DETERMINANTS OF ETHOXYRESORUFIN-*O*-DEETHYLASE (EROD) ACTIVITY IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES CHALLENGED *IN VITRO* WITH DIOXIN

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Introduction

Environmental dioxin contamination from incinerators and unidentified sources persists despite the control of many of the major past contributors. The consumption of contaminated foods and a limited capacity for metabolism results in detectable levels of dioxins in virtually all humans. While industrial or accidental exposures to dioxins have been associated with adverse health effects such as chloracne and cancer in humans (1), the health consequences of low level environmental exposures have been largely uncharacterized until recently. This situation has changed with the ability to measure low levels of dioxins in tissues such as blood and breast milk (2). The detection of altered thyroid and psychomotor function in neonates suggests that the concentration of environmental dioxin is sufficient to affect sensitive individuals (3,4). Findings such as these demonstrate the need for biomarkers to identify susceptible individuals and also to estimate the degree of interindividual variation in dioxin responsiveness among humans. To address this need, we have developed a system that measures dioxin-responsive biomarkers in peripheral blood lymphocytes challenged *in vitro* with 10 nM TCDD during cell culture. This system is used to correlate differences in responsiveness with factors such as age, genetic polymorphisms, gender, diet, cigarette smoking as well as the effect of an individual's dioxin exposure history. However, variation due differences in cryogenic storage and culture conditions may affect the ability to discriminate variation due to individual factors. This paper examines the effect of both individual and culture-related variables on the measured level of the dioxin-inducible enzyme, cytochrome P450 1A1 (ethoxyresorufin-*o*-deethylase (EROD)).

Materials and Methods

The exposure of the three populations chosen for this study differs in magnitude, duration and congener profile. The most highly exposed population is a group of older, primarily male chemical plant workers employed by the German chemical manufacturer Boehringer Ingelheim. Many individuals in this cohort were highly exposed to numerous congeners over the course of their work life. The second population consists of men, women and children living in the vicinity of Seveso, Italy in during an accidental release of tetrachlorodibenzo(*p*) dioxin (TCDD) in 1976.

For details, see Landi *et al*., this volume. Adult North Carolina volunteers, with presumably only environmental exposures, constitute the third population.. Each study subject supplied plasma for dioxin analyses and mononuclear cells for which were cryogenically stored for subsequent *in vitro* culture. Dioxin congener levels were analyzed in plasma using high resolution gas chromatography/mass spectrometry (2). In preparation for the measurement of EROD activity, lymphocytes were rapidly thawed and cultured for 3 days at 37 C with either mitogen only or mitogen and 10 nM TCDD as described by Spencer et al. 1999 (5).

Using the method of Clark (6), EROD activity was measured within two hours after the termination of cell culture. Briefly, reactions containing 10^6 lymphocytes, 20 μg NADPH and 50 pmoles ethoxyresorufin were incubated for 30 minutes at 37 C. The reactions were stopped by the addition of methanol. Fluorescence was measured on a Perkin Elmer LS-50B fluorescence plate reader with excitation at 550 nm and emission at 585 nm. Activity (pmoles/min) was expressed as activity per 10^6 lymphocytes. Activity per mg protein was also estimated when cultures yielded sufficient number of lymphocytes for protein quantitation. EROD activities from cells cultured only with mitogen were below the assay detection limit and not included in further analyses.

The culture variables chosen for evaluation were related to either storage conditions or cell health. "Duration of cryogenic storage" is the time interval between sample collection and thawing prior to cell culture. For the North Carolina volunteers, cells were stored from 15 to 676 days (median 525 days). The duration of storage was longer in both the Seveso (1025 to 1024 days; median 1524 days) and Boehringer cohorts (1176 to 1892 days; median 1756 days). Indicators of cell health are "preculture viability" which was measured after thawing but prior to culture and "post-culture viability" measured shortly after the termination of cell culture. Viabilities were measured by Trypan blue exclusion. "Cell growth" is based on cell counts before and after culture. "Smoking" status in the Boehringer and North Carolina cohorts was classified as never, current and past smokers. In the Seveso cohort, subjects were categorized according to current smoking levels into nonsmokers, 1-19 cigarettes/day and >20 cigarettes/day. Nonparametric methods, including the Kruskal Wallis test, were used to compare EROD activities. Spearman's rank correlation coefficient was used as a nonparametric measure of the correlation between continuous variables. Multiple regression analyses assessed the effect of variables judged likely to be related to EROD activity. The variables, plasma TCDD (lipid adjusted), plasma total toxic equivalents (T-TEQ) (lipid adjusted), Seveso EROD/10^6 lymphocytes (EROD-L), and Seveso EROD/mg protein (EROD-P), required log transformation to achieve normality. SAS and SPSS 8.0 were used for statistical analyses.

Results and Discussion

Table 1. shows the demographic variables and crude results for exposure (TCDD and T-TEQ) and response (EROD activity) in the cohorts. Plasma T-TEQ and TCDD levels are lower in the North Carolina than either the accidentally exposed (Seveso) or occupationally exposed (Boehringer) cohorts. Compared to the North Carolina cohort, the Seveso cohort had significantly lower crude EROD activities while the Boehringer cohort had significantly higher activities.

Spearman's correlation coefficients between EROD activities and population variables are not significant except for a strong negative correlation between TCDD/T-TEQ and both EROD-P and EROD-L. See Table 2. In contrast, EROD values from all cohorts are significantly affected by at

least one of the culture variables. In particular, a pattern of correlation between culture variables and EROD-L is evident. A similar pattern is not seen between EROD-P and culture variables. This is consistent with the expectation that EROD-L values incorporate the effect of lymphocyte health. The effect of "duration of cell storage" is most significant in the Boehringer cohort where the cells were stored the longest (up to 1756 days) compared to North Carolina cohort where no significant correlation with storage duration was found and the cells were stored a maximum of 676 days. The strongest relationship is seen in the Seveso cohort where the correlation between postculture viability and EROD-L is 0.56 ($p<0.001$). This relationship may partially account for inverse correlation between TCDD and EROD because lymphocytes from several of the highly exposed individuals also had longer durations of cell storage.

Table 1. Summary of cohorts

Cohort	North Carolina	Seveso	Boehringer
# subjects	43	121	104
age (SD, max, min)	34.5 (9.5, 52, 21)	47.3 (16.7, 75.8, 20.4)	55.6 (9.6, 81, 27)
males, females	24, 19	58, 60	98, 6
smoking	never: 15	nonsmoker: 93	never: 23
	current:10	1-19/day: 17	current: 34
	past: 18	>20/day: 11	past: 46
TCDD[1] (25-75%-ile, n)	1.3 (1.3-3.5, 29)	8.0 (4.9-14.9,110)	30 (7.7-77.1, 95)
T-TEQ[1] (25-75%-, n)	14.2 (12.2-18.6,29)	7.9 (4.6-14,110)	122 (57-239, 95)
EROD-P[2] (25-75%-ile, n)	1.84 (0.98-2.08,15)	1.50* (1.16-2.40,67)	2.36**(1.79-3.36,85)
EROD-L[3] (25-75%-ile, n)	0.08 (0.05-0.10,39)	0.06*** (0.03-0.10,73)	0.13***(0.08-0.19,85)

[1]median plasma ppt (lipid adjusted); [2]median EROD-P (EROD/mg protein); [3]median EROD-L (EROD/10^6 lymphocytes); Compared to North Carolina: *$p<0.05$, **$p<0.01$, ***$p<0.001$, Mann-Whitney or Wilcoxon rank sum test

Multiple regression analysis was used to examine the combined effect of plasma dioxins, smoking, gender, postculture viability and age. In both the Boehringer and Seveso cohorts, post-culture viability is a significant contributor to EROD-P. Post-culture viability is not a significant contributor in the North Carolina cohort when activity is measured in lymphocytes as EROD-L. EROD-P values were not available for this cohort. Cigarette smoke was expected to be an inducer of EROD activity (7). In the Boehringer cohort, cigarette smoking status (current, past, never) neared significance for EROD-P ($p=0.07$) but not for EROD-L. Daily cigarette smoking was estimated in the Seveso cohort and was a significant inducer of EROD-P ($p<0.001$). Gender, age, and the magnitude of dioxin exposure were not significant in the three cohorts.

In conclusion, the *in vitro* cell challenge system presented in this paper is useful for measuring factors associated with dioxin responsive only when variables related to cell storage and culture are incorporated into the analysis of results. We found that post-culture viability, an indicator of lymphocyte health, is a major contributor to EROD activity. The lack of impact of dioxin exposure status on EROD activity in populations with such distinctly different magnitudes of exposure suggests that other approaches to exposure quantitation, such as integrated total exposure, need to be incorporated into future analyses.

Table 2. Correlation coefficients for EROD activity and selected variables.

Population variables:		North Carolina	Seveso	Boehringer
age	P[1]	-0.38 (15)	-0.19 (67)	0.02 (85)

	L[1]	-0.31 (39)	-0.34 (73)	-0.10 (85)
TCDD[2]	P	0.15 (6)	-0.39** (59)	0.04 (78)
	L	0.19 (28)	-0.38** (65)	-0.06 (78)
T-TEQ[2]	P	0.20 (6)	-0.41*** (59)	0.03 (78)
	L	0.22 (28)	-0.38*** (65)	0.01 (78)
Culture variables:				
duration of	P	-0.21 (14)	-0.19 (68)	-0.12 (85)
cell storage	L	-0.29 (38)	-0.24* (74)	-0.40*** (85)
preculture	P	0.40 (14)	-0.07 (66)	-0.03 (85)
viability	L	0.24 (38)	0.08 (72)	0.06(85)
postculture	P	0.33 (12)	0.42*** (67)	0.19 (83)
viability	L	0.42* (35)	0.56*** (73)	0.26* (83)
cell growth	P	0.32 (9)	0.01 (61)	0.15 (85)
	L	0.37* (33)	0.00 (67)	0.12 (85)

[1]P= EROD/mg protein; L=EROD/10^6 lymphocytes; [2]Plasma levels adjusted for lipid
* $p<0.05$; ** $p<0.01$; *** $p< 0.001$

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