Results of in Vivo and in Vitro Studies for Assessing Prenatal Toxicity

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Examples of a combined approach using in vivo as well as in vitro methods for the assessment of prenatal toxicity are presented. The topics discussed include the analysis of the possible embryotoxic potential of valproic acid (VPA), female sex hormones, bis(tri-n-butyltin) oxide (TBTO), and acyclovir and the problem of supplementing in vitro systems with drug-metabolizing activity.

Introduction

Supplementation of the classical in vivo tests with recently developed in vitro techniques has greatly extended the scientific possibilities in prenatal toxicology (1–3). In this presentation we shall give some examples of such a combined approach as extensively used in our institute in recent years. We strongly feel that only the concerted use of in vivo methods, in vitro techniques, pharmacokinetic studies, and additional specific morphological (especially electron microscopic) and biochemical investigations will allow us to successfully tackle the important problems in revealing the principles and special aspects of prenatal toxicity in the years to come.

On the other hand, one should be aware of the possibility of producing “artifacts” in vitro; misinterpretation of the data may occur easily and must be avoided (4). One of the major difficulties still connected with in vitro methods is to distinguish general cytotoxic effects, which may be produced with any substance at a high enough concentration, from specific interferences with differentiation processes.

None of the culture methods available today are, alone, adequate for tackling the variety of problems and all of them have considerable limitations as well as advantages; the most suitable method has to be selected for solving a special problem.

Here we present results of studies supplemented with different culture techniques on some aspects of abnormal prenatal development induced by valproic acid studied with the “whole-embryo” culture technique which we have modified for use on a larger scale; the question of a possible induction of abnormal heart development by some female sex hormones (“whole-embryo” culture technique); the problem of assessing a possible embryotoxic potential of bis(tri-n-butyltin) oxide (TBTO) from in vivo and in vitro data (limb bud assay); the problem of supplementing in vitro experimental test procedures with systems allowing (at least some type of) metabolic activation (organ cultures); assessing the possible embryotoxic potential of the virostatic agent: acyclovir, using a combined in vivo/in vitro approach (“whole-embryo” culture technique).

Modification and Standardization of the Whole-Embryo Culture Technique

Over the last 5 years we have systematically modified and standardized the technique of cultivating early post-implantation rat or mouse embryos as described by New and co-workers (5) so that it is now easier to use on a larger scale for toxicological studies. The main advantage of our method is the use of bovine serum as a culture medium instead of rat serum (6). This modification allows the study of a large number of samples under different experimental conditions using the same batch of serum. The conditions for whole-embryo culture have been modified in such a way (adjustment of gassing conditions, etc.) that the differentiation

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achieved with bovine serum as culture medium is comparable to that obtained with rat serum (Table 1). We have also developed and extensively used a simple scoring system for documentation of the data in a computerized form and for semi-quantifying normal or abnormal development in culture (6).

The outcome of the development in vitro and the conditions for analyzing the effect of chemicals in this system may be further improved and standardized by incubating the embryos at a temperature of 38.8°C instead of 37°C (Table 2); by adding 1 mL of buffer to 6 mL of serum (thus facilitating the addition of chemicals to the medium); and supplementing the bovine serum with methionine and purified hemoglobin (final concentrations: 7.5 mg/100 mL and 2.5 mg/mL, respectively). The addition of hemoglobin to the medium greatly improves the formation of hemoglobin within the developing embryos in culture.

Although a comparison of the development achieved in culture with that occurring in the intact organism is difficult because of the considerable variability in vivo, it seems fair to state that the technique now used—embryonic development achieved in culture—essentially resembles that seen in vivo with respect to crown-rump length, number of somite pairs, and score; the median protein content is about 85% of that of corresponding embryos developed in the living organism.

### Abnormal Development Induced by Valproic Acid in Whole-Embryo Culture

From a large series of experiments performed with valproic acid (VPA) we shall present here only a few data to illustrate the versatile applicability of the

Table 1. Comparison of rat and bovine serum as culture medium and different gassing procedures.

| Gassing I      | Medium                  | YS, mm* | CR, mm* | Som  | Prot, µg/embryo | Score | ABN, % |
|----------------|-------------------------|---------|---------|------|----------------|-------|--------|
|                | 0 hr                    | 5% O₂   | 5% CO₂ | 27.0 | 313.5          | 40.0  | 0      |
|                | 20 hr                   | 20% O₂  | 5% CO₂ | 26.0 | 223.0          | 40.0  | 0      |
|                | 32 hr                   | 40% O₂  | 5% CO₂ | 26.0 | 206.5          | 39.0  | 0      |
| 100% Rat serum, n = 15 | 3.78                  | 5% O₂   | 5% CO₂ | 37.0 | 241.5          | 37.0  | 25     |
| 4.26b          | 4.02b                   | 3.48    | 26.0    | 27.0 | 190.0*         | 36.5* | 0      |
| 3.84b          |                         | 3.12    | 26.0    | 23.0 | 190.5          | 27.0  | 0      |

*YS = yolk sac diameter; CR = crown-rump length; Som = number of somite pairs; Prot = protein content.

Gassing II

|                | Medium                  | YS, mm* | CR, mm* | Som  | Prot, µg/embryo | Score | ABN, % |
|----------------|-------------------------|---------|---------|------|----------------|-------|--------|
|                | 0 hr                    | 10% O₂  | 5% CO₂ | 28.0 | 304.0          | 40.0  | 5      |
|                | 36 hr                   | 50% O₂  | 5% CO₂ | 27.0 | 230.0          | 38.0  | 0      |
| 100% Rat serum, n = 20 | 4.68                  | 3.48    | 28.0    | 27.0 | 250.0          | 39.0  | 0      |
| 4.20          | 3.90                    | 3.18    | 27.0    | 27.0 | 197.5          | 35.0  | 0      |
| 4.86          | 3.54                    | 27.0    | 25.0    | 26.0 | 230.0          | 38.0* | 0      |
| 5.00          | 3.36                    | 24.0*   | 26.0    | 23.0 | 176.0          | 37.0  | 0      |

*YS = yolk sac diameter; CR = crown-rump length; Som = number of somite pairs; Prot = protein content.

Table 2. Comparison of development of rats in whole-embryo culture at 37.0 ± 0.2°C and 38.8 ± 0.2°C incubation temperature (bovine serum).

| Incubation temperature | YS, mm* | CR, mm* | Som* | Prot, µg/embryo | Score | ABN, % |
|------------------------|---------|---------|------|----------------|-------|--------|
| 37.0 ± 0.2°C n = 20    | 4.20b   | 3.21    | 24.0 | 172.3          | 35.0  | 0      |
| 3.96b                  | 2.94†   | 24.0*   | 141.0| 33.0†          | 10    |
| 3.78b                  | 2.65    | 23.0    | 111.5| 28.0           |       |        |
| 36.8 ± 0.2°C n = 12    | 4.26    | 3.44    | 26.0 | 218.8          | 37.0  | 0      |
| 4.08                   | 3.30    | 25.0    | 185.5| 35.0           |       |        |
| 3.90                   | 2.91    | 24.0    | 151.0| 34.0           |       |        |

*YS = yolk sac diameter; CR = crown-rump length; Som = number of somite pairs; Prot = protein content.

b Middle rows are median values; the numbers in the bottom row represent the first quartile, the numbers in the top row represent the third quartile.

†p = 0.01–0.05 (Mann-Whitney test).

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method. Abnormal development in whole-embryo culture induced by valproic acid was first described by Kao et al. (7) and has been extensively studied by Brown (8). Since the teratogenic action of VPA has predominantly been demonstrated in vivo with mice, it is interesting to note that typical defects seen in mice (predominantly defects in the head region) can also be observed in vitro with rat embryos—a species which has not been found so far to respond readily to VPA with the formation of CNS abnormalities in vivo. The susceptibility of the rat embryo to VPA in vitro can be shown on the macroscopic (Fig. 1) as well as the microscopic level (Fig. 2), especially at the head region and the somites.

We have shown (9) that one of the VPA metabolites (4-en-VPA) is similarly active in inducing abnormal development in vitro as VPA itself (Fig. 3 and Table 3), whereas another chemically similar derivative (2-en-VPA) is completely inactive in this respect. 4-en-VPA induces an additional type of gross structural abnormality in vitro, i.e., cardiac defects (Table 4). This example clearly shows that in vitro techniques are especially suited to assess toxic effects of metabolites in the absence of the original compound and under defined experimental conditions.

Since embryos are routinely standardized with re-

**Figure 1.** Effect of valproic acid (VPA) on rat embryos in whole-embryo culture (48 hr incubation): (A) control medium (6 mL bovine serum + 1 mL Tyrode's buffer); (B) + 0.6 mM VPA; (C) + 1.2 mM VPA; (D) + 1.8 mM VPA. Δ = head; ↑ = somites; □ = pericardial dilatation; * = incurvation of the spine.

**Figure 2.** Effect of valproic acid (VPA) on rat embryos in whole-embryo culture (48 hr incubation): lateral sagittal section of an embryo; the alteration of the somite structures is obvious: (A) control medium; (B) + 0.6 mM VPA; ↑ = somites.

**Figure 3.** Effect of 4-en-VPA on the development of rat embryos in whole-embryo culture (48 hr incubation): (A) control medium; (B) + 1.8 mM 4-en-VPA; Δ = head; ↑ = somites; * = heart tube.
Table 3. Influence of 4-en-VPA (0.6 mM, 1.2 mM, and 1.8 mM) on the development of rat embryos in “whole-embryo” culture.

| 4-en-VPA, mm | YS, mm | CR, mm | Som | Prot, μg/emb. | Score | Abnormalities, % | Abnorm. per embryo |
|--------------|--------|--------|-----|--------------|-------|------------------|-------------------|
| Control      | 4.77b  | 3.61   | 26.2| 249          | 37    | 0                | 0                 |
| n = 18       | 4.47b  | 3.36   | 25.0| 203          | 36    | 0                | 0                 |
| 0.6 mM       | 4.75   | 3.37   | 26.0| 175          | 36.2  | 0                | 0                 |
| n = 18       | 3.97b  | 2.97   | 22.7| 105          | 33    | 0                | 0                 |
| 1.2 mM       | 4.20   | 3.18   | 24.0| 162          | 35    | 0                | 0                 |
| n = 15       | 3.66   | 2.16   | 20.0| 65           | 20    | 3 × 2 Abn.       |                   |
| 1.8 mM       | 4.05   | 2.11   | 14.0| 78           | 20    | 2 × 1 Abn.       |                   |
| n = 14       | 3.84   | 1.50   | 12.0| 51           | 15    | 8 × 3 Abn.       |                   |

YS = yolk sac diameter; CR = crown-rump length; Som = number of somite pairs; Prot = protein content.

*Significant difference (p = 0.01).
†Significant difference (p ≤ 0.01) to the control group.

Table 4. Type of abnormalities observed after treatment with VPA and 4-en-VPA in the whole embryo culture.

| Concentration, mM | Abnormality, % | n | Neural tube | Shape | Heart |
|-------------------|----------------|---|-------------|-------|-------|
| Valproic acid     | 1.8            | 15 | 60          | 100   | 0     |
| 4-en-Valproic acid| 1.8            | 14 | 85          | 92    | 64    |

spect to the developmental stage for in vitro studies when initiating the incubation, the variability of the development in culture is much smaller than the considerable variability of embryonic development within the living organism. This fact holds true for many culture methods.

Table 5 gives an example of the variability observed in mouse embryos in vivo. Such studies have been performed with various strains of rats and mice (10) and have given very similar results: there was always a considerable inter- and intralitter variation in the stages of normal prenatal development. This may be an explanation for the variability of outcome regularly seen when assessing embryotoxic effects in vivo. The example given in Table 5 concerns an inbred strain in which none of these strains would be expected the highest degree of synchronization. The variability seen—as indicated by the somite stages—is considerable. For the 62 embryos evaluated, a median value of 40 somite pairs was found for 11-day-old embryos with a maximum of 44 and a minimum of 29 somites.

In contrast to this, the high degree of reproducibility of an in vitro system is shown, for example, for the morphogenetic differentiation of mouse limb buds in culture (Fig. 4). Because the explants can be standardized at the initiation of the culture (according to the somite stage of the embryos: ±1 somite stage) all the limbs can be made to develop in a synchronized manner.

The in vitro techniques allow the exposure of the explants to the toxic agent for a limited period only; before and after this period the cultivation is allowed to proceed in normal culture medium (12). We have used the whole-embryo culture technique to reveal the period of the highest susceptibility of the rat embryo to VPA. The results of our studies indicate that 9.5-day-old rat embryos are most susceptible to VPA 12 to 18 hr after the initiation of the culture (Table 6). This would cor-
Table 5. Example of the intra- and inter-litter variability of a developmental stage (pairs of somites) in C57Bl mice.

| Litter no. | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | Litter size |
|-----------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----------|
| 1         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 3        |
| 2         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 3        |
| 3         |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 4        |
| 4         |    |    |    |    |    |    |    |    |    | 1  |    |    |    |    |    |    | 5        |
| 5         |    |    |    |    |    | 1  |    |    |    |    |    |    |    |    |    |    | 6        |
| 6         |    |    |    |    |    |    | 1  | 1  | 1  |    |    |    |    |    |    |    | 6        |
| 7         |    |    |    |    |    |    | 1  | 2  | 2  | 1  |    |    |    |    |    |    | 7        |
| 8         |    |    |    |    |    |    | 1  | 2  | 2  | 3  |    |    |    |    |    |    | 6        |
| 9         |    |    |    |    |    |    | 3  | 2  | 1  |    |    |    |    |    |    |    | 6        |
| 10        |    |    |    |    |    |    |    | 2  | 1  |    |    |    |    |    |    |    | 7        |
| 11        |    |    |    |    |    | 3  | 3  | 1  | 2  |    |    |    |    |    |    |    | 9        |

Percentage distribution: 2  2  5  10  13  7  15  16  18  7  8%  (n = 62)

*Somites were counted on day 11 of gestation (between 13<sup>th</sup> and 15<sup>th</sup>).

Table 6. Effect of a short-term exposure (6 hr) to valproic acid (1.8 mM) in the culture medium at different developmental phases on growth and development of rat embryos in vitro.

| Exposure | YS, mm<sup>a</sup> | CR, mm<sup>a</sup> | Som<sup>a</sup> | Prot, µg/embryo<sup>b</sup> | Score<sup>a</sup> | Abnormalities,* % | Abnorm. per embryo |
|----------|---------------------|--------------------|----------------|-----------------------------|------------------|-------------------|-------------------|
| Control  | 4.68                | 3.42               | 27.3           | 220                         | 37               | 0                 | 0                 |
| n = 35   | 4.44                | 3.24               | 26.0           | 175                         | 36               | 0                 | 0                 |
| 0–6 hr   | 4.14                | 3.12               | 24.0           | 134                         | 35               | 0                 | 0                 |
| n = 21   | 4.50                | 3.18               | 26.0           | 159                         | 36               | 0                 | 0                 |
| 12–18 hr | 4.38                | 2.94               | 24.0           | 143                         | 34               | 14                | 3 x 1 Abn.        |
| n = 15   | 4.17                | 2.76               | 23.0           | 110                         | 29               | 6 x 1 Abn.        | 5 x 2 Abn.        |
| 12–18 hr | 4.26                | 3.12               | 24.0           | 159                         | 27               | 80                | 5 x 2 Abn.        |
| n = 15   | 3.78                | 2.70               | 20.7           | 103                         | 23               | 1 x 3 Abn.        |                   |

*YS = yolk sac diameter; CR = crown-rump length; Som = number of somite pairs; Prot = protein content.

<sup>a</sup> Middle row are median values; the numbers in the bottom row represent the first quartile, the numbers in the top row represent the third quartile.

<sup>b</sup> Significant difference (p < 0.01) between 0–6 hr and 12–18 hr.

respond to day 10.0 to 10.3 of gestation in the living organism.

**Studies on the Possible Potential of Female Sex Hormones to Induce Abnormal Cardiac Development in Culture**

There has been a considerable argument over the question whether estrogens or gestagens have a teratogenic potential in man outside the genital tract and, especially, whether they may induce cardiac abnormalities (13–17). The data of one of the main studies providing some evidence in this direction (17) have been doubted in their reliability (18) and apparently cannot be used to provide evidence in this direction.

Since the problem cannot be solved by epidemiological studies we attempted to obtain further clues on a possible teratogenic potential by testing the effects of such female sex hormones on the heart development of rat embryos in the whole-embryo culture. With this system the entire period of early heart development can be monitored. Furthermore, extremely high concentrations of these substances may be evaluated in vitro, whereas in vivo the doses to be tested are very limited due to the early occurrence of embryomortality in rodents. The whole-embryo technique has been successfully used before to analyze the action of retinoic acid on heart development in culture (19).

With the presently available techniques, it is difficult to study development beyond day 12.5 of gestation in the rat. Therefore, possible effects on later stages of cardiac development (e.g., closure of the ventricular septum) cannot completely be ruled out.

When testing a large number of estrogens and gestagens using whole-embryo culture (Tables 7 and 8), we did not find any indication of the ability of these agents to interfere with cardiac development in rat embryos. The studies were performed with 9.5- as well as with 10.5-day-old rat embryos cultured for 48 hr. All the embryos have been evaluated histologically, in each case assessing serial sections. At extremely high concentrations (> 1 µg/mL) most of these substances produced necrosis in the embryo, predominantly at the CNS sites. Even in these cases cardiac development was unimpaired (Fig. 5). Taken together with all the epidemiological evidence available to date (20,21), our data do
Table 7. Evaluation of the effect of female sex hormones on cardiac development in whole-embryo culture (48-hr incubation; culture initiation: day 9.5).

|                | YS, mm | CR, mm | Som* | Normal | Retarded | Abnormal |
|----------------|--------|--------|------|--------|----------|----------|
| Controls       | 5.0    | 3.5    | 28.0 | 25     | 1        | 1^b      |
| n = 27         | 4.5    | 3.3    | 27.0 |        |          |          |
|                | 4.2    | 3.0    | 26.0 |        |          |          |
| β-Estradiol    | 4.7    | 3.3    | 26.5 | 8      | 1        |          |
| (3 µg/mL) n = 9| 4.6    | 3.2    | 26.0 |        |          |          |
|                | 4.4    | 3.0    | 24.0 |        |          |          |
| 17α-Ethinylestradiol | 7.8  | 3.6    | 26.0 | 10     | 3        |          |
| (10 µg/mL) n = 15 | 4.5 | 3.2     | 25.0 |       |          |          |
|                | 4.4    | 3.0    | 24.0 |        |          |          |
| DES            | 4.8    | 3.1    | 26.5 | 8      | 4        |          |
| (10 µg/mL) n = 14 | 4.1 | 2.8     | 26.0 |       |          |          |
|                | 3.9    | 2.4    | 25.2 |        |          |          |
| Progesterone   | 4.5    | 3.5    | 28.0 | 10     | 1        |          |
| (1 µg/mL) n = 11 | 4.4  | 3.3     | 27.0 |       |          |          |
|                | 4.2    | 3.2    | 26.0 |        |          |          |
| 19-Norethisteron| 5.1   | 3.9    | 30.0 | 8      |          |          |
| (3 µg/mL) n = 8 | 4.5  | 3.2     | 27.0 |       |          |          |
|                | 4.3    | 3.0    | 27.0 |        |          |          |

*YS = yolk sac diameter; CR = crown-rump length; Som = number of somites.
^b No cardiac abnormalities.

Table 8. Evaluation of the effect of female sex hormones on cardiac development in whole-embryo culture (48-hr incubation; culture initiation: day 10.5).

|                | YS, mm | CR, mm | Som* | Normal | Retarded | Abnormal |
|----------------|--------|--------|------|--------|----------|----------|
| Controls       | 5.5    | 5.0    | 29.0 | 12     |          |          |
| n = 12         | 5.5    | 4.7    | 28.0 |        |          |          |
|                | 5.2    | 4.2    | 27.3 |        |          |          |
| β-Estradiol    | 5.5    | 4.7    | 30.0 | 9      |          |          |
| (3 µg/mL) n = 12| 5.4  | 4.5     | 29.0 |       |          |          |
|                | 5.0    | 4.2    | 28.5 |        |          |          |
| 17α-Ethinylestradiol | 5.6  | 4.6     | 29.3 | 6      |          |          |
| (10 µg/mL) n = 6 | 5.3 | 4.2     | 27.5 |       |          |          |
| DES            | 5.5    | 4.4    | 28.0 | 6      |          |          |
| (3 µg/mL) n = 6 | 5.2    | 4.3    | 27.5 |        |          |          |
|                | 5.0    | 4.1    | 26.8 |        |          |          |
| Progesterone   | 5.5    | 5.0    | 28.5 | 8      | 1        |          |
| (3 µg/mL) n = 9 | 5.2    | 4.4    | 27.0 |       |          |          |
|                | 5.2    | 4.1    | 28.0 |        |          |          |
| 19-Norethisteron| 5.5    | 4.6    | 28.3 | 8      |          |          |
| (3 µg/mL) n = 8 | 5.5    | 4.5    | 27.5 |       |          |          |
|                | 5.4    | 4.2    | 26.8 |        |          |          |

*YS = yolk sac diameter; CR = crown-rump length; Som = number of somites.
^b No cardiac abnormalities.

not support the speculation that female sex hormones interfere with cardiac development.

Studies on Assessing the Teratogenic Potential of TBTO*

Bis(tri-n-butyl) oxide (TBTO) is a mollusccide and fungicide which is widely used. Its application may increase in the future. It is, therefore, of importance to learn whether this substance possesses a potential to interfere with prenatal development. Again, it was attempted to solve this problem using a combined in vivo/ in vitro approach. In collaboration with WHO we have performed in vivo experiments in mice. From these studies (22) it can be concluded that TBTO seems to exhibit embryotoxic and fetotoxic effects only at doses stating which kind of alteration is observed (specific congenital gross structural abnormality, special dysfunction, etc.). If we use the term at all it is for us synonymous with "inducing congenital gross structural abnormalities," not including certain "minor" abnormalities, anomalies, signs of retardation, or dysjunctions.

*The term "teratogenic" is defined differently by different investigators in the field. Therefore, it is so ill defined that its significance for a toxicological risk assessment is small. It may even be misleading (e.g., for lawsuits). If possible, it should be avoided and replaced by
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which are close to those producing maternal toxicity (Table 9).

The embryotoxicity and fetotoxicity of TBTO is compared with maternal toxicity (mortality) in Table 10. From these data it is obvious that embryotoxicity with this substance occurs close to maternal toxicity. The procedure of calculating ratios of relative embryotoxicity—as we have suggested (23)—requires the establish-

Table 9. Embryo/fetotoxicity of TBTO in mice.

| TBTO dose | Controls | 5 µL/kg | 10 µL/kg | 30 µL/kg |
|-----------|----------|---------|----------|----------|
| Live fetuses | 1154 | 248 | 204 | 21 |
| % Resorptions | 9.1 | 9.8 | 9.7 | 58.8 |
| Average weight of fetuses, g | 1.15 ± 0.12 | 1.19 ± 0.08 | 1.10 ± 0.15 | 0.91 ± 0.19 |
| Cleft palates, % | 8 (0.7%) | 4 (2%) | 14 (7%) | 10 (48%) |
| Minor skeletal deformities | 85 (7%) | 28 (11%) | 22 (10%) | 8 (38%) |

Table 10. Relative embryotoxic risk factors for TBTO.

| Risk factor | Fetal LD_{50}/maternal LD_{50} | ED_{50} cleft palate/maternal LD_{50} |
|-------------|---------------------------|----------------------------------|
| Risk factor | < 30/74 | > 30/45 | > 10/45 |
| Fetal LD_{50}/maternal LD_{50} | 0.41 | 0.44 | 0.22 |
| ED_{50} cleft palate/maternal LD_{50} | 0.87 | 0.91 | 1.00 |

Subsequent studies using the limb bud organ culture system revealed an exceptional capability of this substance to interfere with morphogenetic differentiation in vitro (Table 11): concentrations as low as 50 nM (30
Table 11. Effect of TBTO on limb bud differentiation in vitro.

| TBTO concentration, nM | Interference with development* day 11 | day 12 |
|------------------------|--------------------------------------|--------|
| 17                     | 0                                    | 0      |
| 50                     | +                                    | 0      |
| 170                    | ++                                  | +      |
| 500                    | +++                                 | ++     |
| 1700                   | ao = no effect; + = clear-cut effect; ++ = strong effect; +++ = clearly abnormal differentiation; = completely inhibited growth and differentiation. |

ng/mL) strongly inhibited the development in culture (24). This is one of the lowest concentrations of a substance ever found to interfere with development in this organ culture system.

When measuring the tin content within the embryonic tissues of mice injected with TBTO, we found concentrations (22) which, if caused by original TBTO, should be more than sufficient to induce embryotoxicity. We are presently involved in an attempt to analyze the cause of this apparent discrepancy. One explanation could be that a metabolite of TBTO with a lower embryotoxic potential accumulates within the embryo. To demonstrate this, the experiments have to be supplemented with further pharmacokinetic studies and an assessment of the (presumably lower) potential of such...
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Figure 9. Mouse limb buds on day 11 of gestation after 4 days of culture. (Left) limb buds in assays containing a reconstituted cytochrome P-450 system, NADPH, and 100 μg/mL cyclophosphamide; (right) control assays containing the same components minus NADPH or cyclophosphamide; (top row) cytochrome P-450 concentration: 90 pmole/mL; (bottom row) cytochrome P-450 concentration: 9 pmole/mL.

Table 12. Species differences in the development of monooxygenases.

| Developmental stage | Rat\(^b\) | Marmoset\(^b\) |
|---------------------|-----------|----------------|
|                     | Cou. 450  | Res. 40        | Cou. 263  | Res. 179 | Mo. 3000 | Mo. 2000 |
| − 2 days            |           |                |          |          |         | 80–90    |
| 1 day               | 8         |                |          |          |         |         |
| Birth +             |           | 20             |          |          | 46       |          |
| 1 day               |           |                |          |          |         |         |
| 2 days              |           | 78             |          |          | 100      |         |
| 3 days              |           | 20             |          |          | 100      |         |
| 1 week              | 64        | 100            |          |          | 95       |         |
| 2 weeks             | 100       |                 |          |          |         |         |
| 1 month             |           |                | 6        |          | 100      |         |
| 2 months            |           |                |          |          | 100      |         |

\(^a\)Cou. = ethoxycoumarin O-deethylase; Res. = ethoxyresorufin O-deethylase; Mo. = ethylmorphine N-demethylase.

\(^b\)The data are given in (%) of adult enzyme activity. 100% = pmole/mg protein-min.

\(^c\)Already 20% on day 60 of gestation.

— = not detectable.

a TBTO metabolite to interfere with morphogenetic differentiation in vitro.

Problems Involved in Supplementing in Vitro Systems with Xenobiotic-Activating Capacities

One of the disadvantages of many in vitro systems utilizing embryonic rodent tissues is the inability of these explants to respond to metabolic activation. Especially with organ culture systems, the simple addition of microsomal fractions (or S-9-mix) is completely unsatisfactory, since these crude fractions strongly interfere with development in culture. Another approach has been to perform organ cultures in the presence of “feeder cells” capable of at least some functions of drug metabolism. While this latter approach has been successfully used in a few laboratories (25), it also has a number of limitations, especially since the spectrum of the type of metabolic activation attainable with the supplementing cells (including hepatocytes) is at present quite limited.

For a number of years we have been trying two other approaches: to induce the capacity for metabolic activation within the explants to be used for the organ culture studies, and to isolate defined cytochrome P-450 fractions and to add a reconstituted system to the culture medium. The first approach—if feasible—would
Table 13. Effect of acyclovir and physiological deoxynucleosides (2'-deoxyguanosine, 2'-deoxyadenosine) on growth and development of 9.5 day-old rat embryos in culture (2n = 213).

| Acyclovir concentration | Score | ABN, % |
|-------------------------|-------|--------|
| Control                 | 283.5 | 38.0   |
| n = 44                  | 25.0  | 35.25  |
| 25 µM Acyclovir         | 4.50  | 26.0   |
| n = 27                  | 2.88  | 36.0   |
| 50 µM Acyclovir         | 4.51  | 27.0   |
| n = 18                  | 4.35  | 30.0   |
| 100 µM Acyclovir        | 4.85  | 27.0   |
| n = 19                  | 4.53  | 25.5   |
| 200 µM 2'-Desoxyguanosine | 4.33 | 27.0  |

YS = yolk sac diameter; CR = crown-rump length; Som = number of somite pairs; Prot = protein content; ABN = abnormalities.

Table 14. Influence on acyclovir on the ear development of 9.5-day-old rat embryos in vitro.

| Ear development | Control, n = 44 | 10 µM, n = 19 | 25 µM, n = 27 | 50 µM, n = 18 | 100 µM, n = 19 | 200 µM, n = 21 |
|-----------------|-----------------|---------------|---------------|---------------|----------------|----------------|
| Rec. dors. present, otic vesicle closed | 71% | 58% | 26% | 28% | 10.5% | — |
| Otic vesicle closed | 18% | 42% | 30% | 5.5% | 10.5% | — |
| Otic vesicle slightly open | 11% | — | 30% | 33% | 37% | 9.5% |
| Otic vesicle half open | — | — | 3% | 5.5% | 10.5% | 19% |
| Otic pit | — | — | 11% | 28% | 31.5% | 71.5% |

Table 15. Overview of the histological results in the different test groups.

| Tissue | Control; 10 µM Acyclovir; 200 and 500 µM 2'-deoxyguanosine | 25 µM Acyclovir | 50 µM Acyclovir | 100 µM Acyclovir | 200 µM Acyclovir |
|--------|-----------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Neuroepithelium | Multilayered; partly monolayered | Monolayered; Mitosis ↓ Necroses ↑ | Monolayered; Mitosis ↓ Necroses ↑ | — | — |
| Central channel | 0 | 0 | Slightly dilated | Dilated; Necroses ↑ Dilated ↑, Necroses ↑ | — | — |
| Telencephalon | 0 | 0 | Some poorly developed or completely missing | Poorly developed ↑ Poorly developed ↑ | — | — |
| Ear | Otic vesicle closed Cells cylindrical | Otic vesicle not completely closed Cells cylindrical | Otic pit partly Necroses; Cells cylindrical | Otic pit Necroses ↑ Cells partly cubical | Ear plate, little cell material; Necroses ↑↑; Cells cubical |
| Somites | Regular | Regular | Partly irregular; "Frayed"; Necroses | Irregular; "Frayed"; Necroses ↑↑ | Irregular ↑; Necroses ↑↑ |

be superior to all the other approaches, since the potentially toxic substances would be metabolically activated by the target cells themselves. This is especially important if short-lived active metabolites are to be expected.

So far, only attempts that induce a special subtype of monoxygenase activity: AHH = aryl hydrocarbon hydroxylase, a cytochrome P-448-dependent enzyme system, have been convincing. We have been able to induce an AHH type of activity (e.g., benzo[a]pyrene...
IN VIVO AND IN VITRO STUDIES ON PRENATAL TOXICITY

FIGURE 10. Effect of acyclovir in whole-embryo culture: (a) 9.5-day-old rat embryo (↓) in its membranes at the beginning of the culture; (b)–(f) rat embryos after 48 hr of culture in bovine serum with test substances added to the medium. Culture in the presence of: (b) 200 μM 2-deoxyguanosine (development corresponding to controls); (c) 25 μM, (d) 50 μM, (e) 100 μM, and (f) 200 μM acyclovir, respectively, increasing extent of interference with prenatal development.

hydroxylase) at the stage of organogenesis in various rodent embryos (26). It has been found to be much more difficult to induce typical cytochrome P-450 activities in embryonic tissues of rodents. Although some such enzyme activities may be detectable with the highly sensitive methods available today (27), all the activities studied up till now remained so low—even even after attempts to enhance the activities by various inducers—that a reproducible and exploitable monooxygenase activity sufficient for routine test purposes in vivo could not be obtained.

By using very high substrate concentrations in vitro, it is feasible to test the potential to induce abnormal development with explants or cells subsequent to an enzyme induction in vivo. With this experimental setup apparently only some subtypes of cytochrome P-450-dependent activities can be induced within the embryonic tissues of rats or mice. We have been successful with this experimental design (see schematic presentation in Fig. 6) in studying abnormal development in organ cultures with cyclophosphamide and dimethylnitrosamine using explants of embryos after induction of monooxygenase activities with PCBs (Aroclor A60) or β-naphthoflavone (28,29) in utero. Typical examples of such an approach are shown in Figures 7 and 8.

With respect to the second approach, after many years of attempts and failure we have now succeeded in obtaining enzyme fractions of very high specific activities which are tolerated by the explants in culture (30). Using cyclophosphamid as a model substrate, a pronounced and clear-cut metabolic activation is achieved with the addition of 10 nM cytochrome P-450 fractions from various sources and the necessary com-
The activity of cytochrome P-450-dependent monooxygenases is extremely low or absent in rodent embryos and early fetuses, such activity may be detectable in the livers of primates at rather early fetal stages (32).

Table 12 gives some examples of differences in perinatal development of some monooxygenases in rats and marmosets. Because of practical reasons we find the marmoset to be a primate species which is especially suited for special studies in prenatal toxicity, and therefore some data are included here. While typical cytochrome P-450-dependent monooxygenases (e.g., ethylmorphine demethylation) are already detectable at day 60 of gestation (duration of pregnancy 140 days) in the marmoset (Callithrix jacchus), the activity of other enzymes (predominantly such of the cytochrome P-448 type) may develop even later than in the rat.

With the technique of adding reconstituted monooxygenases to in vitro systems, we intend to study systematically the effect of monooxygenases of different species—including those of primates—after induction by different agents in various culture systems.

Assessment of Possible Teratogenic Potential of Acyclovir Using an in Vitro/in Vivo Approach

One of the best examples for the use of a combined in vitro/in vivo approach comes from recent studies performed in our laboratory to elucidate a possible embryotoxic potential of the virostatic agent acyclovir which is used for the treatment of herpes infections. Segment-II tests performed before on a routine basis (treatment on days 6–15 of pregnancy) have not shown any indication for an embryotoxic potential of this agent (33). Since the doses that could be tested in the experimental animals in vivo are limited (because of an interfering nephrotoxicity in these species), we initiated in vitro studies using the whole-embryo culture technique.

The substance was clearly able to interfere with embryonic development in vitro (Table 13 and Fig. 10) at concentrations exceeding 25 μM (34). Serum concentrations measured in man under extreme therapeutic conditions (IV application) approach 100 μM; serum peak concentrations to be expected after oral medication with the dose regime used today are in the range of 2 μM.

Abnormal development as shown in vitro—besides a general retardation, especially at higher doses—predominantly concerns the shape of the head; most susceptible to the action of acyclovir is the development of the ear (Tables 14 and 15 and Fig. 11). Histologically, necrosis can be seen in several parts of the embryos, e.g., neuroepithelium, somites, ear, etc. (Table 15 and Fig. 12).

The question arose why the substance had not been found to induce abnormal development in vivo. Two
**Figure 13.** Effect of acyclovir on 11.5-day-old rat embryos in vivo and in vitro after 48-hr culture: (A) in vivo control; (B) $3 \times 100$ mg/kg acyclovir (treatment on day 10 of gestation); (C) $8 \times 100$ mg/kg acyclovir (treatment on days 9, 10, and 11 of gestation); (a) in vitro control; (b) $+100 \mu M$ acyclovir; (c) $+100 \mu M$ acyclovir.

**Table 16.** Gross structural abnormalities produced by acyclovir in rats *in vivo.*

| Acyclovir treatment | Evaluation on day 11.5 |
|---------------------|------------------------|
| Day of pregnancy    | Dose, mg/kg | Som$^b$ | Prot$^b$ | Score | ABN, % |
| Control $n = 22$    | —           | 26.5    | 353      | 40    | 0      |
| 9, 10, 11 $n = 23$  | $8 \times 50$ | 27      | —        | 40    | 3 % head |
| 10 $n = 13$         | $1 \times 200$ | 25      | 158      | 35    | 100 % head |

$^a$The effect was evaluated on day 11.5 of pregnancy; acyclovir was given SC.

$^b$SOM = number of somites; Prot = µg/protein/embryo; ABN = abnormal embryos.
explanations appeared possible: (a) the substance does not reach the embryo in vivo at high enough concentrations to exert an embryotoxic potency, or, more likely, (b) the dose regime routinely applied was not adequate to allow the demonstration of an effect. Since the period of highest susceptibility could be deduced from our in vitro studies (day 10 of gestation), we applied the substance at a high dose during one day of pregnancy at the presumed susceptible period (Table 16). Surprisingly, the same type of structural abnormality was found under these special conditions in vivo when evaluated on day 11.5 of pregnancy as was seen in vitro. Figure 13 shows a typical picture of the embryos after treatment in vitro and in vivo.

To our knowledge this is the first example of an effect which, after being observed in vitro, has been predicted to also have a potential of occurring in vivo and subsequently has been demonstrated to be inducible in vivo. We feel that this gives a good example of the benefits of combining in vitro and in vivo tests for the assessment of a possible embryotoxic potential of a chemical.

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