Exposure Assessment for Endocrine Disruptors: Some Considerations in the Design of Studies

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In studies designed to evaluate exposure–response relationships in children's development from conception through puberty, multiple factors that affect the generation of meaningful exposure metrics must be considered. These factors include multiple routes of exposure; the timing, frequency, and duration of exposure; need for qualitative and quantitative data; sample collection and storage protocols; and the selection and documentation of analytic methods. The methods for exposure data collection and analysis must be sufficiently robust to accommodate the *a priori* hypotheses to be tested, as well as hypotheses generated from the data. A number of issues that must be considered in study design are summarized here. *Key words:* developing child, endocrine disruptors, environmental epidemiology, exposure assessment. *Environ Health Perspect* 111:1683–1690 (2003). doi:10.1289/ehp.5798 available via *http://dx.doi.org/* [Online 18 March 2003]

The assessment of human exposure to environmental chemicals requires an understanding of the source of the chemical, its transport and environmental fate, and subsequent routes of entry into the body. Once an exposure occurs, it is necessary to have data on its absorption, distribution, metabolism, and elimination. This is a complex undertaking for any environmental chemical and for any potentially exposed population. It is especially challenging in the evaluation of exposures to children at various stages of their development. The activity of the endocrine system is vital throughout all stages of human life, but it is particularly critical during the stages of greatest human development—*in utero*, during infancy, early childhood, and puberty.

Exogenous chemicals such as endocrine disruptors are of special interest because they mimic, block, or in some way alter the activity of endogenous chemicals that are synthesized by the endocrine system (National Research Council 1999). The endocrine system as used here refers to all compounds involved in communicating information that are secreted by cells. These compounds can have autocrine effects (on the same cell), paracrine effects (on cells nearby), and classic endocrine effects (secretion is into the blood stream, potentially exposing all cells to the compound) (National Research Council 1999). The class of endocrine disruptors about which we know the most are those that mimic or block endogenous estrogens. Examples of synthetic chemicals with reported estrogenic activity are listed in Table 1.

Dietary exposures to some potential endocrine disruptors have been quantified for adults. For example, phytoestrogen consumption (expressed as total bioflavoids) is estimated at 1 g/day, whereas 100 g of wheat germ with 2 ppm zearalenone provides 200 μg/day (National Research Council 1999). The estimated exposure to dichlorodiphenyltrichloroethane (DDT) from all dietary sources in the general U.S. population is 0.01 μg/day, and estimated exposure to polychlorinated biphenyls (PCBs) is 0.002 μg/day (National Research Council 1999). However, exposures to environmental contaminants may vary among countries; for example, in areas where DDT is still used extensively, breast milk may be a significant source of DDT exposure for an infant (Kashyap et al. 1991).

Akland et al. (2000) have modeled the various factors influencing dietary exposures of young children. However, at different human developmental stages, not only do the diets vary but also the primary route of human exposure may vary; for example, Hubal et al. (2000) described the challenge of assessing children's residential exposure to pesticides.

In addition to dietary sources, other aspects of exposure assessment that should be considered in developing a research initiative include timing or age at exposure; the rate of exposure (frequency and duration); qualitative versus quantitative assessment; sample collection and storage; sample analysis; and exposures assessed to test hypotheses and those to generate additional hypotheses. Each of these aspects is treated in more detail below.

Routes of Exposure and Mechanisms of Response

The earliest relevant exposures may occur before conception. For example, the number of female births relative to total births to parents who resided in the most highly dioxin-contaminated area around Seveso, Italy, at the time of the dioxin release in 1976 was statistically higher than expected for the 8 years after the accident, presumably because of high dioxin exposures to the father (Mocarelli et al. 2000).

Epigenetic imprinting of genes is the basis for the finding that chromosomes provided to an embryo by the egg and sperm are fundamentally different (Jiang et al. 1998). Epigenetic imprinting refers to the mechanism(s) by which hormones and chemicals that mimic hormones permanently alter cellular functions when exposure occurs during organogenesis in fetal life; this also occurs inappropriately during carcinogenesis, causing a breakdown in cell cycle control systems. One mechanism that is generating considerable interest is the epigenetic modification of DNA by addition of methyl groups to specific bases located in the promoter region of genes. DNA methylation consists of the covalent addition of methyl groups to the 5-position of cytosines that are 5′ to guanine nucleotides in the DNA sequence. CpG dinucleotide sequences can occur as clusters in regions known as CpG islands, which are normally protected from

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DNA methylation (Gardiner-Garden and Frommer 1987). When methylation occurs at these normally protected sites, changes occur in chromosome structure and in the capacity for the gene controlled by the promoter to be activated. Inappropriate methylation of genes can act in a manner analogous to that of a classic genetic mutation and can cause the lack of a functional protein product. The result can be a subsequent breakdown in homeostasis, producing functional changes that could easily be missed in short-term tests for acute toxicity in adults or *in vitro* tests for classic gene mutations involving changes in base sequences (Jost and Saluz 1993). Thus, current toxicologic testing methods might not reveal this type of functional damage.

Epigenetic imprinting of genes is important for endocrine disruption research. For example, genes contributed to the embryo via the sperm are required for placental formation: When an egg has a pronucleus from another female injected into it, no placenta develops; in contrast, an egg that has its pronucleus replaced such that all genes are paternal does not develop into a normal embryo, but the placenta develops. There are intriguing observations that may relate to imprinting effects of chemicals. For example, when pregnant female mice (F_0 generation)

Table 1. Examples of endocrine-disrupting chemicals with reported estrogenic activity and examples of potential sources.

Compound	Source	Reference
Organochlorine compounds		
Chlordecone (Kepone)	Pest control	Gellert 1978; Hammond et al. 1979; Soto et al. 1994, 1995; Flouriot et al. 1995
Dieldrin		Soto et al. 1994, 1995; Sonnenschein et al. 1995; Arnold et al. 1996
Endosulfan		Soto et al. 1994, 1995; Sonnenschein et al. 1995; Arnold et al. 1996
α-Endosulfan		Soto et al. 1994, 1995
β-Endosulfan		Soto et al. 1994, 1995
DDT (technical grades and isomers)		Welch et al. 1969; Bitman and Cecil 1970; Soto et al. 1994, 1995, 1997
Lindane		Soto et al. 1995
Methoxychlor		Tullner 1961; Bitman and Cecil 1970; Bulger et al. 1978; Cummings and Metcalf 1994, 1995; vom Saal et al. 1995; Soto et al. 1995
Toxaphene		Soto et al. 1994, 1995; Sonnenschein et al. 1995; Arnold et al. 1996
PCBs	Dielectric fluids; contaminated soils; fish	Bitman and Cecil 1970; Ecobichon and MacKenzie 1974; Gellert 1978; Jansen et al. 1993
Phenolics		
Alkylphenol ethoxylates (APEs)	Cleaning agents and surfactants	White et al. 1994; Sumpter and Jobling 1995; Jobling et al. 1996; Routledge and Sumpter 1996
4-Alkylphenols	Used in manufacture of APEs	Soto et al. 1991, 1995; Jobling and Sumpter 1993; White et al. 1994; Bicknell et al. 1995; Fluoriot et al. 1995; Jobling et al. 1995, 1996; Sonnenschein et al. 1995; Sumpter and Jobling 1995; Ren et al. 1996; Routledge and Sumpter 1996
Bisphenol A	Epoxy resins; polycarbonate plastic products	Brotons et al. 1995; Krishnan et al. 1995; Sonnenschein et al. 1995; Soto et al. 1995, 1997; Sumpter and Jobling 1995; Olea et al. 1996; Nagel et al. 1998
Biphenylols (three isomers)		Soto et al. 1995, 1997
Chlorinated biphenyldiol (1 isomer)		Korach et al. 1988
Chlorinated biphenylols (> 10 isomers)		Korach et al. 1988; Jansen et al. 1993; Bergeron et al. 1994; Crews et al. 1995; Soto et al. 1995; Arnold et al. 1996
Other synthetic compounds		
Butylated hydroxyanisole (BHA)	Food/drug preservative	Jobling et al. 1995; Soto et al. 1995
Diphenylphthalate		Jobling et al. 1995
Butylbenzylphthalate (BBP)	Plasticizer for polyvinyl and cellulosic resin	Sonnenschein et al. 1995; Soto et al. 1995, 1997
Di-*n*-butylphalate (DBP)		Soto et al. 1998
Isoflavones		
Diadzein	Alfalfa, soybeans	Shutt and Cox 1972; Verdeal et al. 1980; Sathyamoorthy et al. 1994
Formonetin	Clover, alfalfa	Miksicek 1995
Genistein	Alfalfa, soybeans	Shutt and Cox 1972; Verdeal et al. 1980; Miksicek 1995
Biochanin A	Clover, alfalfa	Miksicek 1995
Flavones, flavonols, flavanones		
Apigenin	Chamomile, parsley, plantain	Miksicek 1995
Luteolin[a]	Plantain, chamomile	Markaverich et al. 1988; Miksicek 1995
Kaempferol	Cabbage, nettles, asparagus, raspberry, may apple	Markaverich et al. 1995
Naringenin[a]	Thyme	Miksicek 1995; Ruh et al. 1995
Chalcones	Licorice	
2,4,4´-Trihydroxyisoliquiritigenin		Miksicek 1995
2´,4,4´,6´-Tetrahydroxynaringenin chalcone		Miksicek 1995
2´4,4´6´-Tetrahydroxydihydrophloretin		Miksicek 1995
Indolo[3,2-β]carbazole	Brassicas	Liu et al. 1994
Coumestrol	Alfalfa, clover, soy, lima beans, pinto beans	Martin et al. 1978; Verdeal et al. 1980; Soto et al. 1992; Nagel et al. 1998
Equol		Shutt and Cox 1972; Tang and Adams 1980; Sathyamoorthy et al. 1994; vom Saal et al. 1995; Nagel et al. 1998
Nordihydroguaiaretic acid (NDGA)	Chaparral, desert cactus, edible oil stabilizer	Sathyamoorthy et al.1994
Zearalenone	Produced by *Fusarium fungi* on moldy corn, wheat, barley	Martin et al. 1978; Verdeal et al. 1980; Soto et al. 1992
Zearalanol	Derivative of zearalenone	Martin et al. 1978; Verdeal et al. 1980; Soto et al. 1992
β-Sitosterol	Saw palmetto, aloe vera, soybeans	Rosenblum et al. 1993; MacLatchy and Van Der Kraak 1995

Note: This table has been modified from the original source (National Research Council 1999), and the list of potential sources is not comprehensive.
[a]Also has antiestrogenic activity, as does quercetin (found in chamomile, raspberry, cabbage, nettles, asparagus).

are treated with the estrogenic drug diethylstilbestrol (DES), the male and female offspring (F_1 generation) have a spectrum of abnormalities, including cancers that appear in mid-life. Interestingly, the F_2 offspring produced by the exposed F_1 generation (mated to control mice) showed a significant increase in cancers, similar to F_1 animals that were exposed directly via their mothers (Newbold et al. 1998; Turusov et al. 1992). There is extensive evidence that alterations in maternal physiology can alter the development of her fetus. However, how genes in sperm might transmit a "memory" of prior environmental chemical exposure to an embryo remains to be determined. Although there is intensive investigation of imprinting mechanisms, the mechanisms by which chemicals alter gene activity without causing classic mutations remain unresolved.

In general, the period of highest susceptibility to adverse effects from environmental exposures is thought to be the *in utero* period. *In utero* exposures result from mobilization in the mother's body of chemical agents that can cross the placenta and enter into the fetal blood flow; the mother may have been exposed to these chemical agents before pregnancy or during pregnancy. Postnatally, the infant may be at risk from exposures occurring from foods, such as breast milk, or from mouthing nonfood objects, such as chips of lead-based paint, toys, and stuffed animals, that may have endocrine-disrupting contaminants (EDCs) on their surface, which are solubilized in the mouth and then swallowed. Infant exposures through breast-feeding may result from exposures of the mother before or during pregnancy but also during the breast-feeding period (National Research Council 1999). Research on chemicals found in breast milk centers on the persistent organic pollutants, such as PCBs, polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and organochlorine pesticides (LaKind et al. 2001). Metals, including lead, mercury, and cadmium, have also been reported in breast milk, but they do not usually accumulate to higher concentrations in breast milk than in blood. Chemical exposures to the mother may result from work, the ambient or home environment, diet, or carrying home of contaminants by another person living at the residence (Fenske 1997; Fingerhut et al. 1991; National Research Council 1999; Rogan et al. 1988; Vine et al. 2000; Weisglas-Kuperus et al. 2000).

Human routes of exposure to environmental toxicants include oral, dermal, and inhalation. In children, oral exposure may occur by the direct ingestion of contaminated environmental media (e.g., food, water, surface and carpet dust, and soil) and as a result of the transfer of toxic materials to the mouth from toys and other objects or from the child's hands. Dermal exposure occurs when the skin is in direct contact with the contaminated media, such as water, soil, or surfaces. Inhalation of airborne particulate or gases and vapors may introduce toxic substances into the respiratory tract, where material may exert an effect directly at the site—that is, at the portal of entry into the body—or the material may be transported to another organ where the toxic effects occur.

The route of entry into the body affects the internal dose to the child. Hand-to-mouth activity provides direct oral ingestion of material over an extended period of time, whereas oral exposures to food are more discontinuous. Covering the skin with a dressing that holds in water vapor decreases the ability of the skin to act as a barrier to entry of a material into the body, because the humidity macerates the skin and makes it more permeable. Moreover, some body sites have skin that presents a better barrier than do other sites. For example, Maibach et al. (1971) demonstrated that parathion uptake from dermal exposure resulted in a 2-fold increase if the material was applied to the ball of the foot, abdomen, or dorsa of the hands compared with application to the forearm (the authors did not specify the location of testing on the forearm). Similarly, the increase was 4-fold when applied to the scalp, jaw angle, or forehead, 5-fold at the ear canal, and 12-fold for the scrotum. Studies with malathion showed the same general trends (Maibach et al. 1971).

If the toxic agent is an airborne gas or vapor with relatively constant concentration in the home, the dose delivered to the lung is less during one hour of sleep, when the breathing rate and volume is lower, than during one hour of vigorous play time. Particulate deposition in the respiratory tract, which depends on particle size and breathing rate, may also change because of activity level (Phalen et al. 1988). Solubility of the compound, motility of the gastrointestinal tract, and age of the child are important factors in understanding absorption (dose) from any exposure (Rogan 1980).

Often exposure is considered to be constant over time, with the total dose estimated by the cumulative exposure, calculated as the product of concentration or intensity and time. However, if exposure is not constant over time, the same total cumulative exposure, delivered in different patterns, may produce different biologic effects. For example, in animals exposed to the same cumulative exposure to carbon tetrachloride, differences were seen in the type and distribution of vacuolation and necrosis in stained sections of liver and in liver enzyme activity (Colborn et al. 1993; Fry 1995). In children, it is possible that the pattern of exposure may produce different effects even when the total exposure is equivalent.

Timing, Frequency, and Duration of Exposure

The extensive literature concerning the effects of disruption of normal endocrine function during critical periods in development has been reviewed (National Research Council 1999). The importance of a critical period is that it represents a developmental window during which endocrine signals can program genes, which regulate the functioning of a tissue. If proper programming fails to occur during this time, structural or functional abnormalities can result. The term "endocrine signals" is used broadly here to refer to any signaling molecule, not just those classically considered as being carried via blood.

The developmental consequences of the disruption of endocrine function due to genetic defects in humans are well known. For example, the absence of a functional enzyme (5α-reductase) required to metabolize testosterone to the more potent androgen 5α-dihydrotestosterone results in a failure of masculinization of the external genitalia and prostate in male fetuses. Turner syndrome occurs in individuals with XY chromosomes due to mutation of the androgen receptor gene; this results in the development of female external genitalia. Developmental abnormalities due to exposure to endocrine-disrupting drugs (e.g., DES) are also well documented (George and Wilson 1994; Mittendorf 1995; Swan and vom Saal 2001). The importance of small changes in the concentration of endocrine-signaling molecules during critical periods in development has been studied extensively in laboratory animals and wildlife (Colborn et al. 1993; Fry 1995; Guillette et al. 1995; vom Saal 1989; vom Saal et al. 1997).

One of the most surprising discoveries over the last few decades is the remarkable conservation of genetic and hormonal systems involved in regulating development in multicellular animals. We know from developmental biology that the same genetic and hormonal systems are used in various ways to achieve the final formation of the adult. This argues strongly that the working hypothesis should be that if developmental disruption by a chemical occurs in one species then the chemical can be expected to result in disruption in many (perhaps most) other species. This does not mean that the consequences of disruption by a chemical on adult form and function will necessarily appear similar based on superficial examination.

For humans, there is more information currently available about the consequences of endocrine disruption during critical periods in fetal life than during neonatal life, infancy, childhood, or adolescence (Majdic et al. 1997; Sharpe and Skakkebaek 1993; Winter et al. 1976). This gap in understanding of the consequences of endocrine disruption in postnatal

life makes it difficult to discern appropriate outcomes to relate to exposures during these times.

The fetal period appears to be differentially sensitive to exogenous hormone exposure. The paradigmatic exposure is to DES, a stilbene estrogen used to prevent spontaneous abortion in the 1950s and 1960s. Fetal exposure to DES resulted in genital tract abnormalities in a high percentage, and vaginal clear cell adenocarcinoma in an estimated 1% at a very young age (Herbst 2000). Although it has not been without consequence for the mothers, nothing so dramatic as the effects in children exposed to DES has been described (Titus-Ernstoff et al. 2001).

Many decades ago there was less concern about exposure to toxic chemicals during pregnancy because it was a presumed that a "placental barrier" would block the transport of the toxicant to the fetus. This assumption is now known to be incorrect. Many of the chemicals listed in Table 1 have been shown or are presumed (based on their structure) to cross the placenta and enter the fetal circulation. Exposure during any developmental period is complicated because the factors that influence absorption, circulation in blood, uptake into tissues, and rates of metabolism and clearance undergo changes throughout development. The complexity of development makes it essential to study exposure at each stage rather than to attempt to characterize risk associated with developmental exposure based on data from adults. In the executive summary of the National Research Council report on diets of infants and children (National Research Council 1993), the panel stated: "A fundamental maxim of pediatric medicine is that children are not little adults." Data collected from exposures in adulthood are not likely to predict those of other life stages, such as development or old age.

The difference between males and females regarding endocrine disruption is an important issue about which there is considerable speculation, but typically, direct comparisons of effects in males and females are not made. In general, adult females are more difficult to study than males because the cyclic nature of hormones in females complicates studies of processes that are influenced by these hormones (e.g., estradiol and progesterone). However, there has been speculation that males are more sensitive to the disruptive effects of EDCs during development (Swan and vom Saal 2001). There are theoretical reasons for this hypothesis: Males are the actively differentiating sex and females the default sex, so there is a greater possibility of interference with the process of masculinization than of feminization. Also, the epidemiologic evidence appears to support the prediction that masculinization is being disrupted, based on reports of a secular trend for changes in sperm count, hypospadias, cryptochidism, and testicular cancer, all of which have been linked to disruption during fetal life of the male reproductive organs (Skakkebaek et al. 2001). It is easier to detect abnormalities of the genitals of males than in those of females, and historic data that would be informative about trends in female reproductive dysfunction are not available.

Information must be collected to characterize exposures throughout the span of development, from prenatal life to postnatal life, through adolescence. Postnatal exposure must be characterized separately in each period of time representing substantial change in the activity of the child: from crib to limited play areas to full mobility at home and within the larger community. Specific recommendations have been made to assess dietary exposures yearly from birth to 5 years old, once between 5 and 10 years, and once between 11 and 18 years (National Research Council 1993).

Qualitative and Quantitative Data Needs

Qualitative exposure data are derived from interviews, questionnaires, and self-reports and may be expressed in relative terms (e.g., "exposure was twice as high when I was a child") or indicate the presence or absence of a potential exposure (e.g., "I did not live near a hazardous waste site"). "Quantitative exposure data" generally refers to the numeric results from analyses for a contaminant in a specific media; a typical result might be reported as mass per unit volume (e.g., milligrams per cubic meter).

To capture the information needed for exposure assessment and subsequent risk assessment, a combination of environmental and biologic data are needed (Dietrich et al. 1993; Hammond and Dietrich 1990; Que Hee et al. 1985). Factors that modulate exposure may be identified through appropriate statistical analyses of observational data, use of questionnaires, biologic samples, or environmental measurements (Buncher et al. 1990). For example, exposure to lead could occur in the following situations: A family member works in a lead industry and carries dust home on clothing; the interior paint contains a lead-based pigment; the exterior of the neighbor's home was once painted with a paint containing lead, and it has peeled off and been incorporated into the soil where children play and may enter the home as dust or soil carried on feet or clothing; the family eats vegetables grown in lead-contaminated soil. Depending on the activity of the child, any, all, or none of these sources of exposure may be important. Exposure could be oral only, or also by inhalation. Absorption can be evaluated by measuring the blood lead of the child; however, the source, route, and rate of exposure must also be understood in order to manage risk.

The sources of exposure to a contaminant in the home can be evaluated from samples of airborne, settled, or carpet/floor dust. By knowing the time–activity patterns of the child, estimates of the relative importance of each source can be obtained. These observational (time–activity logs or videotapes) or questionnaire data must be sufficiently detailed to allow evaluation of the amounts of time spent in various locations. The amount of hand-to-mouth activity in each location or time period is essential to understanding the risk of exposure through the oral route, especially in young children (Buckley et al. 2001; Dietrich et al. 1985). The potential for transfer of a contaminant from hand to mouth can be evaluated by collecting and analyzing hand wipes, swipes, or rinses of objects observed to be frequently placed in the mouth. Similarly, detailed studies on sources of exposure to endocrine disruptors are needed.

Data on dietary intake are essential for accurate assessment of exposure to many endocrine disruptors. Methods commonly employed include collection and analysis of breast milk, dietary history questionnaires, and duplicate diet or split-plate collection and analysis of food (National Research Council 1993).

The exposure assessment protocol must be designed to capture information regarding changes throughout development—from birth through adolescence. The timing of these data collection cycles will have to be selected carefully to maximize the detection of changes but not be intrusive for the participants and overly costly.

Sample Collection and Storage for Both Short-term and Future Work

The prenatal period is, as noted, one of great susceptibility to adverse effects from exposures to environmental toxicants. During these 9 months are short periods of time during specific developmental stages when the fetus is especially susceptible to chemical or physical insults. How do we best assess exposure to the developing fetus during these critical periods or subperiods? If a study is prospective, then we can design appropriate questionnaires for the mother and the father, recording potential exposures and lifestyle/activity patterns. These qualitative methods can be used to better understand quantitative exposure measures. To evaluate the association between fetal exposure and a subsequent health outcome, it will be necessary to collect relevant biologic specimens at the proper time, store the specimens, and conduct appropriate analyses. The timing of sample collection is most important. The most significant time for the direct influence of the father's exposure is before conception, but the mother's exposure is relevant before, during, and even after pregnancy. At least in part,

the timing of biologic sample collection, the type of specimen, and the storage conditions, which must be used to maintain sample integrity, are related to the toxicant or toxicants of interest and its duration in the body of a parent (Needham and Sexton 2001).

In some cases the parent compound acts as an endocrine disruptor, whereas in other cases metabolism of the parent compound is required for bioactivation. For example, alkylphenol ethoxylates used as surfactants in many products are bioactivated in water treatment plants by bacterial action that releases the alkylphenol in free form (Jobling et al. 1996). The insecticide DDT is metabolized in the liver to dichlorodiphenyldichloroethylene (DDE), which has a very long half-life and also has the capacity to bind to androgen receptors and act as an antiandrogen (Kelce et al. 1995). The insecticide methoxychlor is not an endocrine disruptor until it is demethylated in the liver, and it is bis-hydroxymethoxychlor that has both estrogenic and antiandrogenic activity, not the parent compound (Gray et al. 1999).

Toxicants can be divided into two basic classes—those with long biologic half-lives (persistent) and those with short half-lives (nonpersistent)—although one should recognize that there is a continuum of half-lives between the very short and the very long lived chemicals in the body. These half-lives may differ from as long as decades for the persistent toxicants to only minutes for many of the nonpersistent toxicants. So the timing of collection of biologic samples for the analyses of persistent toxicants is much less an issue than it is in the collection of biologic samples for the measurement of nonpersistent toxicants. For the assessment of exposure to nonpersistent toxicants, if we sample the mother only one time, we must recognize that we are getting only a "snapshot in time." This is an important limitation unless the exposure is continual. A more effective but costly approach would be to analyze specimens collected numerous times during pregnancy, thus getting several snapshots.

To assess the mother's and fetus's exposure during pregnancy, the most common maternal samples taken and stored are urine, hair, and blood and its components, such as serum. Saliva could also be used, but results from saliva have not been extensively correlated with results from other biologic matrices (Bernert et al. 2000). In general, persistent organic toxicants, such as organochlorine pesticides, PCBs, and polychlorinated dibenzo-*p*-dioxins, are measured in serum. Nonpersistent chemicals or their metabolites, such as the organophosphate pesticides, polyaromatic hydrocarbons, phytoestrogens, and many inorganic elements, are generally measured in urine. Yet a number of exceptions to these guidelines, such as the phytoestrogens, certain polyaromatic hydrocarbons, and inorganic elements such as lead and cadmium, can be measured in blood. Also, hair is often used to assess past exposures to inorganic elements, such as arsenic and mercury, and has also been used to assess exposure to selected organic toxicants (Paschal et al. 1989).

Blood is often analyzed either as whole blood itself, as a liquid or spotted on filter paper, or as one of its components, such as serum or plasma. The blood spots are the most easily stored and have the advantage of having the entire blood sample, including the DNA component, which is important because of the increasing interest in studying the interaction between genetics and environmental exposure. However, the disadvantage is the small volume of blood available from the blood spots. Serum has the advantage of being able to be harvested in relatively large quantities and stored in a homogeneous manner at low temperatures, such as –70°C, at which temperature there is little if any enzyme activity. To obtain serum, no chemicals need to be added during blood collection to induce or prevent clotting. Many persistent, lipophilic environmental chemicals such as dioxins are measured in serum and reported in terms of the whole weight of the serum used as well as the lipid content of the serum.

Beyond blood, the biologic fluid most frequently sampled and analyzed postpregnancy is mother's breast milk. Milk is an ideal matrix for measuring many persistent chemicals, which tend to be lipophilic. Because milk is relatively high in lipid content, these chemicals are found in higher concentrations in milk than in blood. The measurement of persistent toxicants in milk, which is taken postpregnancy, can be used to estimate the infant's intake. Milk can be used as the matrix for assessing what the mother's internal dose levels were during pregnancy as well as the exposure to the fetus and then postnatally to the newborn, whose main exposure to these types of chemicals is through the milk (LaKind et al. 2001).

Direct measures of assessing fetal exposures are difficult and may not represent the entire span of fetal life. Fetal matrices used in such assessments include the amniotic fluid (usually taken in early second trimester); meconium; cord blood, including blood spots; and the umbilical cord. Each of these has advantages and disadvantages, but none reflects the entire fetal life span. However, meconium potentially reflects exposure from the 12th to 16th week of gestation until birth; hence, meconium is being validated as a matrix for assessing cumulative fetal exposure (Whyatt and Barr 2001). The most frequently analyzed of these matrices is the cord blood, usually as serum, but again, blood spots have the advantages of containing DNA and being easy to store.

Sample Analysis

Desirable characteristics of analytic methods for measuring chemicals in samples of small volume or small weight include specificity (the ability to distinguish toxicants from each other and from background levels), sensitivity, accuracy, precision, and ruggedness (Needham et al. 2002). The laboratory worker should handle each biologic specimen as potentially containing infectious agent, and appropriate clothing should be worn; work should be done in a certified hood; and organic solvents should be added to kill the agent. Other goals for the analytic methods include their being fast and inexpensive (Needham and Sexton 2001). Of course, few methods, if any, meet all of these criteria. Generally, the methods and equipment used to meet the first series of requirements do not meet the goals of being fast and inexpensive. However, that does not mean we should not attempt to meet all the criteria. We need to state our objective clearly and realize that there are many times when we can use "chemical class-screening methods," which allow us to get the needed information.

Using dioxin and related compounds as an example, if one is interested only in assessing the total number of toxic equivalents, bioassays and immunoassays have been developed for measuring this class of compounds. For instance, the toxic equivalency approach can be used to assess the presence of chemicals that act like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Birnbaum 1999). However, this class-screening methodology should be validated and calibrated against the "gold standard" method in that field, which in this example would be high-resolution mass spectrometry (National Academy of Sciences 1989a, 1989b). Regardless of the exact method used, we must be able to defend the data, which means that we must have a strict quality control/quality assurance plan in effect. A component of this plan is the necessary information to merge data from ever-improving analytic techniques. Over the duration of a longitudinal study, it is likely that the analytic method and the limits of detection for many toxicants will change, and these improvements should be incorporated into the design.

Exposure Assessment in Hypothesis Testing and in Hypothesis Generation

In the workshop, only broad issues of exposure assessment could be discussed, because the exposure assessment strategy used in a particular study would depend on the specific research questions to be addressed, the population(s) to be studied, and the toxicants initially identified to be of interest.

Here we review the important issue of the constant collaboration that is required at every stage of development of a study design

between exposure assessment and health status/outcome researchers in a study of endocrine-disrupting chemicals. In designing an exposure assessment strategy, it is recommended that some exposures be selected *a priori* for hypothesis testing, and that the overall design be sufficient to allow for hypothesis-generating analyses as more is learned. The exposure assessment protocols must be sufficient to address the *a priori* research questions; however, the collection, storage, and retention of questionnaire information and biologic samples are also important and may permit subsequent examination of other hypotheses.

The research questions to be investigated dictate the selection of the population to be examined. For example, if the goal is to develop data on the population as a whole, random sampling from a population that has the demographics of U.S. residents may be appropriate. However, exposures of interest may not be uniformly distributed throughout the population. For example, 1–3% of women nationwide are considered to be high consumers of fish that may be contaminated with PCBs. A random sample would not include sufficient numbers of these women to detect increased exposure, but a stratified sampling of women in specific geographic regions focusing on fish eaters would provide data from this small part of the overall population distribution. Alternatively, these questions can be posed as specialized studies among higher exposed populations.

Human variation is a topic of increasing interest in risk assessment. Just as we consider multiple exposure pathways (diet, hand-to-mouth activity, dermal, inhalation) and multiple exposure periods (*in utero* exposure due to maternal exposure before and during pregnancy, lactational exposure, and other childhood exposures from birth through adolescence), we can also think of casting a wider net in terms of the diversity of the study population. There has been increasing interest in answering questions about various populations that are not routinely included in epidemiologic studies. Additionally, in these populations we may see high-intensity exposures due to different dietary patterns and cultural practices. A more complete picture of genetic polymorphisms that can alter susceptibility to the effects of endocrine disruptors may also be identified in these populations. Oversampling these populations may be necessary to obtain an adequate sample size for statistical analysis.

Cumulative risk is another topic of increasing importance in risk assessment. A cumulative risk approach goes beyond identifying effects of individual endocrine disruptors by considering the possibly additive, antagonistic, or synergistic activity of simultaneous exposures to many agents that may affect children's health (Rajapakse et al. 2002). Future cumulative risk assessments will consider *a*) how exposures to multiple agents alter the toxicity of endocrine disruptors, *b*) how genes, life stages, lifestyles, and diseases can affect the toxicity of endocrine disruptors, and *c*) how exposure to endocrine disruptors can alter the toxicity of other agents. To address these questions, it is important to collect data on the principal chemical and nonchemical agents that can interact with endocrine disruptors. This will improve our ability to understand associations that will be identified between exposure to endocrine disruptors and children's health, and ultimately will help us develop approaches to reduce exposure.

Summary

The development of an exposure assessment strategy for examining a range of complex chemical exposures to the developing human in a prospective longitudinal study from conception through adolescence will be a challenging task. We have attempted to discuss many of the relevant issues in developing an exposure assessment protocol for such a study. Each is summarized below.

Routes of exposure. Transplacental, lactational, diet, and hand-to-mouth exposures are particularly important for children, and each needs to be examined. Any strategy to characterize dose from external and internal measures of exposure of endocrine-disrupting chemicals should be designed to capture sufficient data to evaluate all relevant routes of exposure.

Timing, frequency, and duration of exposure. Animal studies show that the life stage most susceptible to endocrine disruption is the embryo/fetus. Such studies show that exposure to endocrine-disrupting chemicals during fetal development can exert qualitatively different effects than can the same exposures after birth. We consider it likely that as more data become available, a similar pattern will also be seen from exposure during other developmental periods: neonatal life, infancy, childhood, and adolescence. Research study designs must capture data on timing of exposure in the course of the child's development and the intensity, frequency, and duration of exposures.

Qualitative and quantitative data needs. Qualitative data from observations and questionnaires are needed to interpret measures of exposure and, in the absence of quantitative measurements, to provide surrogate indicators of exposure. For example, the past history of many potential exposures can be elicited during an interview; among some interviewed women, pregnancy will occur, and biologic samples may be collected to estimate body burden of the mother through which fetal exposure may occur. The combination of qualitative and quantitative data will allow analyses to detect associations between the two research methods. These results will become the foundation of developing methods to assess risk in the population at large where only qualitative interview data are available.

Sample collection and storage. Methods for the collection and storage of relevant biologic specimens as well as the timing of the collection must be carefully considered in study design. Careful documentation of the procedures is required. No single protocol will be appropriate for all toxicants or for all matrices in which a single toxicant can be measured. Careful archiving of biologic material will allow evaluation of future hypotheses, at a substantially reduced cost.

Sample analysis. Analytic methods must be fully documented and a quality control/quality assurance plan developed and implemented. Criteria to be considered in selection of a method are specificity, sensitivity, accuracy, precision, ruggedness, time of analysis, and cost.

Exposures assessed for hypothesis testing and hypothesis generation. In any long-term research project, there are initial, *a priori* questions to be investigated. The exposure assessment protocol must provide sufficient data for the evaluation of these research questions. However, the exposure assessment team may go further and develop a protocol to collect additional data, reasonably anticipated to be useful over the course of the study to answer questions not posed at the outset. For example, the collection of a full residential history may be useful in identifying the potential exposure from mobile or fixed-site ambient sources. Special attention will be needed to ensure that enough additional data are collected to enable identification of an appropriate control population for each research question, including both *a priori* questions and those that may arise in the future.

In a collaborative process of research methodology development, we believe that credible exposure assessment protocols can be designed to address *a priori* hypotheses and to allow for investigation of other research questions, as the state of knowledge and level of analytic technology increases. Design of state-of-the art exposure assessment protocols requires resources. Adequate funds for this aspect of a research initiative will provide data for both hypothesis testing and hypothesis generation. Ultimately, the investment will provide the basis for public health improvements.

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