DNA synthesis inhibition, cytotoxicity and their relationship to teratogenesis following administration of a nicotinamide antagonist, aminothiadiazole, to pregnant rats

By WILLIAM J. SCOTT¹, EDMOND J. RITTER¹ and JAMES G. WILSON¹

From the Children's Hospital Research Foundation and Departments of Pediatrics, Anatomy and Developmental Biology, College of Medicine, University of Cincinnati

SUMMARY

Four groups of Wistar rats were treated with 100 mg/kg aminothiadiazole (ATD) i.p. on day 12 of gestation. The first group was allowed to proceed to day 20, when they were sacrificed and the teratogenic effects analyzed in the surviving fetuses. A second group was given 100 mg/kg nicotinamide (NAM) i.p. simultaneously or at intervals of 4, 8 or 12 h following ATD. Third and fourth groups corresponded to the first and second except that [³H]thymidine was administered and one uterine horn removed for histological study or analysis of DNA synthesis rates. The following observations were made:

1. ATD proved teratogenic to 90% of the surviving fetuses, causing one or more malformations of organs such as limbs, tail, palate and heart.
2. Nicotinamide was capable of protecting the embryo against ATD teratogenicity. This effect diminished as the interval between ATD and NAM administration lengthened so that by 12 h little protection remained.
3. ATD produced a severe and prolonged inhibition of DNA synthesis, reaching a low of 12% of control level at 17 h and returning to near normal by 38 h.
4. NAM reversed this inhibition of DNA synthesis in proportion to the length of the interval between ATD and NAM administration.
5. ATD produces severe cytotoxicity especially in the limb buds and neural tube.
6. Based on this and earlier studies the hypothesis is proposed that the time of appearance of maximal cell death plays a major role in determining the degree and pattern of forelimb ectrodactyly.

INTRODUCTION

Previous reports from this laboratory (Ritter, Scott & Wilson, 1971; Scott, Ritter & Wilson, 1971) considered the relationship between the inhibition of DNA synthesis and the production of congenital malformations by cytosine arabinoside (ara-C) and hydroxyurea (HU). The initial biochemical lesion was assumed to be inhibition of DNA synthesis, and it was our original belief that

¹ Authors' address: College of Medicine, University of Cincinnati, Cincinnati, Ohio 45229, U.S.A.
the rate and type of malformation could be correlated with the degree and duration in the depression of DNA synthesis. The pattern of depression following teratogenically equivalent dosages of ara-C and HU was similar as also were the types and numbers of malformations produced. However, small but consistent differences in the types of malformations were evident, particularly as regards missing digits on the forelimbs; for example, HU produced absence of a single digit per forelimb while ara-C produced forelimbs usually missing more than one digit. It was possible to correlate the pattern of forelimb ectrodactyly with the time of appearance of maximal cell death, which led to the conclusion that the teratogenic effects of these agents were the result of a critical lack of cells produced by (1) reduced proliferative rate evidence by depressed DNA synthesis and (2) destruction of proliferative cells evidenced by visible cell death.

To further investigate the role of these factors we chose to examine the effects of a distinctly different class of drugs on embryonic DNA synthesis and development in the rat. Earlier studies had shown that 2-amino-1,3,4-thiadiazole (ATD) suppressed growth in proliferating systems (Burchenal & Dagg, 1956; Ciotti et al. 1960) and produced a pattern of congenital anomalies (Murphy, Dagg & Karnofsky, 1957; Beaudoin, 1971) similar to that seen after use of known inhibitors of DNA synthesis. The reversibility of the action of ATD by administration of nicotinamide (Krakoff, Lacon & Karnofsky, 1959; Oettgen, Reppert, Coley & Burchenal, 1960) makes this drug particularly attractive for the present study. This report will document the teratogenic, cytotoxic and DNA synthesis inhibiting properties of ATD and compare the results with those of previous studies in an attempt to clarify which effects may be involved in the inception of malformations.

METHODS

Pregnancy in rats of Royalhart stock, derived from a Wistar strain, was dated by counting time 0 as 9.00 a.m. on the morning on which sperm were found in the vaginal smears of females caged overnight with males of the same strain. Twelve days (288 h) later, pregnant females were injected i.p. with 100 mg/kg of ATD in aqueous solution. Other pregnant females, received, in addition to ATD, 100 mg/kg of nicotinamide i.p. simultaneously or at various intervals following ATD administration. Pregnancy was terminated on day 20, and implantation sites were counted in situ. Living fetuses were removed, examined for external malformations, weighed and fixed, either in 95 % ethanol preparatory to KOH clearing and alizarin-red staining for skeletal visualization, or in Bouin’s fluid preparatory to study of internal organs by the razor-blade sectioning technique (Wilson, 1965).

Additional females on day 12 of gestation were injected intraperitoneally with 100 mg/kg of ATD, followed by i.p. injection 1, 3, 6, 9, 15, 21, 27, 36, or 45 h later with 20 mg of cold thymidine and 200 μCi of [3H]thymidine per kg. Cold
DNA synthesis inhibition and cytotoxicity

thymidine was used in an attempt to spare maternal metabolism of the tritiated material. Another group of animals received 100 mg/kg of nicotinamide i.p. at 4, 8 and 12 h after ATD treatment and then were given thymidine as above.

Two hours after thymidine administration each rat was anesthetized with ether, a midline abdominal incision was made, and both uterine horns were exposed to view. Implantation sites were counted and one horn was returned to the abdominal cavity. The other horn was removed surgically after ligating its blood supply at the ovarian and cervical ends, and the abdominal wall was closed in routine fashion. Pregnancy was allowed to proceed until day 20, when females were killed, implantation sites in the remaining horn were counted, and living fetuses were examined.

The uterine horn removed surgically at intervals after ATD treatment was immediately opened, some embryonic vesicles were expelled into a dry Petri dish, and the embryos were dissected free from their membranes and rinsed in isotonic saline. Each embryo was carefully blotted with narrow strips of filter paper, weighed in a plastic test-tube, made up to 1.0 ml with cold distilled water, and sonicated at ice-bath temperature. Then 1.5 ml of 0.83 N perchloric acid (PCA) was added and, after standing for 10 min, the homogenate was centrifuged at 1500 rev/min for 5 min in an International PR-2. Supernatant fluid was removed and saved for determination of unincorporated radioactivity. The precipitate was resuspended in 1 ml of 0.3 N PCA followed by filtration through Whatman GF/C glass fiber filter papers on a Hoefer Model FH 101 24 mm filter, followed by two washes with 2.0 ml each of 0.3 N PCA. The filter paper carrying the acid-insoluble fraction was placed in a scintillation vial containing 1 ml of Soluene TM 100 (Packard) and allowed to digest for a minimum of 6 h at 37°C. After addition of a scintillation fluid, radioactivity was determined in a Packard TriCarb liquid scintillation spectrometer with automatic external quenching correction. To measure unincorporated [3H]thymidine, 0.1 ml of the supernatant fluid was prepared and counted as above.

To determine the cytotoxic effects of ATD one embryo from each extirpated uterine horn was immediately dissected from its membranes and placed in Bouin’s fluid, serially sectioned at 6 μm, and stained with hematoxylin and eosin.

RESULTS

The teratogenicity of ATD and the capability of nicotinamide to reverse this effect, both in intact animals and in those from which one uterine horn had been removed, is shown in Table 1. It is evident that ATD is highly embryotoxic to the 12-day rat embryo. The pattern of malformations observed resembled that produced by hydroxyurea and cytosine arabinoside, administered on day 12, consisting mainly of ectrodactyly, brachydactyly, syndactyly, cleft palate, diaphragmatic hernia, short and kinky tail, cardiac and aortic arch defects and anal atresia. Nicotinamide completely reverses the teratogenic effect when admini-
Table 1. Embryotoxicity following treatment with 100 mg/kg of aminothiadiazole on day 12 of rat gestation and its modification by 100 mg/kg of nicotinamide

<table>
<thead>
<tr>
<th>Agent</th>
<th>Total no. of implants</th>
<th>Condition of fetus at day 20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dead resorbed (%)</td>
</tr>
<tr>
<td>None</td>
<td>477</td>
<td>4</td>
</tr>
<tr>
<td>ATD alone</td>
<td>167</td>
<td>11</td>
</tr>
<tr>
<td>ATD + NAM-simult</td>
<td>77</td>
<td>5</td>
</tr>
<tr>
<td>ATD + NAM-4 h later</td>
<td>87</td>
<td>7</td>
</tr>
<tr>
<td>ATD + NAM-8 h later</td>
<td>56</td>
<td>7</td>
</tr>
<tr>
<td>ATD + NAM-12 h later</td>
<td>60</td>
<td>7</td>
</tr>
</tbody>
</table>

Thymidine injected and one uterine horn removed

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Dead resorbed (%)</th>
<th>Control wt. (%)</th>
<th>Survivors malformed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>158</td>
<td>3</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>ATD alone</td>
<td>91</td>
<td>25</td>
<td>69</td>
<td>91</td>
</tr>
<tr>
<td>ATD + NAM-4 h later</td>
<td>41</td>
<td>2</td>
<td>85</td>
<td>38</td>
</tr>
<tr>
<td>ATD + NAM-8 h later</td>
<td>61</td>
<td>11</td>
<td>77</td>
<td>60</td>
</tr>
<tr>
<td>ATD + NAM-12 h later</td>
<td>48</td>
<td>17</td>
<td>80</td>
<td>65</td>
</tr>
</tbody>
</table>

stered simultaneously with or 4 h after treatment with ATD in the routine teratological study. However, when thymidine treatment and partial hysterectomy were added to the regimen, this degree of protection after a 4 h interval was not observed. The 38% rate of malformation after thymidine injection and surgery is considerably at variance with the results of previous experiments in which these procedures accentuated embryotoxic effects only moderately. Some of the discrepancy may be attributed to the relatively small number of implantations examined in this group.

Intervals of 8 and 12 h between ATD and NAM treatment largely eliminated the protective effect of the latter compound, regardless of subsequent surgery and thymidine injection. Embryolethality (Table 1) showed a moderate rise above control values in both groups receiving ATD alone. Fetal weight was reduced to about 70% of control level in both ATD-treated groups, but the weight reduction was generally less when nicotinamide was added to the regimen.

Fig. 1 depicts rates of DNA synthesis as reflected by the amount of radioactivity in the perchloric acid insoluble fraction of embryos harvested 2 h after thymidine administration. Aminothiadiazole produced a marked depression of DNA synthesis that was evident 8 h after administration and was maximal at 17 h, with a gradual return toward normal by 38 h. As regards the interval between treatment and the appearance of significant depression, the results are quite unlike those produced by cytosine arabinoside (Ritter et al. 1971) and hydroxyurea (Scott et al. 1971). At comparable teratogenic doses the latter agents caused inhibition of DNA synthesis much more rapidly, the maximal
DNA synthesis inhibition and cytotoxicity

DNA synthesis was more severely depressed, and the rates of DNA synthesis when ATD was followed at 4, 8 or 12 hours after nicotinamide administration are also illustrated in Fig. 1. As with the teratological response, return of DNA synthesis to approximately normal levels was dependent on the length of the interval before nicotinamide was given. The amount of unincorporated radioactivity in the perchloric acid-soluble fraction of the homogenate was similar in control and in treated embryos, suggesting that ATD did not affect the amount of available thymidine.

Microscopic examination of embryonic tissues taken at various times after ATD treatment revealed delayed but severe cytotoxicity, especially in the limb-buds and neural tube. Pyknotic nuclei representing dead or dying cells were first evident 11 h following ATD administration. The number of dead cells was moderate at 17 h and was high by 23 h. By 29 h following ATD administration the number of pyknotic nuclei began to diminish and was no greater than in
controls by 36 h. Of special interest was the pattern of cell death in the forelimb bud as seen in Fig. 2. When compared to hydroxyurea and cytosine arabinoside, both of which produce more or less diffuse cytotoxicity throughout the limb bud, ATD produced severe necrosis localized mainly to the distal portion of the forelimb bud. Examination of the hindlimb bud revealed no such concentration of the effect, dead cells being scattered throughout. Localized cytotoxicity was also seen in the neural tube, where signs of cell death were noticeably more prevalent in the alar plate than in the basal plate. A similar distribution of cytotoxicity occurred in the neural tube after hydroxyurea and cytosine arabinoside treatment.

A previous paper (Scott et al. 1971) reported a correlation between the onset of cell death in the limb bud on day 12 and the degree of ectrodactyly in near-
Table 2. Correlation between time of maximal cell death seen after day-12 treatment and degree of forelimb ectrodactyly seen in 20-day fetus

<table>
<thead>
<tr>
<th>Drug</th>
<th>Interval between drug administration and appearance of maximal cell death (h)</th>
<th>Forelimbs missing one digit (%)</th>
<th>Forelimbs missing more than one digit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyurea</td>
<td>3</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td>Aminothiadiazole</td>
<td>23</td>
<td>50</td>
<td>13</td>
</tr>
<tr>
<td>Cytosine arabinoside</td>
<td>23–29</td>
<td>28</td>
<td>37</td>
</tr>
</tbody>
</table>

term fetuses. Comparable data obtained here after ATD treatment permit an extension of that correlation (Table 2). Actually ATD tends to occupy an intermediate position between hydroxyurea, which killed cells rapidly and produced absence of one digit per forelimb, and cytosine arabinoside, which killed cells more slowly but produced many fetuses lacking more than one digit per forelimb. The rear limb, known to differentiate later than the forelimb, reflects a similar pattern. Hydroxyurea, which kills cells soon after treatment, produced only 1% ectrodactyly in hindlimbs; ATD, which is cytotoxic after an intermediate interval, produced 12% ectrodactyly; while cytosine arabinoside, which is slowest to produce cell death, produced 39% of such defects of the hindlimb.

DISCUSSION

These data permit the direct conclusions that (1) ATD is teratogenic to the 12-day rat embryo, (2) nicotinamide is capable of reversing this teratogenic effect, (3) ATD inhibits embryonic DNA synthesis and (4) ATD kills embryonic cells after an appreciable latent period of 11–23 h.

These facts while interesting, attain real significance only to the extent that they can be applied to explaining how a particular congenital deformity is produced. Thus the data from this study in themselves offer little new information in regard to the question of how congenital deformities are produced. When compared with other drugs having similar effects, however, these data strengthen the earlier hypothesis concerning a pathway for the production of reduction deformity of the digits.

It was mentioned above that the pattern of forelimb ectrodactyly produced by ATD could be correlated with the time of appearance of maximal cell death. In sharp contrast, the duration and degree of DNA synthesis inhibition showed little relationship to the pattern of forelimb ectrodactyly. Fig. 3 compares the pattern of DNA synthesis inhibition following teratogenically equivalent doses of ATD, HU and ara-C. It can be seen that HU and ara-C produce a rapid and profound depression of DNA synthesis followed by a gradual return to control rates within 23–29 h. Surprisingly, in spite of this similarity in DNA synthesis
inhibition, the temporal pattern of cell death was distinctly different. Hydroxy-
urea produced massive cell death within 3–5 h while cytotoxicity from ara-C
was not evident before 9 h and did not reach a peak until 23–29 h. Aminothia-
diazole, on the other hand, produced a gradual depression in DNA synthesis
which did not become maximal until 17 h. Recovery was also gradual with the
return to near normal rates occurring at 38 h. Despite this difference in DNA
synthesis depression the temporal aspects of cytotoxicity produced by ATD
were very similar to those seen following ara-C, i.e. cell death first evident at
11 h, reaching a peak at 23 h.

This is not to say that reduced DNA synthesis plays no part in the production
of these defects. It appears, however, that the pronounced cytotoxic effect is the
common factor after treatment with HU, ara-C and ATD, and that it acts as the
major determinant in the production of forelimb ectrodactyly possibly by
causing a paucity of cells. Reduced proliferative rate, as evidenced by depressed
DNA synthesis, undoubtedly contributes to this paucity, and thus indirectly
increases the severity of the overall effect. Further evidence to strengthen this
concept was generated by administering HU on day 13 (Ritter et al. 1973) so
that the maximal expression of cytotoxicity would coincide temporally with that
produced by ara-C or ATD administered on day 12. In the latter study the
pattern of missing digits on the forelimbs resembled that produced by day-12
treatment with ara-C or ATD, i.e. absence of more than one digit per limb.

To assess critically the relative merits and faults of this hypothesis it will be
necessary to generate quantitative data of a more localized nature. For instance, information is needed on (1) the rate of DNA synthesis depression in the limb-bud as opposed to that in the whole embryo, (2) the actual proportions of cells dying in different locations, and (3) the comparative rates of repair or regeneration within the limb-bud following treatment with different drugs.

Other aspects of these experiments warrant mention but discussion will be held to a minimum until it is possible more fully to assess their significance. The first of these concerns the protective ability of nicotinamide when administered simultaneously with or 4 h following ATD. The latter is an unusually long interval when compared with other protection experiments and at least two explanations seem plausible at this time: (1) ATD may act slowly to produce its biochemical lesion (i.e. abnormal pyridine nucleotides) (Ciotti, Kaplan, Goldin & Vendetti, 1958) or (2) these abnormal nucleotides are easily reversible in the presence of nicotinamide.

Another area of interest concerns the more localized pattern of cytoxicity in the forelimb bud following ATD, a pattern distinctly different than that produced by HU and ara-C. The reason for this localization and whether it plays any role in determining the form of subsequent digital deformity is unknown. Finally it should once more be noted that the appreciable cell death found in the neural tube after all three agents was followed by little recognizable gross structural change in the central nervous system of the near-term fetus. In an earlier study with HU (Scott et al. 1971) it was postulated that either (1) developmental defect, possibly of a functional nature, may have been present but undetectable by our routine method of examination or (2) defects were truly absent owing to the capacity of the remaining proliferative cells at this stage of development to restore the population to a number compatible with normal development. In an attempt to evaluate these alternatives we have recently examined the behavioral profile of the offspring of rats treated with marginally teratogenic doses of hydroxyurea. A dose-related impairment in the ability of these rats to escape from a water-filled multiple T-maze was evident, suggesting that a functional neural deficit had been produced (Butcher, Scott, Kazmaier & Ritter, 1973). It is thought likely that ATD would effect similar changes.

The authors are grateful to Miss Jamie Campbell, Miss Rochelle Fradkin, Mrs Christine Grant, Mrs Denise Plegeman and Miss Joan Shields for indispensable technical assistance. These studies were supported by NIH grants HD 02792 and HD 05221.

REFERENCES


(Received 14 December 1972, revised 7 March 1973)