Transient inhibition of DNA synthesis by methotrexate, in the rat embryo and foetus

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SUMMARY

Massive doses of methotrexate, a folic acid inhibitor, followed by folinic acid, the specific antagonist, have been used to produce a period in which the embryo and foetus are exposed to tetrahydrofolate deficiency with subsequent inhibition of DNA synthesis. The effects of this inhibition vary at different stages of gestation, and in late foetal life provide a useful method of inducing a delay in the appearance of vertebral body ossification centres. This defect is rapidly repaired, although there may be permanent sequelae. It is hoped that this technique will be useful in the study of cellular events in ‘catch-up’ growth.

INTRODUCTION

Methotrexate, a folic acid antagonist, interferes – among other actions – with the methylation of deoxyuridylate to form thymidylate. This inhibition is brought about by blocking the action of the enzyme dihydrofolate reductase and preventing the formation of tetrahydrofolate, the latter being the coenzyme (as 5,10-methylene tetrahydrofolate) in the conversion of deoxyuridylate to thymidylate. Tetrahydrofolate is also essential for the de novo synthesis of the purine moiety of inosinic acid, the precursor for adenylic and guanylic acid. Folinic acid (5-formyltetrahydrofolate) will counteract the inhibitions caused by methotrexate.

The use of massive doses of methotrexate, followed after an interval by folinic acid, allows the effects of transient, complete inhibition of DNA synthesis to be studied. This technique has been used in investigations of humoral and cellular immunity (Berenbaum & Brown, 1965; Berry, 1969). The present report concerns the effects on the rat embryo and foetus of varying periods of exposure to methotrexate followed by folinic acid. The study was undertaken to determine the length of time that the embryo is able to tolerate inhibition of DNA synthesis, and to examine effects on a developmental marker of a transient period of inhibition. If effective in the induction of developmental delay the technique would be a valuable tool in analysing ‘repair’ processes during foetal life.

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MATERIALS AND METHODS

Wistar rats, kept under standard conditions, were mated overnight. Day 1 of pregnancy was determined by a positive vaginal smear that morning. Injections of methotrexate were given by the intraperitoneal route; the dosage varied from 1 to 50 mg/kg. At varying periods after injection of methotrexate, folinic acid was injected subcutaneously in a dose eight times that of methotrexate, calculated on a molecular basis.

Relatively low doses of methotrexate (1–3 mg/kg maternal body weight) were used in an initial series of experiments with 12-day pregnant rats. Resorption rates were determined on killing on day 14. A similar series of animals was treated with saline injections, as controls. In addition the effect of simultaneous administration of folinic acid and methotrexate was examined.

Skeletal studies, with alizarin staining, were carried out on animals injected on days 17 and 18. Particular attention was directed to the ossification of vertebral centres, and a control series was studied to verify the time of appearance of these centres. There were three experimental groups. Animals injected on day 17 and day 18 were exposed to methotrexate for 8 h and then given folinic acid; 16 h after this they were killed, the viscera were removed and examined and alizarin staining of the skeleton carried out. A further group injected on day 18 were killed 8 h after methotrexate, without folinic acid injection. In a final experiment animals injected with methotrexate on day 17 and exposed to the drug for 8 h before folinic acid injection, were killed on day 21. The number of viable foetuses was determined, the viscera were removed and after separation of tissue components by the technique of Shibko et al. (1967) DNA content was determined spectrophotometrically, and protein by the method of Lowry, Rosebrough, Farr & Randall (1951). The skeletons were examined by alizarin staining.

RESULTS

Injections on day 12

Resorption rates, determined by killing on day 14, increased with the time interval between the injection of methotrexate and folinic acid; saline injections and simultaneous injection of methotrexate and folinic acid did not alter the normal resorption rate (4-6% of all foetuses in our colony at day 14). With doses in excess of 2.5 mg/kg all foetuses were found to be dead at 14 days, after 2 h exposure on day 12 (see Table 1).

Injections on day 17 and 18

The control series of non-injected alizarin-stained rats indicated the uniformity of behaviour of our strain and that studied by Strong (1925) who has documented the time of appearance of vertebral centres in the Wistar rat. Thus at 10 a.m. on day 18, nine thoracic centres (T 13–T 5) were present and four, or
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uncommonly five, lumbar vertebrae showed signs of ossification. On day 19, thirteen thoracic centres, six lumbar, two sacral and two caudal vertebrae were visualized by alizarin staining.

In the experimental series it was found that doses of methotrexate in excess of 3 mg/kg maternal body weight did not increase the degree of retardation of appearance of vertebral ossification centres. This suggests that 3 mg/kg has a maximum inhibitory effect, not increased by raising the dose further; for convenience a dose of 4 mg/kg maternal body weight was used in all experiments described below.

**Table 1. Resorption after exposure to methotrexate**

<table>
<thead>
<tr>
<th>Dose of methotrexate on day 12 (mg/kg maternal body weight)</th>
<th>No. of pregnancies tested</th>
<th>Resorption rate % on day 14 after 1, 2 or 4 h exposure to methotrexate</th>
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<tbody>
<tr>
<td>1</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>1.5</td>
<td>11</td>
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<tr>
<td>2.0</td>
<td>13</td>
<td>5.8</td>
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<tr>
<td>2.5</td>
<td>8</td>
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<tr>
<td>3.0</td>
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In animals injected with 4 mg methotrexate/kg maternal body weight and with 32 mg folinic acid/kg body weight 8 h later, there was a reduction in the number of vertebral ossification centres ossified, compared with controls, when the foetus was examined 16 h after the injection of the folic acid antagonist. Fifty-seven foetuses from animals treated on day 17 and killed on day 18 showed a mean loss of five centres/animal from the 13 centres expected (range 6–12 centres ossified in treated animals). This difference is highly significant by the Rank Sum Test. Treatment of day 18 with killing on day 19 was less effective in reducing ossification rates, a loss of 2.8 centres/animal from the 23 expected was found after examining 49 foetuses (range 16–23 centres ossified in treated animals). This difference is also significant (Rank Sum Test). In no instance were more than the expected number of centres found in either group of animals. On each day ossification occurred in some bifid vertebral body centres, in seven animals treated on day 17 and three treated on day 18 (see Figs. 1, 2).

That group of animals given methotrexate only, were injected when 13 vertebral centres were ossified. After 8 h, injected controls showed 16 ossified centres. In 23 test foetuses, 69 centres might therefore be expected to ossify in 8 h, in fact only 39 centres appeared. A further 14 centres were bifid or extremely small, and four were absent out of sequence, with a gap in the spinal column.

**Animals injected on day 17, killed on day 21**

These animals were injected with 4 mg/kg maternal body weight, followed 8 h later by 32 mg/kg folinic acid. There was no evidence of abnormal ossification
in 46 foetuses, all of which were alive. In these animals mean DNA and protein per g wet weight of liver did not differ significantly from normal – 2·8 mg/g test (range 2·69–2·86), 2·76 mg/g control (range 2·69–2·77) for DNA; 243·3 mg/g test (range 222–266·7 mg/g), 256 mg/g control (range 227–264 mg/g) for protein.

Fig. 1. Drawing of axial skeleton of normal animal, day 18. Alizarin staining, x 15.

Fig. 2. Drawing of axial skeleton of animal at 18 days having been exposed to methotrexate on day 17. Note bifid vertebral body centre. Alizarin staining, x 15.

Abnormalities

One foetus from an animal injected on day 18 and examined on day 19, showed an encephalocele. One further foetus in this group had a bifid thirteenth rib on the left-hand side.
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DISCUSSION

Thiersch (1954) demonstrated the protective effect of folinic acid in pregnant animals treated with folic acid antagonists (2,4-diaminopyrimidine derivatives). The use of a more readily reversible chemical agent, methotrexate, by Berenbaum & Brown (1965) enabled selective injury of a cohort of dividing cells to be used as a method of investigation of the humoral immune response, and of the formation of granulomata (Berry, 1969).

Methotrexate can be given as an LD100 and its inhibitory action on DNA synthesis counteracted by folinic acid. In this experiment total inhibition of DNA synthesis has been shown to have variable effects at different stages of gestation, as might be expected. In the early embryo, only short periods of interference with cell replication are tolerable.

Later, however, a selective period of inhibition may retard the appearance of certain developmental markers (vertebral body ossification centres have been studied in this instance) and may permanently affect their eventual form. It seems probable that the bifid centres seen in these experiments are indicative of reduced size of the vertebral body blastemata, since Griineberg (1963) points out that in genetically determined bone defects bifid vertebral centres occur as a result of reduction of the dorsiventral dimensions of the developing vertebral body.

The capacity of the foetus to repair an induced defect is evident from the fact that animals killed on day 21 showed no skeletal abnormality, and had organs with cell numbers within the normal range – as judged by chemical studies. This ‘catch-up’ ability has been demonstrated under other circumstances (Berry, 1970). It is hoped that the technique described here will permit the more detailed study of cellular events during such ‘repair’ situations.

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REFERENCES


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