APPLICATION OF THE LUCIFERASE RECOMBINANT CELL CULTURE BIOASSAY FOR DETECTION OF DIOXIN-LIKE ACTIVITY OF PAHS IN THE SERUM OF WILDLIFE

Michael H. Ziccardi [1,2], Ian A. Gardner [2] and Michael S. Denison [3]

Wildlife Health Center, School of Veterinary Medicine[1]; Department of Medicine and Epidemiology, School of Veterinary Medicine[2];and Department of Environmental Toxicology, University of California, Davis California USA[3]

Introduction

Halogenated and non-halogenated polycyclic aromatic hydrocarbons (HAHs/PAHs) have long been recognized as significant and widespread pollutants in the environment. In mammalian systems, exposure to such xenobiotics has produced a variety of species- and tissue-specific toxic and biologic effects. The majority of these effects are mediated by the Ah receptor (AhR), and non-mortality-related effects include tumor promotion, birth defects, hepatotoxicity, immunotoxicity, dermal toxicity, and alterations in endocrine homeostasis, as well as induction of many metabolic enzymes such as the cytochrome P450 (CYP) system [1].

Because of the potential for high morbidity and mortality associated with exposure to these compounds, many analytical techniques have been developed to detect their presence in biological samples. These procedures often rely on very accurate methods, such as gas chromatography, high-pressure liquid chromatography or other methods; however, their use also has limitations, such as high analysis costs, lack of rapidity and the need for large sample volumes. All of these factors negatively affect their utility for rapid screening and analysis of large numbers of samples.

To better assess biological responses of such compounds in complex systems, much research effort has been directed toward the development of bioassays. Techniques that examine *in vivo* responses in tissues (such as measurement of CYP1A1 induction by the quantification of ethoxyresorufin-o-deethylase (EROD) activity, aryl hydrocarbon hydroxylase (AHH) activity or CYP1A1 mRNA levels) have been used widely to determine exposure of individuals [2-4]. However, this approach has several disadvantages, including species differences in response to HAHs/PAHs [5], their requiring either the euthanasia of the animal or the use of invasive surgical techniques for sample collection, and that many CYP1A1-inducing chemicals also act as substrates for this activity, thus competitively inhibiting response at high concentrations [6]. Other methods that use detection of enzymatic activity, Ah receptor binding or the induction of reporter genes have been more recently used to evaluate exposure of animals or environments to HAHs/PAHs. The H4IIE rat hepatoma cell bioassay [7] has been used to detect HAHs/PAHs and provides an estimate of the biological potency (expressed as 2,3,7,8- TCDD equivalents or TEQs) of compounds, however, since this method also measures increases in EROD activity, it has many of the same limitations described above. The application of recombinant reporter plasmids, such as the firefly luciferase (*Luc*) gene [8] has proven to be a very effective method to detect these chemicals and to provide an approach to estimate TEQs (through calculation of induction equivalency factors, or I-EFs) in a variety of compounds [9]. The recombinant cell culture luciferase bioassay system has been found

to be extremely sensitive in directly detecting AhR-dependent potential of a variety of pure HAH compounds [10]. However, the determination of luciferase induction associated with PAH compounds has been done in only a few studies [11,12] and very little work has yet been done to characterize and optimize this method for the direct analysis of small volumes of whole serum [13]. Therefore, the aims of this study were to determine the induction potential (described in relation to TCDD) for a variety of PAH compounds, and to use this method to analyze samples from selected wildlife populations with differing known exposure to pollutants containing high levels of PAHs, namely oil spill events.

Experimental Methods

Chemicals: All aromatic hydrocarbons were considered hazardous and appropriate personal protective methods and materials were used in all experiments. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Dr. S. Safe (Texas A&M University). Monocyclic and PAH compounds used in this study are specified in Table 1 and were purchased from Accustandards Co. (St. Louis, MO) and diluted in methanol (MeOH).

Serum. Serum samples were collected from a variety of wildlife species (under appropriate IACUC approval) with differing exposure to PAHs. Free-ranging common murres (*Uria aalge*) were sampled during routine population surveys in the coastal California waters, as well as during several oil spill events that occurred in California during 1997. Northern sea otters (*Enhydra lutris)* were collected either as part of larger health assessment projects or from animals undergoing rehabilitation during the *Exxon Valdez* oil spill.

Cell Line. Recombinant H1L1.1c2 cells were used in these studies. These cells respond to AhR agonists with the induction of firefly luciferase [9]. Treatment of cells in the 96-well microplate format was previously described [13]. Dose-response experiments for PAHs and TCDD were conducted using ten-fold serial dilutions (v/v) of the inducing products in DMSO or methanol. Analysis of all serum samples was done by addition of 75 µl of either control serum (containing DMSO, or TCDD at a 1% final concentration) or sample serum at a 50% dilution in media. After a three-hour induction period, cells were analyzed as previously described [13] and expressed as relative light units (RLUs) per mg protein.

Data Analysis. Median (EC_{50}) and 20%(EC_{20}) effective concentrations of maximal TCDD induction, and induction equivalency factors for each ($I\text{-}EF_{50}$ and $I\text{-}EF_{20}$) were calculated by fitting dose-response data to a four-parameter Hill model by least squares (Sigma Plot; SPSS, Chicago, IL). Spearman's rank order correlation comparing EC and I-EF values from other published data were calculated using Statistica (Statsoft, Tulsa, OK).

Results and Discussion

Of the sixteen PAH evaluated, no statistically significant luciferase induction was observed for cells treated with monocyclic, 2- or 3-ringed compounds (all P values > 0.05), most likely due to the compound's inability to bind to and activate the AhR [11,14]. In contrast, most four- to six-ring structures tested (with the exception of benzo(g,h,i)perylene) showed high levels of induction, and had relatively low EC_{50} and EC_{20} values, and relatively high corresponding $I\text{-}EF_{50}$ and $I\text{-}EF_{20}$ values (Table 2). Calculated EC_{50} and I-EF values correlated somewhat with values determined using a rat cell line [11]; Spearman r = 0.71, P = .11) and a human cell line [12]; Spearman r = 0.84, P

= .04), and improved using the EC_{20} and I-EF_{20} values (Willett: Spearman r = 0.77, P = .07; Jones: Spearman r = 0.93, P = .003). Induction equivalencies determined for each of the individual congeners were from 9.3 to 144 times greater than that reported in the human cell line, and 0.2 to 7.5 times greater than with the rat luciferase bioassay using the I-EF_{50} values, and 10.3 to 1,350 times greater and 0.2 to 501 times greater, respectively, using the I-EF_{20} data. These differences are most likely attributable to the reduced incubation time (three hours) compared to these other assays (16 to 24 hours). Since reduction of the incubation period would decrease PAH metabolism and increase its inducing potency relative to TCDD, our estimated I-EF values would be greater than those where there was significant PAH metabolism. These data support the fact that the luciferase bioassay method is a sensitive technique to detect PAH compounds, Furthermore, our more rapid analytical technique suggests that previous cell bioassay analyses may have underestimated the induction potency of PAHs.

Using this information, the bioassay was evaluated whether it could detect PAH exposure in wildlife species, specifically that due to exposure to a marine petroleum spill. Statistically significant differences were noted between oiled and unoiled common murres and Northern sea otter populations ($P < 0.05$). While it is understood that this assay is not analytically specific (in that it cannot differentiate petroleum exposure from other CYP1A1-inducing compounds such as HAHs and other AhR ligands), its use as a rapid screening tool for samples from large numbers of animals can prove extremely beneficial for decision making during spill events. Should quantitation of true petroleum exposure be necessary, it can allow for significant cost reduction in order to identify those to be further characterized by GC/MS. This bioassay can also allow for large-scale assessment of toxin loads in wildlife populations exclusive of oil spill events by the analysis of either small amounts of serum collected during an ongoing effort, or through retrospective serological surveys of banked samples. Thus, through the ability to analyze large numbers of samples in an effective and sensitive manner, this method has the potential to be a very powerful tool for the epidemiological assessment of wildlife population and ecosystem health.

Acknowledgments

This work was supported by the US National Institutes of Environmental Health Sciences (ES07685 and ES04699) and the California Department of Fish and Game's Oil Spill Response Trust Fund through the Oiled Wildlife Care Network at the Wildlife Health Center, School of Veterinary Medicine, University of California, Davis.

References

1. Whitlock, J.P. (1999) Ann Rev Pharm Tox 39, 103-25.
2. Burke, M.D. and Mayer, R.T. (1974) Drug Metab Disp 2, 583-8.
3. Nebert, D.W. and Gelboin, H.V. (1968) J Biol Chem 243, 6242-9.
4. Vanden Heuvel, J.P., Clark, G.C., Thompson, C.L., McCoy, Z., Miller, C.R., Lucier, G.W. and Bell, D.A. (1993) Carcinogenesis 14, 2003-6.
5. Denison, M.S. and Wilkinson, C.F. (1985) Eur J Biochem 147, 429-35.
6. Kennedy, S.W., Lorenzen, A., James, C.A. and Collins, B.T. (1993) Anal Biochem 211,102-12.
7. Bradlaw, J.A. and Casterline, J.L. (1979) J Assoc Off Anal Chem 62, 904-16.
8. Denison, M.S., El-Fouly, M.H., Aarts, J.M.M.J.G., Brouwer, A., Richter, C. and Giesy, J.P. (1993) Organohalogen Compounds 13, 365-8.
9. Garrison, P.M., Tullis, K., Aarts, J.M., Brouwer, A., Giesy, J.P. and Denison, M.S. (1996) Fund Appl Tox 30, 194-203.

10. Sanderson, J.T., Aarts, J.M., Brouwer, A., Froese, K.L., Denison, M.S. and Giesy, J.P. (1996) Tox Appl Pharm, 137, 316-25.
11. Willett, K.L., Gardinali, P.R., Sericano, J.L., Wade, T.L. and Safe, S.H. (1997) Arch Env Contam Tox 32, 442-8.
12. Jones, J.M. and Anderson, J.W. (1999) Env Tox Pharm 7, 19-26.
13. Ziccardi, M.H., Gardner, I.A. and Denison, M.S. (2000) Tox Sci 54, 183-93.
14. Piskorska-Pliszczynska, J., Keys, B., Safe, S. and Newman, M.S. (1986) Tox Lett 34, 67-74.

Table 1: Calculated median (EC_{50}) and 20% maximal (EC_{20}) luciferase induction, and corresponding induction equivalency factors ($I\text{-}EF_{50}$ and $I\text{-}EF_{20}$) for polycyclic aromatic hydrocarbons analyzed using the luciferase bioassay.[1]

Chemical	EC_{50} (M)	$I\text{-}EF_{50}$ (M)	EC_{20} (M)	$I\text{-}EF_{20}$ (M)
TCDD	1.22×10^{-10}	1	3.46×10^{-11}	1
Chrysene	1.12×10^{-6}	1.08×10^{-4}	1.82×10^{-8}	1.90×10^{-3}
Benzo(a)pyrene	1.62×10^{-6}	7.53×10^{-5}	3.89×10^{-8}	8.89×10^{-4}
Benzo(b)fluoranthene	6.52×10^{-7}	1.87×10^{-4}	2.76×10^{-9}	1.25×10^{-2}
Benzo(k)fluoranthene	6.54×10^{-9}	1.87×10^{-2}	3.30×10^{-10}	1.05×10^{-1}
Indeno(1,2,3-cd)pyrene	4.23×10^{-8}	2.88×10^{-3}	1.31×10^{-9}	2.64×10^{-2}
Dibenz(a,h)anthracene	7.82×10^{-8}	1.56×10^{-3}	6.39×10^{-10}	5.41×10^{-2}

[1] PAHs that did not produce luciferase activity: benzo(g,h,i)perylene, naphthalene, 2-methylnaphthalene, acenaphthylene, acenaphthene, Fluorene, anthracene, phenanthrene, pyrene

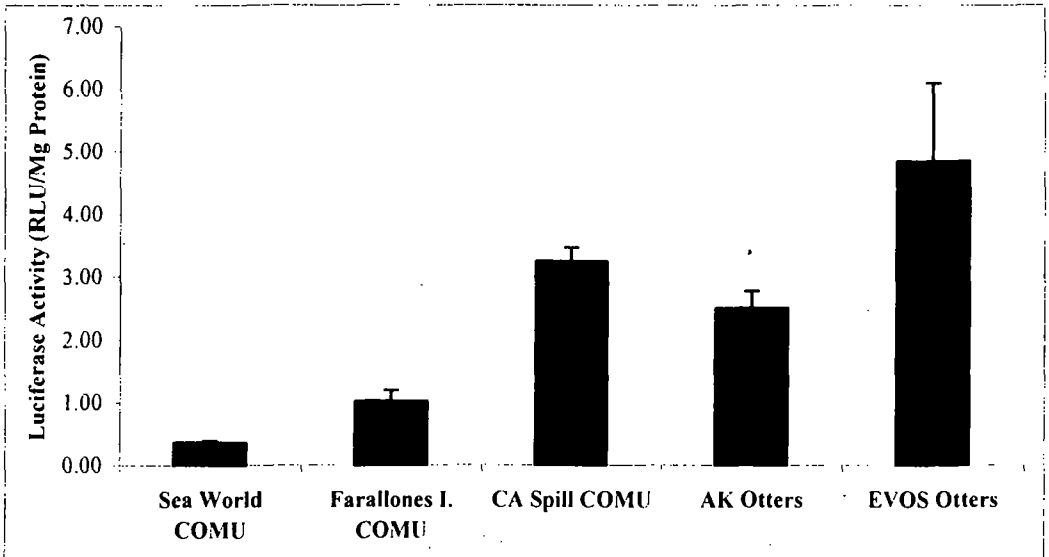

Figure 1: Luciferase activity in serum from common murres (COMU) from Sea World (n=23), the Farallones Islands, (n=20), and during a 1997 oil spill in California (n=94), and sea otters from southeast Alaska (n=46) and the Exxon Valdez oil spill (n=16)