The susceptibility of the offspring of alloxan-diabetic mice to a teratogen

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Maternal diabetes, or pre-diabetes, has been considered as a possible factor in the aetiology of human congenital malformations by several investigators (De Costa, 1955; Hoet et al., 1960; Hiekkala & Koskenoja, 1961; Nicholson & De Maria, 1963; Pederson et al., 1964; Lenz & Maier, 1964). However, a few authors have reported no increase in the incidence of anomalies among infants of diabetic women (Given et al., 1950; Hall & Tillman, 1951; Rubin & Murphy, 1958). Alloxan-induced maternal diabetes in animals has also been studied, and an increase of stillbirth and foetal macrosomia has usually been recognized (Davis et al., 1947; Lindan & Morgans, 1950; Bartelheimer & Kloos, 1952; Angervall, 1959; Solomon, 1959; Kim et al., 1960; Love et al., 1960; Koskenoja 1961). The incidence of malformations, however, was not increased in most cases, the exception being those reported by Ross & Spector (1952), Fujimoto et al. (1958) and Watanabe & Ingalls (1963).

Recently, Runner & Dagg (1960) and Wilson (1964a, b) suggested that an interaction of multiple environmental factors might be important and that a potentiation or reinforcing effect might occur between two or more agents in regard to teratogenesis. In this respect there have been several animal experiments in which the influence of a maternal factor on the action of a certain teratogen was investigated. A hypocaloric diet was shown to have a synergistic effect on the teratogenicity of cortisone (Kalter, 1960); simultaneous administration of certain vitamins or hormones influenced the activity of chemical teratogens such as cortisone (Stream & Peer, 1956; Nishihara et al., 1960), vitamin A (Millen & Woolam, 1958), urethan (Nishimura, 1961) or triethylene thiophosphoramide (Tanimura, 1962). Most of those factors were short acting, and do not correspond to such chronic modification of metabolic states as occurs in humans.

The present study is an attempt to investigate the susceptibility of the foetuses

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in alloxan-diabetic mice to a known teratogen, as well as the effect of maternal alloxan treatment, itself, on the development of the offspring.

MATERIALS AND METHODS

Japanese colony bred ddS stock mice maintained at Animal Center, Kyoto University were used. Nulliparous females at 12 to 17 weeks of age were placed in the evening with mature males from the same colony. Copulation was checked by the presence of a vaginal plug next morning. The day when the vaginal plug was found was designated as 0 (zero) day of gestation. On the 8th day of gestation, pregnancy of the animals was ascertained by inspection of the vaginal smear. Animals were kept on the diet of pellets N.M.F. (Crude protein, 26·5 per cent.; crude fat, 6·1 per cent.; crude fibre, 4·1 per cent. N.F.E., 49·8 per cent.; Total ash, 6·5 per cent.) supplied by Oriental Co., Tokyo, and fresh tap water ad libitum. The pregnant animals were treated with either or both of the two chemical compounds being tested.

A preliminary experiment showed that intraperitoneal injection of 4 per cent. solution of alloxan at 200 mg. per kg. of body weight induced an increased blood sugar level and glucosuria within 2 days in most animals. In the main experiment this dose was injected once, immediately after the preparation of the solution, intraperitoneally to about half the available pregnant mice either on the 8th or 9th day of gestation.

As the teratogen, triethylene thiophosphoramide (thio-TEPA) was used: its potent teratogenicity to mouse foetuses had been ascertained in our laboratory (Tanimura & Nishimura, 1962). The agent was administered by a single intraperitoneal injection at 5·0 or 2·5 mg. per kg. of body weight in 0·1 or 0·05 per cent. solution in physiological saline to about two-thirds of the alloxan-treated and a similar number of non-treated pregnant mice, on the 10th or 11th day.

A further twenty-five animals were used as controls without any treatment. The pregnant mice were sacrificed on the 18th day of gestation and the foetuses were examined for intrauterine death and for external deformities.

In a separate investigation of placental transmission of thio-TEPA, an additional experiment using 32P-labelled thio-TEPA was carried out. Alloxan (200 mg./kg., i.p.) was injected into twenty pregnant mice on the 9th day. Twenty non-treated pregnant mice were used as controls. Freshly prepared aqueous solutions of the labelled compound were injected intraperitoneally to both groups of pregnant mice on the 11th day at 5·0 mg. per kg. of body weight. Five animals of the treated and five of the control group were sacrificed at times, 1½, 3, 6 and 24 hr. after the injection, and two live foetuses and their placentae were removed from each litter at random. After finding the fresh weight of each foetus and placenta, they were homogenized and diluted to 0·5 ml. with distilled water. One-fifth, i.e. 0·1 ml., of the sample was put on a
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sample dish and desiccated. The radioactivity of each sample was counted by a gas flow counter (Shimadzu Seisakusho Ltd., Kyoto). The specific activity of the labelled thio-TEPA was about 6.95 mc. per g. at the time of its injection and 5.20 mc. per g. at the time of counting.

RESULTS

All the mice survived until the 18th day of gestation, when they were sacrificed. All uteruses were found to have live foetuses or nodes of placenta. The results are summarized in Tables 1, 2 and 3.

Effect of alloxan

The mortality rates of the foetuses were 12.9 per cent. in group 1e and 9.7 per cent. in group 2e respectively (Tables 1, 2). The former rate is significantly higher than that in the normal control \( N(p<0.01) \), but the latter is not significantly higher.

The incidence of malformation in surviving foetuses was 7.1 per cent. (ten cases) in group 1e, and of these three showed polydactylyia and seven had clubfeet. In group 2e, 12.2 per cent. (seventeen cases) were malformed of which one showed oligodactylia, thirteen clubfeet, and three cleft palates. Both of these incidences are significantly higher than those in the control without treatment \( (p<0.01) \).

The average body weight of living foetuses in the group 1e was slightly lower than that in the control \( N \) (\( p<0.05 \)). In group 2e, however, the foetal weight was almost the same as that in the control \( N \).

Effect of thio-TEPA upon normal animals

Although not statistically significant, the foetal mortality rate in groups 1c and 2c tended to be higher than that in the control \( N \). In groups 1d and 2d such tendencies were not shown.

The incidences of malformed foetuses among survivors were significantly higher in every experimental group than in the normal control. In regard to the types of malformation, polydactylyia of the hindfeet was the most common in the group 1c and 1d. In the experimental groups 2c and 2d such digital malformations were not shown, but clubfoot was a common abnormality.

The average body weight of live foetuses was significantly lower in the experimental groups than in the controls, especially when treated on the 9th day.

Effect of thio-TEPA upon animals pre-treated with alloxan

The foetal mortality rate was remarkably high in the injected groups with pre-treatment: the rates were from 23 to 38 per cent. In all these experimental
### TABLE 1
Effect of intraperitoneal injection of thio-TEPA at 10 days of gestation on the foetuses of mice with or without pre-treatment at 8 days of 200 mg./kg. alloxan

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-treatment with alloxan</th>
<th>Dose of thio-TEPA (mg./kg.)</th>
<th>No. of mice</th>
<th>Total implants</th>
<th>Dead* (%)</th>
<th>Malformed† (%)</th>
<th>Weight (alive) average ± Sx (range) (g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>+</td>
<td>5.0</td>
<td>19</td>
<td>158</td>
<td>38.0</td>
<td>52.1</td>
<td>0.957 ± 0.014 (0.74–1.25)</td>
</tr>
<tr>
<td>1b</td>
<td>+</td>
<td>2.5</td>
<td>23</td>
<td>201</td>
<td>25.4</td>
<td>47.3</td>
<td>1.020 ± 0.016 (0.61–1.62)</td>
</tr>
<tr>
<td>1c</td>
<td>—</td>
<td>5.0</td>
<td>25</td>
<td>204</td>
<td>9.3</td>
<td>11.9</td>
<td>1.136 ± 0.008 (0.74–1.33)</td>
</tr>
<tr>
<td>1d</td>
<td>—</td>
<td>2.5</td>
<td>19</td>
<td>164</td>
<td>5.5</td>
<td>13.5</td>
<td>1.161 ± 0.011 (0.82–1.64)</td>
</tr>
<tr>
<td>1e</td>
<td>+</td>
<td>—</td>
<td>20</td>
<td>162</td>
<td>12.9</td>
<td>7.1</td>
<td>1.119 ± 0.021 (0.61–1.74)</td>
</tr>
<tr>
<td>N</td>
<td>—</td>
<td>—</td>
<td>25</td>
<td>210</td>
<td>4.8</td>
<td>1.0</td>
<td>1.185 ± 0.008 (0.84–1.60)</td>
</tr>
</tbody>
</table>

* Percentage of total implants. † Percentage of total living foetuses.

### TABLE 2
Effect of intraperitoneal injection of thio-TEPA at 11 days of gestation on the foetuses of mice with or without pre-treatment at 9 days of 200 mg./kg. of alloxan

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-treatment with alloxan</th>
<th>Dose of thio-TEPA (mg./kg.)</th>
<th>No. of mice</th>
<th>Total implants</th>
<th>Dead* (%)</th>
<th>Malformed† (%)</th>
<th>Weight (alive) average ± Sx (range) (g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>+</td>
<td>5.0</td>
<td>21</td>
<td>173</td>
<td>25.4</td>
<td>34.1</td>
<td>0.938 ± 0.021 (0.42–1.33)</td>
</tr>
<tr>
<td>2b</td>
<td>+</td>
<td>2.5</td>
<td>21</td>
<td>177</td>
<td>23.0</td>
<td>17.6</td>
<td>1.123 ± 0.009 (0.63–1.43)</td>
</tr>
<tr>
<td>2c</td>
<td>—</td>
<td>5.0</td>
<td>20</td>
<td>178</td>
<td>7.3</td>
<td>13.3</td>
<td>1.043 ± 0.010 (0.70–1.35)</td>
</tr>
<tr>
<td>2d</td>
<td>—</td>
<td>2.5</td>
<td>20</td>
<td>163</td>
<td>4.3</td>
<td>9.6</td>
<td>1.105 ± 0.012 (0.82–1.47)</td>
</tr>
<tr>
<td>2e</td>
<td>+</td>
<td>—</td>
<td>20</td>
<td>154</td>
<td>9.7</td>
<td>12.2</td>
<td>1.164 ± 0.011 (0.80–1.55)</td>
</tr>
<tr>
<td>N</td>
<td>—</td>
<td>—</td>
<td>25</td>
<td>210</td>
<td>4.8</td>
<td>1.0</td>
<td>1.185 ± 0.008 (0.84–1.60)</td>
</tr>
</tbody>
</table>

* Percentage of total implants. † Percentage of total living foetuses.
TABLE 3

Incidence of malformations induced by thio-TEPA injection with or without alloxan pre-treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Day of pre-treatment with alloxan</th>
<th>Dose of thio-TEPA (mg./kg.)</th>
<th>No. of live foetuses</th>
<th>Foetuses* with malformed digits (%)</th>
<th>Type and number of digital malformations†</th>
<th>Foetuses* with clubfoot (%</th>
<th>Foetuses* with cleft palate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>8</td>
<td>5.0 (day 10)</td>
<td>98</td>
<td>45.9</td>
<td>8.0:1.0:9.0:33:10:14.3:2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>8</td>
<td>2.5 (day 10)</td>
<td>150</td>
<td>36.7</td>
<td>21.1:1.0:4.0:55:19:11.3:2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1c</td>
<td>—</td>
<td>5.0 (day 10)</td>
<td>185</td>
<td>7.0</td>
<td>0.0:0.0:1.0:15:1:4.9:0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1d</td>
<td>—</td>
<td>2.5 (day 10)</td>
<td>155</td>
<td>8.4</td>
<td>0.0:0.0:0.0:0.0:15:1:5.1:0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1e</td>
<td>8</td>
<td>—</td>
<td>141</td>
<td>2.1</td>
<td>0.0:0.0:0.0:3:0:5.0:0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>9</td>
<td>5.0 (day 11)</td>
<td>129</td>
<td>24.0</td>
<td>40.50:2.4:0.0:0:10:0:6.2:13.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td>9</td>
<td>2.5 (day 11)</td>
<td>136</td>
<td>10.3</td>
<td>4.11:9.3:0.2:0.0:15:1:5.1:2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2c</td>
<td>—</td>
<td>5.0 (day 11)</td>
<td>165</td>
<td>2.4</td>
<td>0.0:2.4:0.0:0:0:0:0:0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2d</td>
<td>—</td>
<td>2.5 (day 11)</td>
<td>156</td>
<td>0</td>
<td>0.0:0.0:0.0:0.0:0:0:0:0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2e</td>
<td>9</td>
<td>—</td>
<td>139</td>
<td>0.7</td>
<td>0.1:0.0:0.0:0.0:0:9.3:2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>—</td>
<td>—</td>
<td>200</td>
<td>0</td>
<td>0.0:0.0:0.0:0.0:0:1.0:0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Percentage of total living foetuses.
† Bd, Brachydactylia; f, forefeet; h, hindfeet; Mdh, Macrodactylia at hindfeet; Od, oligodactylia; Pdh, polydactylia at hindfeet; Sd, Syndactilia; ±, with or without.

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groups (1a, 1b, 2a, 2b) the rates exceeded the sum of the rates in the corresponding groups to which either alloxan or thio-TEPA alone were administered.

It is interesting that the tendency to potentiation was also clearly shown in the incidence of malformations. As shown in Tables 1 and 2, the percentage of malformed foetuses among the survivors in the groups to which both agents were applied significantly exceeded the sum of the percentages shown in the groups to which one agent alone was administered, except in the group 2b. In this group, however, a conspicuous potentiation was revealed in regard to the incidence of digital malformations as shown in Table 3. Potentiation was not
consistently seen in respect of the incidences of clubfoot or of cleft palate. As far as the malformed digits are concerned, a high susceptibility to teratogenesis is presumed in the foetuses of all groups pre-treated with alloxan.

The growth of the surviving foetuses was severely suppressed in groups 1a and 2a, where 5.0 mg. per kg. body weight of thio-TEPA was applied after the alloxan pre-treatment.

**Placental transmission of labelled thio-TEPA**

Ten foetuses and placentae at a time were examined for their radioactivity in each group. Text-fig. 1 represents the mean value of counts per min. per mg. of wet weight.

Noticeable radioactivity was already shown in both groups 1 ½ hr. after the administration of the labelled agent and the amount in the embryos was significantly higher than that in the placentae. However, no significant difference of the radioactivity either in foetuses or in placentae was shown between the pre-treated and the control group.

**DISCUSSION**

The following adverse effects on foetuses of alloxan diabetes in pregnant mothers have earlier been reported: increase of intrauterine or neonatal death, abnormal growth of surviving foetuses, and the induction of congenital malformations.

High foetal mortalities were reported in mice (Koskenoja, 1961; Watanabe & Ingalls, 1963) and in rats (Davis et al., 1947; Bartelheimer & Kloos, 1952; Angervall, 1959) in which alloxan diabetes was induced during pregnancy. The lethal effect to offspring was also recognized when the alloxan treatment had occurred before pregnancy in rabbits (Fujimoto et al., 1958) and in rats (Lindan & Morgans, 1950; Kim et al., 1960; Lawrence & Contopoulos, 1960; Love et al., 1960). Lazarow et al. (1960) reported that the foetal mortality increases even in sub-diabetic rats to as much as three times the normal control level. Love et al. (1960) in rat and Koskenoja (1961) in mice, however, did not find such tendencies in pre-diabetic or mildly diabetic animals.

Overweight has been reported in the live born of mother animals with patent diabetes (Angervall, 1959) or with mild diabetes or sub-diabetes (Solomon, 1959; Lazarow et al., 1960; Koskenoja, 1961). Kim et al. (1960) and Koskenoja (1961) recognized that the variation in body weights of the offspring of diabetic animals was wider than that in the controls, although a similar average body weight was shown. In other words, the frequency of neonatal overweight might be higher in the offspring of diabetics.

As regards congenital malformations induced by maternal alloxan diabetes, Bartelheimer & Kloos (1952) found only two anomalies (short tail and cataract).
among fifty-nine living newborn rats whose mothers had been given alloxan at various stages of pregnancy. Fujimoto et al. (1958) found ten cases of malformed foetuses, mostly affected in the central nervous system, among ninety-three live-born from alloxan-diabetic rabbits. In mice, Ross & Spector (1952) reported that the incidence of malformed foetuses was 29 per cent. from mothers which had received alloxan on three successive days during pregnancy, and most of the malformed foetuses were likely to have died by the time of observation. However, Koskenoja (1961) found only a few cases with zonular cataract from alloxan-diabetic mice. Recently, Watanabe & Ingalls (1963) treated female Phipps mice with a single injection of alloxan between 8·5 and 13·5 days after copulation and found a fairly high incidence of malformed foetuses: 21·6 per cent. and 12·9 per cent. of live foetuses were deformed after treatment at 8·5 and 9·5 days after copulation respectively. The 8·5 days after copulation in their report corresponds to the 8th day in our usage.

In comparison with the reports mentioned above, the foetal mortality in our results with alloxan-treated mice was lower, and the mean weight of living foetuses was only slightly affected. The frequency of malformed foetuses was, however, significantly increased. The low incidences of congenital malformations in most of the former reports might be due to the fact that the offspring were only examined after delivery.

The raised susceptibility of alloxan-pre-treated mice to teratogenesis by thio-TEPA might make one suspect that the metabolism of thio-TEPA in them and/or the permeability of their placentae to the agent were modified by the induced diabetes of their mothers and that the amount of the agent transferred to the foetuses in diabetic mothers, was larger than in the control animal. In this respect, Dixon et al. (1961) have found that diabetes induced by alloxan decreases the activity of certain hepatic drug-metabolizing enzymes in rats. However, the concentration of thio-TEPA in foetuses and in placentae from alloxan-pre-treated mothers was almost the same as that in the control so far as our present research using 32P-labelled thio-TEPA was concerned. Therefore, it might be assumed that the foetuses in both groups were equally exposed to thio-TEPA. Considering also the fact that the types of deformity induced by both treatments were similar to those caused by the single administration of thio-TEPA, it seems likely that the susceptibility of the foetuses to the agent was intensified in the alloxan-diabetic mice.

Furthermore, it might be suggested that high incidence of foetal death and of malformations in diabetic mothers ascertained by several investigators is due to the combined effects of intensified susceptibility and of unknown exogenous factors.

**SUMMARY**

1. Pregnant ddS stock mice were treated in three ways: the first group of animals was given a single intraperitoneal injection of 200 mg. of alloxan per kg. of body weight on the 8th or 9th day of gestation; the second group was
given 2·5 mg. or 5·0 mg. of thio-TEPA per kg. of body weight on the 10th or 11th day of gestation; the third group received both treatments with alloxan and with thio-TEPA at the same times as the first and the second group. The fourth, untreated, group was used as the control. The foetuses from 233 pregnant mice of all groups were examined near term.

2. After alloxan treatment alone a significantly raised incidence of gross deformities, including clubfoot, cleft palate, polydactylia and oligodactylia, was observed, while the foetal mortality rate and the mean weight of live foetuses were only slightly affected in comparison with the normal control.

3. After successive administration of the two agents, the incidence of digital malformations and of foetal death significantly exceeded the sum of the effects of their separate actions, and the mean weight of surviving foetuses was notably diminished.

4. In a supplementary experiment to examine placental transmission of \( {\text{32P-labelled thio-TEPA}}, \) detectable radioactivity was found in foetuses and placentae 1½ and 3 hr. after the administration of the labelled agent both in the alloxan-pre-treated mothers and in the control animals. However, no significant difference in the level of radioactivity either in foetuses or in placentae was shown between the pre-treated and the control group.

RÉSUMÉ

Sensibilité à un agent tératogène de la descendance de souris rendues diabétiques par injection d'alloxane

1. Des Souris gravides de souche ddS ont été soumises à trois traitements différents. Les animaux d'un premier groupe ont reçu en une seule injection intrapéritonéale 200 mg. d'alloxane par kg. de poids au 8ème ou 9ème jour de la gestation. Dans le second groupe, les Souris ont reçu 2,5 ou 5 mg. de thio-TEPA par kg. au 10ème ou 11ème jour de la gestation. Les animaux du troisième groupe ont subi les deux injections, alloxane et thio-TEPA, en même temps que les premier et deuxième groupes. Un quatrième groupe témoin est formé d'animaux non traités.

Les foetus de 233 Souris gestantes des différents groupes ont été examinés peu avant la naissance.

2. Après le seul traitement à l'alloxane, on a constaté une augmentation significative de graves malformations, telles que pieds-bots, fentes palatines, polydactylie et oligodactylie. En revanche, le taux de mortalité foetale et le poids moyen des foetus vivants sont peu affectés par rapport aux témoins.

3. La fréquence des malformations des doigts et de la mortalité foetale après injection successive des deux agents est significativement supérieure à la somme des effets obtenus avec l'un et l'autre des agents. Le poids moyen des foetus vivants est sensiblement diminué.

4. Une autre série expérimentale a été faite pour examiner la transmission
placentaire du thio-TEPA marqué au $^{32}$P. On a décelé de la radioactivité dans les foetuses et les placentas 1½ et 3 hr. après administration de l'agent marqué, aussi bien chez les mères traitées préalablement à l'alloxane que chez les témoins. Toutefois, on n'a observé aucune différence significative dans le taux de radioactivité présenté par les foetus ou les placentas dans les groupes témoin et traités.

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