Effects of cyclophosphamide treatment before implantation on the development of rat embryos after implantation

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SUMMARY

After treatment of pregnant rats 24 h before implantation with a single injection of cyclophosphamide (20–80 mg/kg), a dose-dependent increase in resorption was observed at term but no malformed fetuses could be found. The lowest cyclophosphamide dose that caused 100% resorption was 60 mg/kg. Somite number and wet weight indicated retardation of about 24 h during organogenesis. Determination of the time of implantation revealed that the developmental retardation in treated embryos was not due to delayed implantation. At implantation, 24 h after cyclophosphamide treatment, a significant and dose-dependent decrease of the cell number of blastocysts was found. Embryo transplantation experiments showed that early cyclophosphamide treatment interfered with the subsequent development of both the embryo and the mother. The decidual reaction seemed to be more affected by the treatment than the embryos. Most teratologists hold that mouse embryos after treatment in the preimplantation period either die before implantation or survive to term without being malformed. The present study, however, proves that the reaction of drugs at this early stage of pregnancy is more complex than is generally assumed.

INTRODUCTION

The treatment of cleaving rabbit eggs with purine analogues in vivo had no effect on development until at or after implantation (Adams, Hay & Lutwak-Mann, 1961). In general, however, preimplantation embryos are remarkably resistant to teratogens; the resulting rarity of birth defects following treatment during this period has been explained as follows (Austin, 1973): ‘The effect of teratogens on the cleavage embryo depends on the number of cells killed or inhibited: above a certain portion, the embryo dies; below that figure, the remaining cells multiply to replace those lost and subsequent development is essentially normal’. This view is still shared by most teratologists (e.g. Wilson, 1973).

A different approach to this problem has been used by Gottschewski and

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coworkers (Gottschewski, 1963, 1964; Gottschewski & Zimmermann, 1963). After treatment during the preimplantation period they inspected rabbit embryos during organogenesis. When cyclophosphamide (CPA) (40 mg/kg intravenously) was given to pregnant rabbits before implantation, a high percentage of deformed fetuses was found on days 11, 17 and 30 p.c. (post-coitum), suggesting that the substance may penetrate into the blastocysts even before implantation and interfere with normal development.

Gottschewski's studies were later extended to the rat (Brock & von Kreybig, 1964). When CPA was given on day 3 p.c. and the uteri were examined on day 12, the number of resorbed and malformed embryos was significantly increased. On day 15 p.c. all malformed embryos were resorbed so that only resorbed or completely normal embryos could be seen.

Mice injected with CPA on day 3 p.c. showed an increased resorption rate, but no malformed embryos at the end of gestation (Gebhardt, 1970). Administration of CPA to pregnant rabbits at about the time of implantation (day 6 and 7) led to an increase in the number of foetal deaths (Fritz & Hess 1971) and about 10 % of the foetuses from dams treated on day 7 exhibited malformations at term.

To get further information on the mechanism of action of teratogens given during the preimplantation period we repeated and extended earlier studies (Brock & von Kreybig, 1964) with CPA on the rat. We tried to determine the time of death of such embryos and whether embryos that die are malformed or only retarded in development. We also attempted to study the development of treated embryos before and around the time of implantation. Finally we used the embryo transfer technique to see whether CPA during the preimplantation period predominantly affects the embryo or the mother.

**MATERIALS AND METHODS**

Wistar rats of the strain SW 72 weighing 200 g (breeder Winkelmann, Kirchborchen, Germany) were kept under a normal day/night cycle and placed with males overnight. The presence of spermatozoa in vaginal smears indicated day 0 of pregnancy. Implantation occurs in this strain between 120 and 132 h after the midpoint of the overnight mating period, this is between day 4 and day 5. Blastocysts were usually obtained at 2 p.m. on day 4, 24 h after the time of treatment. CPA, which was a gift from Prof. N. Brock (Asta-Werke AG, Chemical Factory Brackwede, Germany), was dissolved in distilled water and injected subcutaneously.

In the transplantation experiments and in the course of the determination of cell number, embryos were handled in Whitten's medium (Whitten, 1971), freshly prepared from three-times quartz-distilled water. The cell number of the preimplantation embryos on day 4 was determined by the method of Tarkowski (1966) and the mitotic index (number of cells in mitosis as percentage of total
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Cells) was calculated from the same preparations. To follow the decidual reaction, 0.5 ml of a 1% Pontamine sky blue solution was injected into a tail vein (Finn & Martin, 1972).

In the embryo transfer experiments blastocysts were surgically transplanted in groups of five to one uterine horn of pseudopregnant females on day 4 of pseudopregnancy. The pseudopregnant recipients were anaesthetized with Evipan (hexobarbital) which was a gift from the Bayer AG (Pharmaceutical Company, Germany). Pseudopregnancy was induced by mating normal females with vasectomized males. The foster mothers were killed on day 20 of pregnancy and the success rate of the transplantations was determined by the number of resorbed and live embryos. The embryos were weighed, inspected for growth retardation and malformations, and stained for skeletal abnormalities with Alizarin red S (Lorke, 1965).

In the morphological studies the treated mothers were killed at different times during pregnancy, the uteri were excised and the embryos were removed from the implantation sites on days 11, 14, 17 and 20. The embryos were checked for developmental anomalies under a stereo microscope (Carl Zeiss, Oberkochen, Germany) and their somite numbers determined.

For histological examination uterine horns of pregnant mice of day 11 or 14 of gestation were excised, fixed with embryo undisturbed in Carnoy’s solution, and embedded in paraffin. Serial sections in two directions (cross and longitudinal) were prepared. Sections were routinely stained with haematoxylin and Eosin, PAS, or Azan. Only material which clearly showed an implantation site without too heavy haemorrhage was chosen for histological studies.

RESULTS

1. Lethality rate at term after CPA treatment on day 3

As described by Brock & von Kreybig (1964), groups of ten pregnant rats received a single s.c. injection of 20, 40, 60 or 80 mg/kg CPA on day 3. The maternal LD$_{50}$ in our strain is 180 mg/kg. The resorption rates at term for the four doses were 48%, 82%, 100% and 100%, compared with 10% for control SW 72 embryos (see Fig. 1).

The wet weight of the living embryos at term was not reduced in the four treated groups, and no malformations were observed.

2. Changes in the number of dead and living fetuses after CPA treatment (60 mg/kg) on day 3

Pregnant animals were treated with one injection of 60 mg/kg CPA on day 3 and the number of embryos recorded at different times after treatment. After 24-48 h, i.e. on days 4 and 5, the total number of embryos was calculated from the number of implantation sites visible after Pontamine sky blue injection and the number of embryos (blastocysts) that could be flushed from the same
uterus. On days 8, 11, 14 and 20 the number of implantation sites could easily be determined. On days 11, 14 and 20 all implantation sites were dissected under the stereo microscope and inspected for living or dead embryos. Ten treated and ten control animals were investigated on each day.

The results are given in Fig. 1. There are no significant effects of CPA treatment on the total number of implantations per female. In both groups there is a reduction of the number of embryos at the time of implantation. The most striking reduction in the number of living embryos in the treated group occurred during organogenesis, as indicated by the decrease from 70% to 10% between days 11 and 14.

3. Development of rat embryos during organogenesis (day 11 and 14) after maternal treatment with 60 mg/kg CPA on day 3

We tried to confirm earlier observations (Brock & von Kreybig, 1964) on malformations of rat embryos during organogenesis caused by CPA treatment during the preimplantation period. Among 30 litters observed under the stereo microscope we never found any malformations of the brain or heart as described previously (Brock & von Kreybig, 1964). The embryos seemed to be retarded in development rather than grossly malformed (Spielmann, 1976).

The somite number per embryo is significantly lower in treated embryos (Fig. 2). According to the developmental stage given by the somite number per
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Fig. 2. Influence of cyclophosphamide treatment on day 3 of gestation on the somite number of embryos during organogenesis. Values are given ± standard deviation, the numbers indicate the amount of embryos used in each determination. •—•, Control embryos; *—*, treatment on day 3 with 60 mg/kg cyclophosphamide.

Embryo in Fig. 2, the treated embryos seem to be retarded by about 24 h. Dry weight determinations of treated and untreated embryos gave the same results. In contrast to previous investigators (Brock & von Kreybig, 1964), we conclude that rat embryos treated with CPA in the preimplantation period die during organogenesis without being malformed and that these embryos are retarded in development by about 24 h. Histological inspection of control and treated uteri during organogenesis revealed a disturbed development of the placenta, e.g. on day 14 (Fig. 3).
Fig. 3. For legend see facing page.
Table 1. Number of embryos per female on days 4-5 after treatment with 60 mg/kg cyclophosphamide on day 3*

<table>
<thead>
<tr>
<th>Time of determination</th>
<th>Control embryos</th>
<th>Treated embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not implanted</td>
<td>Implanted</td>
</tr>
<tr>
<td>Day 4</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>4-5</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>10</td>
</tr>
</tbody>
</table>

* Based on means from 10 animals for each time of determination.

4. Development of rat embryos at the time of implantation (day 4 and 5) after maternal treatment with 60 mg/kg CPA on day 3

The developmental retardation of 24 h found in treated embryos during organogenesis could be explained by delayed implantation. To determine the exact time of implantation for treated and untreated animals, the implanted and unimplanted embryos were counted in both groups on day 4, day 4-5 (12 h later) and day 5. Table 1 shows that there were only slight differences in the time of implantation between the treated and untreated groups. There is no indication of a delayed implantation in the treated group.

5. Effects of CPA treatment on pregnant rats on day 3 of embryonic development before implantation

To find out whether the abnormal embryonic development of rat embryos during organogenesis after maternal treatment on day 3 with 60 mg/kg CPA is caused by an action of the drug predominantly on the mother (decidual reaction) or on the embryo or on both, we studied the development of treated embryos before implantation. Groups of ten pregnant females were treated with single...
Injections (subcutaneously) of 20, 40 or 60 mg/kg. CPA on day 3 and the uteri were flushed 24 h later, on day 4. The number of blastocysts per female was not reduced in any of the treated groups.

However, the cell numbers of the treated blastocysts (see Table 2) were significantly lower than those of control blastocysts ($P < 0.001$), and the cell numbers of the blastocysts in the 60 mg/kg group were significantly lower than those of the 20 mg/kg group ($P < 0.001$). The cell number as well as the mitotic index of the blastocysts treated with the highest CPA concentration (60 mg/kg) is the same as that of normally developing control morulae of the same strain (see Table 2). The considerable reduction of the cell number of the treated blastocysts indicates that CPA or one of its metabolites interfered with embryonic development during the 24 h of preimplantation development between day 3 and day 4.

Table 3. Influence of maternal cyclophosphamide treatment (day 3; 60 mg/kg) on development of rat blastocysts after transplantation to pseudopregnant recipients on day 4*

<table>
<thead>
<tr>
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<th>Untreated control</th>
<th>Blastocyst treated recipient untreated</th>
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<tr>
<td><strong>(A)</strong> Number of embryos transferred</td>
<td>85</td>
<td>115</td>
<td>115</td>
</tr>
<tr>
<td><strong>(B)</strong> Implantations (percentage of A)</td>
<td>72 (85 %)</td>
<td>103 (90 %)</td>
<td>75 (65 %)</td>
</tr>
<tr>
<td><strong>(C)</strong> Living embryos (percentage of A)</td>
<td>40 (47 %)</td>
<td>14 (12 %)</td>
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</tr>
<tr>
<td><strong>(D)</strong> Resorptions (percentage of A)</td>
<td>32 (38 %)</td>
<td>89 (78 %)</td>
<td>61 (53 %)</td>
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* Five blastocysts transferred to one uterine horn of a recipient which was killed on day 20 for determination of success rate.

Mitotic index: Cells in mitosis expressed as percentage of total cells of the embryos.

* = significantly lower than cell number of controls on day 4 at $P < 0.001$. 

Table 2. Cell number of rat embryos at implantation (day 4) 24 hours after cyclophosphamide treatment

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<tr>
<th>Time of determination</th>
<th>Dose (mg/kg)</th>
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<td>60</td>
<td>14.2 ± 5.8*</td>
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Fig. 4. Light microscopy (magnification approximately × 15) of the transplantation experiment during organogenesis (day 11).

(a) Control, day 11 (× 12). On day 11 the normal decidua (D) fills half of the space internal to the unstriated muscles of the uterine wall. The amniotic cavity (A) with the relatively small embryo is occupying the other half. The uterine lumen is almost completely compressed or obliterated in the periphery of the decidua. P = placenta.

(b) Transplantation control, day 11 (× 12). After transplantation of blastocysts to pseudopregnant hosts on day 4, the light microscopic picture of most of the embryos on day 11 corresponds to the morphological situation of the control group. The decidua (D) of some animals, however, seems to be slightly smaller. Moreover, part of the uterine lumen (L) can still be demonstrated.

(c) Transplantation of a treated embryo (60 mg/kg CPA on day 3) to an untreated pseudopregnant recipient, day 11 (× 17). Under these conditions the decidua (D) is normally developed. However, the placenta anlage (P) and the embryonic-maternal transitional region are smaller than in control animals. In some instances the amniotic cavity (A) is smaller and the decidua fills two thirds of the area in the central section. The decidua capsularis (X), which is normally compressed to a narrow band, is still comparatively wide.

(d) Transplantation of an untreated embryo to a treated pseudopregnant recipient (60 mg/kg CPA on day 3), day 11 (× 17). When the recipient is treated 24 h before transplantation, the decidua (D) is considerably smaller. The size of the placenta of most of the embryos is clearly diminished. The uterine lumen (L) is in all cases widely open. It is usually filled with blood and fibrin. Here and there cells of maternal and embryonic origin penetrate into these cavities. Fibrous filaments and numerous trophoblast giant cells can still be observed.
Fig. 5. For legend see facing page.
6. Transplantation experiments after maternal treatment with CPA during the preimplantation period

Following treatment during the preimplantation period, the transplantation of preimplantation embryos distinguishes between effects of an agent on the mother and direct effects of the agent on the embryo (Finn & Bredl, 1973; Spielmann, 1976). We therefore transplanted blastocysts on day 4 (24 h after maternal treatment) to pseudopregnant recipients on day 4 of pregnancy. We also transplanted untreated blastocysts on day 4 to pseudopregnant recipients on the same day of pregnancy which had been treated with CPA on day 3 (24 h before transplantation). Untreated blastocysts transplanted on day 4 to untreated pseudopregnant recipients on day 4 of pregnancy served as controls. Table 3 shows that in untreated control experiments 47% of the blastocysts developed into viable fetuses at term. The percentage of living fetuses at term was reduced to the same extent in both treated groups (12%). No indication of malformations could be found among the living term fetuses from the control.
transplantations nor from the transplantations with pretreated embryos or recipients. We therefore conclude that CPA given to rats on day 3 of gestation interferes with the development of the embryo before implantation and also with the decidual reaction of the uterus.

7. Histological examinations of the transplantation experiments during organogenesis (day 11, Fig. 4 and Fig. 5)

The uterine histology of untreated transplantation controls (Figs. 4b and 5b) did not differ from that of normal controls on the same day of pregnancy (Figs. 4a and 5a). When a treated embryo is transplanted to an untreated foster mother (Figs. 4c and 5c), the embryo-derived placental anlage and the embryo-maternal transitional region are smaller than in controls. Finally, when an untreated embryo is transferred to a pretreated recipient (Figs. 4d and 5d), the maternal part of the implantation site, the decidua, is considerably smaller than in controls. This again suggests an action of the CPA treatment on both the embryo (Figs. 4c and 5c) and the uterus.

DISCUSSION

The present study clearly demonstrates that full information on the effects of different agents on preimplantation embryos cannot be obtained when evaluation of the resulting developmental abnormalities is performed at term only, since the damaged embryos rarely survive up to birth. We confirmed the finding of Brock and von Kreybig (1964) that after CPA treatment of pregnant rats during the preimplantation period the embryos die during organogenesis. In contrast to the earlier findings, treated embryos were not malformed and were retarded in development by about 24 h, as judged by the somite number and the dry weight of the embryos. After our treatment, even though it is performed before implantation, the embryos neither die during implantation nor survive to term, but survive up to organogenesis. The common view on drug action before implantation has, therefore, to be modified.

CPA was shown to have an effect on embryonic development prior to implantation, as revealed by the reduced cell number of the treated blastocysts. There are several reports on embryos that degenerated after in vivo treatment during the preimplantation period, e.g. when using X-rays (Russell, 1950) or a zinc-deficient diet (Hurley & Shrader, 1975). However, this is to our knowledge the first report on preimplantation embryos that appeared morphologically normal after maternal drug treatment but had a reduced cell number.

The embryo transplantations, and also the histological examination of the uteri of the pretreated recipients, demonstrated an effect of the alkylating drug on the decidual reaction of the uterus. The survival rate of pretreated blastocysts was also reduced at term. These results provide additional evidence for a direct effect of the drug or one of its metabolites on the embryo. On day 11 the histo-
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logical examination of the uteri of untreated hosts to which treated embryos had been transplanted point in the same direction, since the placentae show signs of retarded or abnormal development but the decidua is not affected. The present data give no indication whether the retarded development of the embryos is due to an action of the drug on the embryo or to secondary effects caused by the inhibited development of the decidua. The effects of the early treatment on later stages of pregnancy might be explained by a retarded clearance of the teratogenic agent from the blastocoele.

Investigations on the teratogenic effect of CPA during organogenesis indicate that the unmetabolized compound seems to be teratogenic (Gibson & Becker, 1968, 1971). This is in contrast to the alkylating activity of the drug, which is due to metabolites (Brock, 1967; Sladek, 1973).

Chemically induced chromosome aberrations in the mouse produce embryonic mortality in the pre- as well as in the postimplantation period (Basler, Buselmaier & Röhrborn, 1976). Malformations could not be found in these embryos during organogenesis, though chromosome anomalies were easily detectable. Since CPA is a very potent mutagenic agent (Röhrborn & Buckel, 1976), the abnormal development of our treated embryos could be caused by somatic mutations and subsequent chromosomal imbalance.

This work was supported by grants of the Deutsche Forschungsgemeinschaft awarded to the Sfb 29-Embryonale Entwicklung und Differenzierung. We thank Dagmar Nagel, Ursula Jakob, Imke Dillmann and Helga Stürje for their technical assistance, Barbara Steyn for her editorial assistance and Thomas KwasiGroch for his comments on the manuscript.

REFERENCES


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