Molecular Epidemiology Study in Seveso: Ah Receptor and CYP1A1-Dependent Enzymatic Activity in Individuals from Dioxin Exposed and Control Areas

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Introduction:

In July of 1976 a runaway reaction during the production of trichlorophenol in a chemical plant in Seveso, Italy, resulted in the discharge of products containing 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or simply "dioxin")(1). As a surrogate for exposure status the contaminated area was divided into exposure zones based on analysis of soil in the area. Zone A had average levels of 15.5 to 580 $\mu g\ m^{-2}$ TCDD, zone B had average levels of 1.7 to 4.3 $\mu g\ m^{-2}$ TCDD, and zone R had scattered points of contamination with average levels of 0.9 to 1.4 $\mu g\ m^{-2}$ TCDD (2). An extensive follow up study of clinical parameters in these exposed people found little association of TCDD exposure with abnormal clinical results except for the skin disease chloracne, though exposure was confirmed through analysis of TCDD concentrations of up to 56,000 ppt in the blood (3)(4). However, epidemiological studies of the Seveso area population found increased risks for developing certain forms of neoplasms including hepatobiliary cancer, lymphoreticular sarcoma, multiple myeloma, myeloid leukemia, non-Hodgkin's lymphoma and soft tissue tumors (5)(6)

In an attempt to better understand the human effects of dioxin exposure we are investigating the possibility of using biomarkers of effect and susceptibility in the ongoing studies on long term

effects of dioxin exposure in Seveso. Since human blood samples can be collected from these individuals, we have developed methods to analyze biomarkers present in peripheral blood lymphocytes. These studies are part of a large collaborative effort between the University of Milan, Desio Hospital, NCI, CDC and NIEHS. The general study plan for the Seveso population has been reported (7) and results of a pilot investigation of gene polymorphism's and inducibility by TCDD have been published (8) We are investigating the expression of these biomarkers in volunteers that had resided in Zone A & B in Seveso or individuals that were outside these exposure zones.

Two potential biomarkers for dioxin susceptibility or effect are the levels of the Ah receptor in human lymphocytes and induction of the dioxin responsive gene CYP1A1. The Ah receptor is a cytosolic high affinity binding protein for 2,3,7,8-tetrachlorodibenzo-p-dioxin that is thought to mediate the biochemical and toxic effects of TCDD (9). One ot the most characterized responses to TCDD exposure is the induction of the drug metabolizing enzyme cyp1a1 produced by transcriptional activation of the CYP1A1 gene by the TCDD-Ah receptor complex. Assay of induction of cyp1a1 protein can be done by measuring the cyp1a1 associated enzyme activity, ethoxyresorufin-O-deethlylase (EROD) which demonstrates large interindividual differences for induction by TCDD in lymphocytes from various individuals (10).

In this preliminary report we have analyzed Ah receptor levels and EROD induction in cultured lymphocytes from 10 individuals outside the contaminated area and 6 individuals that resided in Zone B. This is a preliminary report from a larger study that will include 126 individuals, with 63 residing in Zone A or B and 63 residing in non-polluted surrounding areas. It will be interesting to determine the distribution of these biomarkers in the exposed and non-exposed human populations, the effect of TCDD levels on the biomarkers, and the inter and intra-individual variation of the biomarkers and whether they are predictive for human health effects.

Materials and Methods:

Blood was drawn into heparinized tubes from randomly selected healthy individuals that had resided in Zone A and B in Seveso or individuals that were outside the contaminated area, that agreed to participate in the study by signing an informed consent. Peripheral blood lymphocytes were purified using the Accuspin System (Sigma Chemical Co.) and cells were cryopreserved using standard procedures and shipped to the USA on dry ice. Cells were then stored in the vapor phase of liquid nitrogen until assayed. Cells were thawed quickly at 37C in a water bath and cultured in RPMI 1640 containing 10% Fetal Calf Serum at 1 x 10^6 cells/ml in the presence of the mitogens PHA and PWM with or without 10 nM TCDD as previously described (10). Within each experimental group of cells from Seveso a similar sample of cells from an individual from North Carolina was included as a control for method variability. Cells were cultured for 3 days and

harvested. Cells were counted and aliquoted to various assays. Ethoxyresorufin-O-deethylase (EROD) activity was assayed by a fluorimetric procedure as previously described (10). Cytosol fractions were isolated by sonicating the cells in Hepes buffer containing 20 mM $NaMO_4$ and centrifugation at 100,000 X g for 30 minutes. Alternatively, whole cell lysates were produced and assayed similar to cytosol fractions. Lysates were assayed for protein with the Pierce BCA protein assay with rabbit gamma globulin as standard and all samples adjusted to 100 µg/ml. Lysates were photoaffinity labeled with I^{125}-2-azido-3-iodo-7,8-dibromodibenzo-p-dioxin as previously described by Poland et al (11). The samples were electrophoresed in polyacrylamide gels, the gels were dried and autoradiographs made. Specific binding was detected in a 104 kDa band as demonstrated by inhibition of binding in the presence of a 200 fold molar excess of 2,3,7,8-tetrachlorodibenzofuran. In addition, cytosols and whole cell lysates were quantified using western blotting procedures with an antibody specific for amino acid residues 12-31 of the murine Ah receptor using an ECL method of detection. Within each set of samples a standard cytosolic preparation of C57BL/6J mouse liver Ah receptor was included as an external standard (binding activity of 50 fmoles/mg protein). The autoradiographs were scanned with an LKB laser densitometer and absorbance quantified for the 95 kDa band from standard C57BL/6J mouse liver as well as the 104 kDa band observed in human samples. Dilutions of this standard prep were used to produce a standard curve from which extrapolation of receptor concentrations were estimated assuming that human preparations demonstrate similar binding as well as antigenic activity with the antibody. Limits of detection for the assay of Ah receptor levels was approximately 60 atamoles receptor per 20 µg of protein.

Results:

Out of 10 individuals from non-contaminated areas 6 had detectable receptor levels in their cytoplasm with an average of 79 ± 25 atamoles receptor per 20 µg protein while in whole cell lysates, 8 of 10 individuals had detectable receptor levels with an average of 82 ± 16 atamoles receptor per 20 µg protein. Out of 6 individuals from zone B, 5 of 6 had detectable cytoplasmic receptor levels with an average of 81 ± 23 atamoles receptor per 20 µg protein while in whole cell lysates the average was 105 ± 40 atamoles receptor per 20 µg protein.

Shown in table 1 are the results of EROD determinations for the these individuals as well as for our method control subject from North Carolina. There was a significant amount of inter-individual variation among the individuals from both the exposed and unexposed areas surrounding Seveso. Intra-individual variation was not that great in our method control subject under induced conditions (coefficient of variation, 10.8%) suggesting that the method may be useful in discerning responsiveness to dioxins.

Table 1:

Ethoxyresorufin-O-deethylase Activity (pM/min*mg protein) in Cultured Human Lymphocytes:

Treatment	Exposed Area B [a] (mean ± SD)	Unexposed Area N [b] (mean ± SD)	NC Individual [c] (mean ± SD)
Uninduced	1.28 ± 0.78	0.93 ± 0.71	1.14 ± 0.76
Induced (10 nM TCDD)	3.36 ± 2.37	3.02 ± 2.23	3.88 ± 0.42

a Mean of EROD activity from 6 individuals from Zone B.

b Mean of EROD activity from 10 individuals from areas outside of Zone ABR.

c Mean EROD activity of 3 replicate determinations from the same individual from NC.

Discussion:

Humans have demonstrated large interindividual differences in their response to exposure to TCDD and its structural analogs from in vitro studies of CYP1A1 induction (10)(12)(13). Epidemiological evidence also suggest large interindividual differences in human responsiveness to dioxin exposure, in that some individuals exposed to equivalent levels of TCDD in the Seveso exposure incident developed chloracne while other individuals did not. The reason for these interindividual differences in susceptibility may be due to variation in receptor number or receptor affinity if the receptor is the rate limiting event in the final biological response. We are currently investigating receptor expression in Seveso populations to determine if there is a relationship of receptor expression to biological responses observed in humans. At this time the number of individuals analyzed is small and the power is inadequate to detect anything but the largest differences in these biomarkers. It is also possible that there is a misclassification in using geographic origin as a substitute for TCDD exposure. In the complete study we will compare these biomarkers to exposure levels by GC/MS analysis of dioxin serum levels as well as clinical and epidemiological endpoints to determine whether these biomarkers may be predictive for human health effects.

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