Alterations in Fetal Thymic and Liver Hematopoietic Cells as Indicators of Exposure to Developmental Immunotoxicants

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Recent studies indicate that immune development in humans and other species may be altered after perinatal exposure to immunotoxic environmental contaminants. However, limited information is available regarding appropriate tests that may adequately detect developmental immunotoxic compounds. Experiments in which pregnant laboratory rodents were exposed to a variety of immunotoxic environmental agents indicate that fetal thymus and liver immune cells may be quantitatively and qualitatively altered by immunotoxicant exposure and, thus, may serve as sensitive markers of developmental immunotoxicant exposure. In particular, depression of fetal thymic cell counts appears to be a common event following gestational exposure to immunotoxicants that produce this response in adult animals. Total hematopoietic cell counts in fetal liver, however, may be a poor indicator of immunotoxicant exposure. Altered marker expression in both fetal thymus and liver appears to be a highly sensitive indicator of gestational immunotoxicant exposure. Together, these reports suggest that immune tests with high predictability for immunosuppression in adults may also be appropriate for the detection of developmental immunotoxic agents. — Environ Health Perspect 104(Suppl 4):809–813 (1996)

Key words: developmental immunotoxicity, immunotoxicant biomarker, fetal liver, fetal thymus

Background

Limited information is available on the postnatal immune alterations that may result from developmental (i.e., gestational) exposure of humans and other species to environmental immunotoxicants. However, studies in laboratory rodents suggest that perinatal treatment of animals with a variety of immunotoxic compounds may have more dramatic and persistent effects on subsequent immune function than does exposure during adult life [see review by Holladay and Luster (1)]. For example, studies show that prenatal exposure of mice to the organochlorine insecticide chlordane results in significant depression of cell-mediated immune responses in offspring that persist for at least 100 days after birth (2,3). Alterations in the formation of bone marrow colonies in these mice have been observed to persist up to 200 days of age (4). This latter effect in particular has not been observed in adult mice after similar dosing with chlordane. Similar to chlordane, the offspring of pregnant mice treated with the polycyclic aromatic hydrocarbon (PAH), benzo[a]pyrene (B[a]P), showed long-term (e.g., still present at 18 months of age) depression of antibody, graft-versus-host, and mixed lymphocyte responses (5). Similar changes in mice after gestational exposure to additional immunotoxic compounds have been associated with reduced ability of the animals to withstand immunologic challenge (e.g., syngeneic tumor cells or bacterial, viral, or parasitic agents) later in life (6–8). Together, these reports suggest that in utero immunotoxicant exposure may alter development of immunity, and that such exposure during development may lead to severe and sustained postnatal immunosuppression.

Currently available reports suggest that immune changes, such as those described above, may occur in rodents after exposure to a variety of diverse environmental immunotoxicants, including numerous pesticides, fungicides, heavy metals, carcinogenic PAHs, and polycyclic halogenated hydrocarbons (PHAH) (the most notable and studied member of the latter group being 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)) (9,10). The sensitivity of the developing immune system to estrogenic compounds (7,8,11) has raised additional questions regarding potential developmental immunotoxicity after gestational exposure to environmental pollutants that are referred to as endocrine disrupters [e.g., 1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane (DDT), kepone]. Mycotoxins, such as the trichothecene fungal metabolite T2 toxin, may also warrant concern as developmental immunotoxicants (12,13).

Use of Routine Immune Tests to Detect Developmental Immunotoxicants

Although perinatal exposure to immunotoxic agents has been found to alter immune development in experimental studies, little information is available regarding appropriate tests that may adequately detect developmental immunotoxic agents. Recently, data collected and analyzed from rodent studies in adult mice employing over 50 immunotoxicants indicated that the performance of only two or three immune tests is usually sufficient (>90% concordance) for immunotoxic compound identification (Figure 1). Certain of these immune tests (e.g., thymus/body weight ratio; 68% predictability) apply well to the developmental model, while other tests (e.g., plaque-forming cell assay) are not readily adaptable. One of the tests with a high individual predictive value for immunotoxicity, surface marker expression (85% predictability), applies readily to developmental models.
However, before these assays are adapted as part of routine developmental toxicity studies, it will be necessary to determine their sensitivity, reproducibility, and predictive value.

**Indicators of Developmental Immunotoxicity**

Recent experiments in which pregnant laboratory rodents were exposed to a variety of immunotoxic compounds indicate that fetal thymus and liver immune cells are quantitatively and qualitatively altered by immunotoxicant exposure and, thus, may serve as sensitive markers of developmental immunotoxicant exposure. For instance, pregnant mice treated via gavage from gestational days (gd) 13–17 with 50 or 100 mg/kg B[a]P had markedly reduced numbers of fetal thymocyte cells on gd 18 (15). The investigators in these studies did not conduct additional experiments with decreased dose levels of B[a]P to identify an exposure that would not affect thymic cellularity nor was the time of dosing varied to evaluate the effects of chemical administration at other (earlier) periods of gestation. However, considering the ability of PAH to readily cross human skin, and in light of the recent demonstration that total PAH in skin oil of road paving crews may reach 1400 ng/cm² (16), these data raise questions about possible effects of PAH on fetal immune development in humans exposed occupationally. Dosing of pregnant mice with an additional PAH, 7,12-dimethylbenz[a]anthracene (DMBA), was also found to produce hypocellularity of the fetal thymus during late gestation (17). Additional studies showed that total numbers of fetal thymic cells decrease in mice after maternal dosing with a broad range of chemically diverse immunotoxic compounds, under a variety of dosing regimens (Table 1).

Certain of these compounds (e.g., TCDD and diethylstilbestrol [DES]) produce fetal thymic atrophy after exposure to extremely low levels, supporting the consideration of this parameter as a biomarker of environmental immunotoxicant exposure.

The primary hematopoietic compartment of the fetus is the liver and functions similar to adult bone marrow (23). Qualitative and quantitative effects of developmental exposure to immunotoxic compounds on fetal liver cellularity have also been examined. Fetal liver hematopoiesis is first detectable in mice by day 10 of gestation and in humans by the sixth week of gestation (24,25). This compartment, containing multiple lineages of rapidly proliferating immune cell types, soon thereafter replaces the yolk sac as the primary center of fetal hematopoiesis, a change that persists for the remainder of gestation (23). Treatment of adult animals with a number of immunotoxic agents, including T2 toxin (26), ethylene glycol monomethyl ether (EGME) (27), and B[a]P (28) significantly reduces cell counts in the bone marrow. However, recent results indicate that exposure of pregnant animals to immunotoxicants that produce fetal thymic atrophy may or may not result in decreased total hematopoietic cell number in the fetal liver. For instance, dosing of mice with EGME from gd 10–17 reduced cellularity of the gd 18 fetal thymus without altering total cell counts in the fetal liver (22). Similarly, mice exposed to DES from gd 10–16 displayed severe reduction of fetal thymic cell counts without altering fetal liver cellularity (17). Treatment of mice with 1.5 mg/kg T2 mycotoxin from gd 14–17 also reduced thymic cellularity to 32.5% of the control level, without altering fetal liver cellularity (12). In contrast, B[a]P reduced both fetal

![Figure 1](image_url). Individual and pairwise concordance to establish predictability using the immune panel. Values are presented as percentage concordance (calculated as the sum of specificity and sensitivity). Abbreviations: NK, natural killer; MLR, mixed lymphocyte response; CTL, cytotoxic T lymphocyte; BW, body weight; LPS, lipopolysaccharide. Individual concordance values are shown in boldface on the diagonal of the matrix and combinations, using two tests, on the off-diagonal element. Values in parentheses are the number of chemicals tested for the assay. p Values are given for individual concordance only. Data from Luster et al. (14).

| Plaque-forming cells | 76 | 0.0001 |
|----------------------|----|--------|
| NK-cell activity     | 94 | 0.0014 |
| T-cell mitogens      | 85 | 0.0003 |
| MLR                  | 87 | 0.0058 |
| DHR                  | 89 | 0.0034 |
| CTL                  | 100| 0.0017 |
| Thymus/BW ratio      | 92 | 0.0009 |
| Spleen/BW ratio      | 85 | 0.0395 |
| Spleen cellularity   | 86 | 0.0894 |
| LPS response         | 81 | 0.0260 |

Table 1. Immunotoxicants producing fetal thymic atrophy.  

| Immunotoxicant                  | Exposure regimen | References |
|---------------------------------|------------------|------------|
| 2,3,7,8-Tetrachlorodibenzo-p-dioxin | 1.5-3.0 µg/kg/day | gd 6–14 (18,19) |
| 2,3,7,8-Tetrachlorodibenzo-p-dioxin | 10 µg/kg | gd 14 (20) |
| 3,3',4,4'-Tetrachlorobiphenyl   | 6–16 mg/kg | gd 12 (21) |
| Diethylstilbestrol              | 3.0–6.0 µg/kg/day | gd 10–16 (11) |
| Ethylene glycol monomethyl ether | 100–200 mg/kg/day | gd 10–17 (22) |
| Benz[a]pyrene                   | 50–150 mg/kg/day | gd 14–17 (15) |
| 7,12-Dimethylbenz[a]anthracene  | 10–25 mg/kg/day | gd 14–17 (17) |
| T2 mycotoxin                    | 1.2–1.5 mg/kg/day | gd 14–17 (12,13) |

gd, gestational days.
thymic and fetal liver cellularity in mice (15). However, the reduction in fetal thymus cellularity by B[a]P appears to be greater than that seen in fetal liver at corresponding doses. These studies suggest that fetal liver cellularity may not be altered following exposure to moderate, or, in some cases, relatively high levels of known immunotoxicants. Thus fetal liver cellularity may not be the most sensitive indicator of developmental immunotoxicity.

Chemical Effects

Although the total number of fetal liver hematopoietic cells appears insensitive, qualitative changes within this compartment may occur and provide sensitive indicators of exposure. For example, after a single maternal dose of 10 mg/kg TCDD in mice on gd 14, fetal liver lymphoid cells have only 40% of the control capacity to biosynthesize the DNA polymerase, terminal deoxynucleotidyltransferase (TdT) (20). TdT+ cells in fetal liver include prolymphocytes, the immediate precursors of thymocytes (29), and values for TdT biosynthesis correlate with total numbers of TdT+ cells observed by immunofluorescence (30).

Thus, the reduced capacity of fetal liver from TCDD-exposed animals to synthesize TdT suggests an altered stem cell population in treated mice that may be responsible for thymic atrophy. This hypothesis was supported by experiments in which irradiated hosts reconstituted with stem cells from vehicle or TCDD-exposed animals demonstrated reduced capacity of the chemically-exposed stem cells to reconstitute the host thymus (20). It is worth noting that thymic TCDD levels ranged from 1 to 31 ng of TCDD/mg tissue in these experiments, emphasizing the extreme sensitivity that both fetal thymic cell counts and depressed fetal liver TdT biosynthesis may have as markers of exposure to this widespread environmental contaminant.

DES shares with TCDD the ability to produce fetal thymic atrophy at dose levels in the low micrograms per kilogram range, without altering cellularity of the fetal liver. Analysis of cell-surface antigen expression on gd 18 fetal liver hematopoietic cells of mice exposed to DES from gd 10–16 demonstrated no changes relative to vehicle-treated animals in the percentage of CD44+ (hematopoietic precursors), CD45R+ (B-lineage lymphocytes), or Mac-1+ (granulocytic) cells (11). However, TdT+ cells were reduced, again suggesting possible prolymphocyte targeting. Experiments in which irradiated hosts were reconstituted with stem cells from vehicle or DES-exposed animals demonstrated reduced capacity of the chemically-exposed stem cells to reconstitute the host thymus, indicating that thymic atrophy after exposure to DES may result, at least in part, from a fetal liver lesion at the level of the T-cell progenitor. These data again suggest the use of fetal liver TdT as a sensitive marker of exposure to DES, and possibly other endocrine disrupters.

EGME and T2 mycotoxin represent additional immunotoxic agents that alter the expression of immune cell antigens in fetal liver in the presence of unaltered total organ cellularity. Additional reports indicate developmental exposure to B[a]P and DMBA alters fetal liver antigen expression, in the presence of reduced fetal liver cellularity. A summary of fetal liver antigens for which data have been collected after gestational immunotoxicant exposure is presented in Table 2. Of note, many of these studies have demonstrated relatively selective targeting of prolymphoid cell subpopulations in fetal liver by the immunotoxic agents. In particular, studies employing CD44 demonstrate that fetal liver TdT+ cells from TCDD-exposed animals have measurable TdT levels (12,31,32), consistent with chemical targeting of prolymphoid cells in fetal liver. When examined, expression of CD45R, an antigen expressed on pre/pro-B lymphocytes and some prolymphocytes (31), was also reduced in the fetal liver. Taken together, these reports indicate that prolymphoid cells in fetal liver represent sensitive targets of immunotoxic chemicals, and therefore may serve as sensitive biomarkers.

In addition to altering surface antigen expression in fetal liver, developmental immunotoxicants have been found to alter surface antigens expressed on fetal thymocytes. Ontogenic development in the fetal thymus originates when prolymphocytes of fetal liver origin seed the murine thymic rudiment on gd 10–11. Thymocytes then differentiate and mature in a process that can be followed by CD4 and CD8 cellsurface antigen expression. Initially, thymocytes are double negative (DN) with respect to the CD4 and CD8 antigens. Subsequently, they develop sequentially through immature CD8− single positive (SP) and CD4+8+ double positive (DP) stages in the thymic cortex, to mature CD4+ SP or CD8+ SP thymocytes in the thymic medulla by gd 18–19 (33,34). Changes in the normal pattern of thymocyte development have been observed after gestational exposure to a variety of environmental immunotoxicants. For example, mice exposed prenatally to TCDD display altered CD4 and CD8 antigen expression at gd 18 (Table 3). In particular, TCDD-treated mice displayed reduced percentages of DP thymocytes (the most mature major population present at gd 18) and increased percentages of DN and CD8+ SP thymocytes (the most immature phenotypes present at gd 18), consistent with inhibition of thymocyte maturation. Interestingly, a similar

| Table 2. Fetal liver markers altered by gestational immunotoxicant exposure. |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Immunotoxicant             | TdT  | CD44 | CD45 | CD45R | Mac-1 |
| DES                        | +    | ND   | ND   | ND   | ND   |
| EGME                      | ND   | +    | ND   | ND   |
| B[a]P                      | +    | +    | +    | ND   |
| DMBA                      | ND   | +    | ND   | +    |
| T2 toxin                   | ND   | +    | ND   | +    |

Abbreviations: ND, not determined; +, the compound altered expression of the particular marker; −, the compound did not alter expression of the marker.

| Table 3. Effect of TCDD on fetal thymocyte CD4 and CD8 antigen expression. |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Treatment                  | CD44−| CD44+ | CD48− | CD48+ |
| Vehicle                  | 1.8±0.2 | 69.1±1.2 | 21.1±0.7 | 8.1±0.7 |
| 1.5 µg/kg                 | 1.5±0.1 | 52.6±2.5* | 30.3±1.8* | 15.5±0.9* |
| 3.0 µg/kg                 | 2.0±0.2 | 43.2±4.5* | 37.3±3.7* | 17.5±0.9 |

CD4 and CD8 surface antigen expression determined in gestational day 18 fetal mice after maternal exposure to 1.5 or 3.0 mg/kg/day TCDD from gestational days 6–14. Values represent mean ± SEM of five mice per treatment group. *p<0.05 vs vehicle controls. Adapted from Holladay et al. (18).
Table 4. Effect of gestational exposure on the percentage of CD4^+8^+ (DP) and CD4^+8^- (DN) phenotypes from gestational day 18 fetal thymocytes.

| Agent     | Exposure regimen | Control |  |  |  |  |  |  |  |  |  |  |  |
|-----------|------------------|---------|---|---|---|---|---|---|---|---|---|---|---|
|           |                  | DP      | DN | DP | DN | References |
| TCDD      | 3.0 µg/kg/day     | 69.1    | 21.1 | 43.2 | 37.3 | (18) |
| DES       | 3.0-8.0 µg/kg/day | 71.4    | 22.0 | 63.6 | 29.2 | (17) |
| EGME      | 50-150 mg/kg/day  | 78.0    | 18.1 | 33.4 | 62.0 | (15) |
| B[a]P     | 1.2-15 mg/kg/day  | 71.6    | 23.6 | 55.0 | 40.2 | (12) |

Abbreviations: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; DES, diethylstilbestrol; EGME, ethylene glycol monomethyl ether; B[a]P, benzo[a]pyrene; gd, gestational day(s). *indicates numbers were reported as significantly different from control, *p<0.05.

pattern of altered thymocyte differentiation, including reduced percentages of DP thymocytes and increased percentages of DN thymocytes, has been reported after gestational exposure to a diverse variety of immunotoxic compounds (summarized in Table 4). Although specific mechanisms responsible for such altered thymocyte differentiation after exposure to these compounds have not been elucidated, apoptosis of DP cells may be involved (10-12,15,18,22).

In summary, depression of fetal thymic cell counts appears to be a common event after gestational exposure to immunotoxic agents that produce this response in adult animals. The relation of fetal thymic atrophy to postnatal immunosuppression is not known. In contrast, total hematopoietic cell counts in the fetal liver may represent a poor indicator of immunotoxic exposure. Regardless of whether an immunotoxic compound altered fetal liver cellularity, however, immune cell markers in fetal liver were found to be altered following immunotoxicant treatment in all cases where such data were available. These reports indicate that fetal liver cells may represent highly sensitive targets of immunotoxic chemicals, and, as such, may represent a sensitive indicator of exposure. Similar to fetal liver, altered fetal thymocyte marker expression appears to be a sensitive indicator of gestational immunotoxicant exposure. The findings in fetal liver and thymus agree well with the recent observation in adult rodents that altered immune cell marker expression is a good indicator for immunotoxic agents (14).

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CHEMICAL ALTERATIONS IN FETAL THYMUS AND LIVER

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