ligand binding assays for screening of unknown extracts for dioxins and related HAHs. Consequently, use of GRAB or ligand binding assays as environmental screening tools will result in large numbers of non-HAH false positives and, as such, they are inappropriate for use in chemical screening procedures. Clearly, the preferred approach for identification of dioxins and related HAHs in extracts containing unknown mixtures of chemicals is initial screening using the CALUX (or a related cell-based bioassay) although some false positive will still result (albeit significantly less that obtained with GRAB analysis). The coupling of the CALUX bioassay with a rapid chemical extraction/separation procedures will not only reduce these false positives, but it will improve the sensitivity and specificity of detection and facilitate the use of the CALUX bioassay as a prescreening tool for large numbers of samples in order to identify those for subsequent analysis by GC/MS.

Acknowledgments

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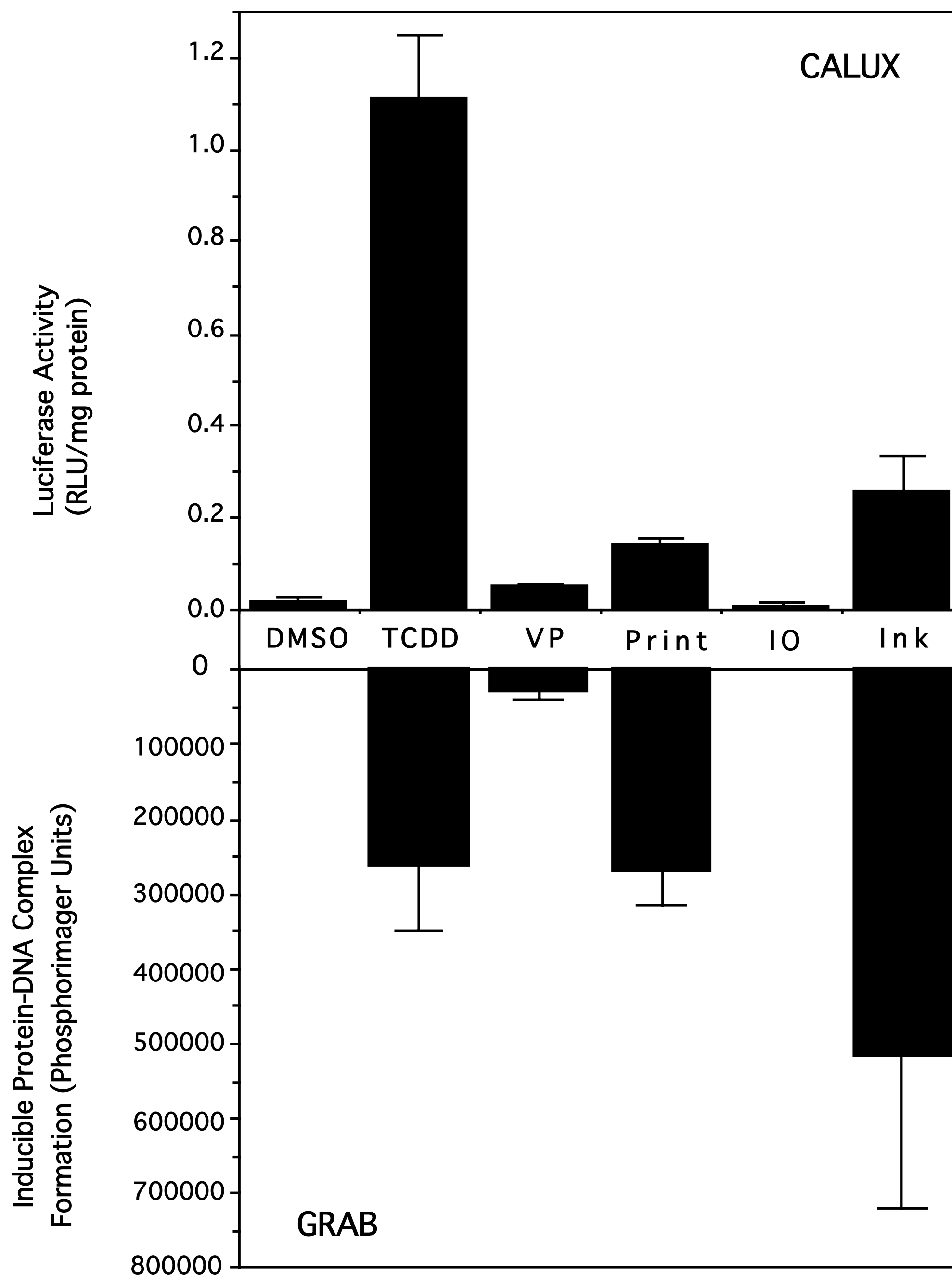

Figure 1. CALUX and GRAB analysis of DMSO exracts of newspaper (Print), virgin unprinted paper (VP) and isooctane (IO) extracts of printers ink.