Early indicators of developmental toxicity

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Abstract The hazards for humans and the environment are readily apparent in chemical emergencies. On the other hand, the risks from non-genotoxic effects of chronic exposures to low levels of chemicals are poorly understood. In particular, developmental toxicity studies should pay greater attention to the identification and biological evaluation of subtle effects as early indicators of damage and to the definition of dose-response relationships and risk estimates, particularly the "benchmark" dose. In this present paper we briefly discuss two examples of subtle developmental alterations of the axial skeleton and the kidney which are induced by the herbicides Bromoxynil and Dinoseb, respectively.

INTRODUCTION

Each stage of development is a potential target for adverse effects of chemicals (Mantovani, 1993). The possible outcomes range from minor alterations to the death of the fetus. Disturbances of maternal hemostasis may play a role, which may create difficulties in the evaluation of the results from developmental toxicity studies. Moreover, the adverse effects of chemicals on development may be influenced by both the actual amount of substance coming into contact with the fetus and/or by the duration of the exposure of a particular organ bud during organogenesis. The identification of subtle developmental alterations is particularly important at exposure levels where teratogenesis is not observed by means of standard prenatal toxicity studies. Such low-dose effects could be significant as early indicators of the risk for more severe developmental impairments (Beck, 1990). Subtle effects may also be important to estimate the hazard from exposure pathways other than ingestion. For example, percutaneous absorption may also be relevant for xenobiotics present in water for domestic uses (Maxwell et al., 1991). In this paper, the available data on two herbicides have been considered as examples of compounds which might be consumed at low amounts and induce subtle developmental effects. The two herbicides discussed are Bromoxynil, which induces supernumerary ribs in rodent fetuses (Rogers et al., 1991), and Dinoseb, which may alter the renal morphogenesis and differentiation in the rat (Daston et al., 1988).

Moreover, low-dose effects can be studied through the definition of dose-response curves. Low-dose effects are relevant when small amounts of xenobiotics come into daily contact with a population. The use of the "benchmark dose" approach (Crump,
1984) may identify the most sensitive end points and increase the accuracy of risk estimates.

MINOR SKELETAL CHANGES: BROMOXYNIL

During prenatal toxicity studies in the rat, minor alterations of the axial skeleton are frequently observed in both control and treated groups. A double staining method helps to distinguish between actual structural changes and calcification delays (Stazi et al., 1992). In a number of studies, a trend of increasing alterations is observable, even at low dose levels. However, these alterations may be defined as either anomalies, variations or deviations, depending on whether their frequency of occurrence is lower or higher in the background control groups. Thus, a standardized classification of minor skeletal changes will be useful for evaluating their relevance as early indicators of developmental impairment (Beck, 1990).

Bromoxynil (3-5 dibromo-4-hydroxyphenyl cyanide) (Br) is a broad-spectrum inhibitor of photosynthesis, with an environmental half-life of about 10 days. Its ester octanoate is the active ingredient of the herbicide Buctril, commonly employed in the USA. As a characteristic effect, Br increases the incidence of supernumerary lumbar ribs (SLR) in both rat and mouse fetuses. In a study by Chernoff et al. (1991), 15.0 and 96.4 mg (kg body weight)\(^{-1}\) of Br were administered p.o. to pregnant SD rats and Swiss Webster mice, respectively, on gestation days (GD) 6-15. GD20 fetuses of both species showed a significant increase in the prevalence of SLR. In mouse pups, this specific change was only partly reversible. Its prevalence decreased, but it did not disappear, between postnatal days (PD) 20 and 40. On the other hand, the PD40 prevalence in bromoxynil-exposed rat pups was not different from controls. The thoraco-lumbar border is a particularly unstable and sensitive region in the rodent fetus, although the supernumerary segments tend to be remodeled during postnatal life (Wickramaratne, 1988). According to Kimmel and Wilson (1973), a dose-related increase of SLR could indicate a teratogenic effect at higher exposure levels. In most cases, this change has been related to maternal toxicity or stress, though genetic factors may also play a role (reviewed in Stazi et al., 1992). However, based on the postnatal persistence in the bromoxynil-exposed mice, Chernoff et al. (1991) concluded that SLR might have some significance in developmental toxicology. Later in this paper we use the results from another study on bromoxynil-induced supernumerary ribs (Rogers et al., 1991) to compare the reference doses derived from two approaches, the benchmark dose and the NOAEL/uncertainty factors, with regard to both SLR and maternal toxicity.

IDENTIFICATION OF SUBTLE KIDNEY ALTERNATIONS: DINOSEB

A number of xenobiotics with nephrotoxic potential may be present in the environment (WHO, 1991). The adverse effects on prenatal and/or postnatal development of urinary system, particularly the kidney tissue, have been the focus of considerable study. Structural changes of the different renal functional areas may be considered transitory or persistent according to their severity. Even reversible alterations can be biologically important if they occur during a critical phase, such as the neonatal period. However,
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the standard segment-II (organogenesis) prenatal toxicity studies usually take into account only evident morphological parameters (renal papilla, pelvis and ureters, for example) which may be insensitive to disturbances of the functional development. Moreover, such studies do not cover the entire period of renal morphogenesis and histogenesis (which, in the rat, extends to early postnatal life) (Monie, 1977).

Dinoseb (6-1-methylpropyl 1-2,4 dinitro phenol) (Di) is an intermediate synthesis product which is also utilized as a herbicide. Di is nephrotoxic in the adult rat. Like other nitrophenols, it is well absorbed both orally and percutaneously and slowly excreted. Di induces maternal toxicity and embryo toxicity in the rat at oral dose levels $\geq 10 \text{ mg (kg body weight)}^{-1}$. Daston et al. (1988) studied the experimental approaches to detect effects on the development of kidney functionality. Di injected i.p. to SD pregnant rats on GD 10-12 or 11-13 induced a steep, dose-related increase in maternal mortality at dose levels $\geq 8.0 \text{ mg kg}^{-1}$, with an approximate LD50 at 10.5 mg kg$^{-1}$ (for comparison, rat oral and dermal LD50s are 25-60 mg kg$^{-1}$ and 80-200 mg kg$^{-1}$, respectively). Fetal weight was reduced at $\geq 7.5 \text{ mg kg}^{-1}$ in litters examined on GD20. However, there was no increased post-implantation loss. The incidence of fetuses with reduced development of the renal papilla was significantly increased only in the groups treated with 6.0 and 12.0 mg kg$^{-1}$ on GD 10-12. Thus, the morphological examination of the kidney was an insensitive indicator of possible Di-induced effects.

In the following experimental step, SD pregnant rats were treated i.p. on GD 10-12 with 0, 8.0 and 10.5 mg (kg body weight)$^{-1}$ of Di. Litters were examined on PD 2-3, 6, 14-17 and 30 for in vitro cortical functionality, nitrogen and creatinine clearance and urinary parameters. Pup weight was reduced at 10.5 mg kg$^{-1}$. A few PD 30 pups with dilated pelvis were observed in both control and treated groups, with no clear relationship with the treatment. Significant functional effects were observed only in the 10.5 mg kg$^{-1}$ pups (i.e. at the approximate maternal LD50). A deficit in the urine concentration ability was detected initially, but it was reversed on PD 30. This may be related to a transitory impairment of medullary function. Indicators of an increased secretion of organic ions from proximal tubules were provided by urinary excretion of Cl and PAH uptake by cortical slices. These changes persisted on PD 30. However, even such subtle effects were detected only at an overtly maternally toxic dose level. Thus, the functional testing approach was not a sensitive indicator of possible prenatally Di-induced renal alterations. A limited histological examination was performed in an earlier study (McCormack et al., 1980). Histogenesis of kidneys and ureters was altered in newborn rats from dams treated with $\geq 8.0 \text{ mg (kg body weight)}^{-1}$ on GD 10-12 (NOAEL: 6.4 mg kg$^{-1}$). Changes were no longer observable in weaned rats examined on PD 42. A relationship between morphohistological changes and functional impairment was not apparent in this study.

The data presented do not allow us to assess the Di-induced hazard for renal development. However, they suggest that histological and histomorphometrical changes should not be overlooked as sensitive early indicators of damage. A detailed histological examination has been attempted for prenatally-induced effects of nephrotoxic drugs like aminoglycosides. The examination considered quantitative parameters, such as glomerular density and cortical-medullary area ratio (see Mantovani et al., 1992). If properly validated with other classes of chemicals, such an approach might provide an early detection of developmental toxicity in target organs.
NEW APPROACHES TO THE ASSESSMENT OF DOSE-RESPONSE RELATIONSHIPS AND REFERENCE DOSES (RfD) FOR DEVELOPMENTAL TOXICITY

To estimate a RfD for non-genotoxic compounds, the approach generally utilized is the application of uncertainty factors (UF) to the most appropriate NOAEL. A different approach, originally proposed by Crump (1984), is based on the definition of the benchmark dose (BD). Such an approach gives a greater role to the dose-response (D/R) relationship. The BD is defined as the lower (e.g. 95%) confidence limit of a dose level inducing a determined increase (e.g. 1% (ED01)) of the frequency of a given effect. An attempt to standardize the application of BD to embryo toxicity data has been proposed by Auton (1994). Other authors (Faustmann et al., 1994; Allen et al., 1994a, 1994b) have compared the LOAEL/NOAELs and various levels of BD (1%, 5%, 10%) for a very large data base of developmental toxicity studies. In general the NOAEL and LOAEL values tended to cluster close to the 5% and 10% level of risk, respectively, although the LOAELs were generally 1.3-1.6 times greater than the corresponding BD10. It should be noted that Faustmann et al., (1994) and Allen et al., (1994a, 1994b) examined the experimental data for severe effects (embryolethality, malformations), but not for minor abnormalities. The use of the BD approach may provide such advantages as:

(a) A well-performed study which does not lead to a clear NOAEL could be utilized to define a BD.

(b) The residual risk at NOAEL is unknown and may vary according to the quality of the data, whereas the BD is associated with an invariant level of risk, by definition.

(c) The identification of a BD is less dependent on the statistical power (e.g. number of animals in the experimental group) and on the selected dose levels of a study. Also, the confidence limits incorporate uncertainty associated with small sample size whereas the NOAEL approach does not (Allen et al., 1994a, 1994b). For these reasons, the derivation of a RfD would not normally need the use of very high UF (≥ 500);

(d) Finally, the derivation of a RfD could take into account the slope of the D/R curve, either through the confidence limits or, possibly, the use of an appropriate UF.

A paper by Calabrese and Baldwin (1994) presents a concept of NOAEL which is closer to the BD approach. The definition of "no effect" should take into account the presence of increases in the response, which are not statistically significant, but may have a biological significance. Thus, the true NOAEL should be defined as the dose level which (a) is not statistically different from the control group and (b) is statistically different from the LOAEL. A NOAEL not fulfilling both criteria should be considered a quasi-NOAEL; either a lower dose level or a further UF (e.g. 5) should be utilized to determine a RfD.

It is apparent that the BD approach is based on the definition of D/R curves. Several attempts to define such models for developmental toxicity are described below.

The relationship between maternal body burden and methyl mercury-induced congenital abnormalities of the central nervous system (CNS) was studied by Clarkson et al. (1981). The authors used a regression method, which assumed a background frequency of alterations. A practical threshold of exposure was demonstrated, above which the D/R was steep and approximately linear (a "hockey stick" curve).

A general model of D/R in developmental toxicity has been proposed by Faustman
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et al. (1989). The model takes into account (a) the risk of a litter having ≥ 1 fetus with a given abnormality, and (b) the risk for an individual fetus of the litter of having that alteration, which is conditioned by parameter (a). According to the model, with increasing exposures, (a) tends to increase slowly in progressive increments, while (b) tends to steeply increase only at the higher does levels. According to Allen et al. (1994a, 1994b), litter size can be a significant covariable in predicting response rates. Nevertheless, similar BD estimates were obtained using different D/R models, including generic ones, (models not specifically designed for developmental toxicity testing) (Allen et al., 1994a, 1994b; Auton, 1994).

Probit analysis could be a practical and reliable approximation to describe the D/R relationship of prenatal toxicity effects, which, in most cases, is steep and linear (Neubert et al., 1987). Ogata et al. (1984) applied probit analysis to malformations induced by the fungicide thiabendazole in mice to identify the most sensitive effect (i.e. that effect with the lowest ED01). According to Auton (1994), probit analysis gives slightly more conservative estimates of BD than the Weibull model for D/R relationships.

We applied probit analysis to the estimate of BD from experimental data on Bromoxynil, derived from a study by Rogers et al., (1991). Pregnant Swiss Webster mice and SD rats were treated p.o. on GD 6-15 with dose levels ≤ 96.4 mg (kg body weight)⁻¹ and ≤ 15 mg (kg body weight)⁻¹, respectively. Maternal toxicity and increased prevalence of supernumerary lumbar ribs were observed in both species (Table 1). In mice, the skeletal change showed a dose-related trend parallel to that of maternal toxicity.

<table>
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<th>Table 1</th>
<th>Reference doses (RfD) according to the NOAEL and benchmark dose (BD) approaches for bromoxynil-induced maternal toxicity and supernumerary ribs in the mouse and rat (data from Rogers et al., 1991).</th>
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**Mouse**

Dose levels (mg (kg body weight)⁻¹) 0 10.7 32.1 96.4

Maternal mortality 0/60 0/60 4/60 22/62

NOAEL: 10.7 mg kg⁻¹; RfD (UF = 100): 0.1 mg kg⁻¹; slope: 2.53; ED01 and ED10 (95 % confidence limits): 16.08 (5.54-25.64) and 41.61 (26.33-53.89) mg kg⁻¹ respectively; RfD (UF = 100): 0.06 mg kg⁻¹.

Supernumerary ribs (fetal incidence) 25/166 43/371 53/283 91/178

NOAEL: 10.7 mg kg⁻¹; RfD (UF = 100): 0.1 mg kg⁻¹; slope: 2.83; ED01 and ED10 (95 % confidence limits): 16.32 (5.89-26.15) and 38.23 (22.68-49.57) mg kg⁻¹ respectively; RfD (UF = 100): 0.06 mg kg⁻¹.

**Rat**

Dose levels (mg (kg body weight)⁻¹) 0 1.7 5.0 15.0

NOAEL for maternal toxicity (effects on body weight and liver weight) is 5.0 mg kg⁻¹; RfD (UF = 100): 0.05 mg kg⁻¹.

Supernumerary ribs (fetal incidence) 23/264 40/287 50/224 160/254

Not possible to define a NOAEL (incidence at 1.7 mg kg⁻¹ significantly higher than in controls); slope: 2.64; ED01 and ED10 (95 % confidence limits): 1.65 (0.78-2.56) and 4.10 (2.68-5.31) mg kg⁻¹, respectively; RfD (UF = 100): 0.008 mg kg⁻¹.
(as expressed by increased mortality and by concurrent effects on body weight and liver weight). The NOAELs and LOELs for both effects were close to ED01 and ED10, respectively. The BD01-derived RfD was slightly more conservative than the NOAEL-derived RfD (0.06 vs 0.1 mg kg\(^{-1}\)). In rats, the slope of the probit-derived D/R curve for supernumerary ribs was similar to that in mice. However, an increased prevalence of the skeletal alteration appeared at a lower exposure level, compared to maternal toxicity (effects of liver weight and body weight). In fact, the ED01 (1.65 mg kg\(^{-1}\)) was close to the lower dose level (1.7 mg kg\(^{-1}\)). A NOAEL could not be identified, whereas the BD approach allowed the determination of a RfD.

CONCLUSIONS

Risk assessment for non-genotoxic chemicals is becoming a process as complex as that for mutagenic/carcinogenic effects. In particular, developmental and reproductive toxicity are already recognized as critical end points in the determination of RfDs for many chemicals present in drinking water (Mantovani, 1993). Their role may be further increased by improved experimental approaches. Strategies to detect subtle effects as early indicators of damage should be considered, to better characterize the D/R relationship, to define more sensitively a NOAEL or a BD and to elaborate chemical class-specific testing protocols.

The BD approach stresses the importance of the D/R relationship to derive an RfD. It is, therefore, important to consider the data from all dose levels of a study. Such a procedure may be particularly relevant for developmental toxicology, where modest increases in spontaneous abnormalities or developmental delays, or small frequencies of unusual malformations are important to define low dose effects.

REFERENCES


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