Preimplantation development of gynogenetic diploid mouse embryos

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SUMMARY

Diploid gynogenetic mouse embryos were produced in a three-step procedure: fertilization in vitro, suppression of the 2nd polar body formation by Cytochalasin B, and microsurgical removal of the male pronucleus. The operated eggs were transplanted to the oviduct of recipient females for 72 or 96 h. The overall recovery rate was 73%, but compacted morulae and blastocysts constituted only 28.6% of transplanted eggs. After 72 h blastocysts were rare (3.5%) but 24 h later their incidence increased to 21.2%. In eggs homozygous for T6 chromosome it was possible to prove karyologically that the male pronucleus was effectively removed and that the diploid genome was of purely maternal origin.

INTRODUCTION

Diploid gynogenetic and androgenetic development in the mouse has been recently obtained by microsurgical removal of one of the pronuclei followed by diploidization by suppression of the 1st or 2nd cleavage division (Markert & Petters, 1977; Hoppe & Illmensee, 1977; Modliński, 1980). Because diploidization was achieved by suppression of the mitotic rather than meiotic division, the embryos and adults were fully homozygous.

The diploid gynogenetic embryos described in this paper have been obtained differently, namely by removal of the male pronucleus from the fertilized eggs in which the formation of the second polar body has been suppressed. From the genetic point of view the embryos produced in this way resemble those parthenogenones whose diploidy is also restored by the inhibition of the second meiotic division. Contrary to embryos diploidized during cleavage and thus fully homozygous, both gynogenones and parthenogenones diploidized by suppression of the 2nd polar body retain the heterozygosity of the mother in those loci that have undergone crossing-over. This creates the opportunity of using them for gene mapping (see Discussion). While genetically similar, the diploid gynogenones differ from parthenogenones in that they originate from

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fertilized rather than artificially stimulated eggs. Thus any differences in their developmental potential can be attributed only to the mode of egg activation (sperm versus artificial agent).

MATERIALS AND METHODS

CBA-T6T6 and Swiss albino females from an outbred colony were induced to ovulate by intraperitoneal injection of 10 i.u. of PMSG followed by 5 i.u. of HCG 48 h later.

Fertilization in vitro was carried out in Whittingham's medium containing 32 mg of bovine serum albumin per 1 ml (Fraser & Drury, 1975). Aliquots of 1 ml of medium were kept overnight in embryological watch glasses under liquid paraffin, at a temperature of 37-5 °C in an atmosphere of 5% CO₂ in the air. Sperm was obtained from caudae epididymes of adult F₁ (C57BL × CBA-H) males. Before mixing the gametes, the sperm suspension was pre-incubated for 1 h at 37-5 °C. Sixteen hours after the HCG injection the eggs were released from ampullae directly into the fertilization medium. A small amount of sperm was added to eggs but no effort was made to estimate the concentration of the sperm suspension. Two hours after mixing the gametes, eggs were transferred to Whitten's medium (Whitten, 1971) containing 10 μg of Cytochalasin B (CB)/ml. All subsequent manipulations were carried out in this medium.

Monospermic eggs containing three pronuclei were selected for operation at the stage when the pronuclei were clearly visible, but were yet of small or medium size (about 3–5 h after mixing the gametes). Micromanipulations were performed with the help of Carl Zeiss micromanipulator under an Ergaval (Carl Zeiss Jena) microscope (magnification 400 x). The eggs were operated on in a hanging drop of medium in a chamber filled with liquid paraffin. The male pronucleus was removed by the technique of Modliński (1975), i.e. by moving the pipette to the pronucleus and by pulling out the pronuclear membrane by suction (Figs. 1 and 2).

Five hours after transferring eggs to Whitten's medium containing CB the operated eggs were washed thoroughly in CB-free medium and cultured for another 1 h before being transplanted to the oviduct of cycling mice (Tarkowski, 1959a). To prevent possible passage of eggs into the uterus and their loss, the oviduct was ligated at the tubouterine junction.

72 or 96 h after transplantation the eggs were recovered from the ligated oviduct and classified in the living state. Morulae and blastocysts which had developed from CBA-T6T6 eggs were processed for air-dried preparations (Tarkowski, 1966). In addition, chromosomal preparations were made from a small number of eggs at the time of the first cleavage division.
Table 1. In vitro fertilization of Swiss albino and CBA-T6T6 eggs with F1 (C57BL x CBA-H) sperm and suppression of the second meiotic division with Cytochalasin B

<table>
<thead>
<tr>
<th>Origin of eggs</th>
<th>Total number of eggs</th>
<th>Fertilized eggs (%)</th>
<th>2nd polar body suppressed</th>
<th>Monospermic eggs with 3 pronuclei (%)</th>
<th>Polyspermic eggs (%)</th>
<th>2nd PB present Monospermic eggs with 2 pronuclei (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swiss albino</td>
<td>840</td>
<td>601 (71.5)</td>
<td>458 (76.2)</td>
<td>93 (15.5)</td>
<td>50</td>
<td>93 (15.5)</td>
</tr>
<tr>
<td>CBA-T6T6</td>
<td>251</td>
<td>221 (88.0)</td>
<td>172 (77.8)</td>
<td>27 (12.2)</td>
<td>22</td>
<td>27 (12.2)</td>
</tr>
</tbody>
</table>

RESULTS

Over 1000 eggs were used in this study. The fertilization rate was 71% in Swiss albino eggs and 88% in CBA-T6T6 eggs. 14.6% of fertilized eggs were polyspermic, i.e. contained 4 or more pronuclei.

Formation of the second polar body (2PB) was suppressed in about 90% of eggs. In the remaining eggs the 2PB had probably been extruded in the fertilization medium, i.e. before they were transferred to Whitten's medium containing CB. Altogether over 75% of fertilized eggs were suitable for producing diploid gynogenones i.e. contained three pronuclei (Table 1). In these eggs two pronuclei were situated closely together at the egg periphery and were scored as female pronuclei while another pronucleus was situated apart from them but also near the egg surface and this was scored as male pronucleus (Fig. 3). In some eggs the male pronucleus was larger and possessed more nucleoli than the female pronuclei.

The survival rate of operated eggs was 66% in Swiss albino eggs and 75% in CBA-T6T6 eggs (Table 2). Degeneration usually occurred immediately after enucleation (during the first 10 minutes) and was a consequence of damaging the cell membrane at the site of withdrawal of the male pronucleus. Only those eggs from which the whole male pronucleus was removed were transplanted to recipient females (Fig. 4); eggs in which pronuclear membrane disrupted during the operation were discarded (Table 2).

A dozen of CBA-T6T6 gynogenetic eggs were examined karyologically at the time of the first cleavage division and all proved to contain the diploid set of chromosomes of maternal origin only (two T6 marker chromosomes) (Figs. 6, 7).

The overall recovery rate from the ligated oviducts was 73.6%, but compacted morulae and blastocysts constituted only 28.6% of all transplanted eggs. The remaining eggs were degenerated or fragmented or arrested in
### Table 2. Development of diploid gynogenetic embryos

<table>
<thead>
<tr>
<th>Origin of eggs</th>
<th>Operated eggs</th>
<th>Survived (%)</th>
<th>No. of transplanted eggs</th>
<th>Recovered eggs</th>
<th>Degenerated in cleavage or fragmented</th>
<th>Total</th>
<th>No. of transplanted eggs</th>
<th>Recovered eggs</th>
<th>Degenerated in cleavage or fragmented</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swiss albino</td>
<td>300</td>
<td>198 (66.0)</td>
<td>105</td>
<td>5</td>
<td>30</td>
<td>21</td>
<td>17</td>
<td>73</td>
<td>69</td>
<td>13</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>CBA-T6T6</td>
<td>65</td>
<td>49 (75.4)</td>
<td>35</td>
<td></td>
<td>8</td>
<td>5</td>
<td>6</td>
<td>19</td>
<td>11</td>
<td>4</td>
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Figs. 1 and 2. Microsurgical removal of the male pronucleus from a monospermic tri-pronucleate egg. ×230.

Fig. 1. The pronucleus is sucked to the tip of the micropipette.

Fig. 2. The pronuclear membrane is pulled out through the plasma membrane and the zona pellucida.

Fig. 3. A tri-pronucleate monospermic Swiss albino egg before the operation. Two female pronuclei situated closely together and near the egg surface (top); the male pronucleus lies on the opposite side of the egg (bottom). Differential interference contrast; ×360.

Fig. 4. The same egg as in Fig. 3 after removal of the male pronucleus. Differential interference contrast; ×360.

Fig. 5. Gynogenetic Swiss albino blastocyst recovered 96 h after transplantation to the oviduct of one-cell operated eggs such as the one shown in Fig. 4. ×400.

Fig. 6. Diploid plate of chromosomes from a gynogenetic CBA-T6T6 egg entering the first cleavage division. ×350.

Fig. 7. Detail of chromosome spread shown in Fig. 6 – two T6 chromosomes (arrows). ×1000.
cleavage at the best at the 6-cell stage. After 72 h the majority of the developing embryos were compacted morulae and only five embryos were early blastocysts. After 96 h the picture was reversed: out of 20 developing embryos only three were morulae and others were medium or late blastocysts (Table 2, Fig. 5). The majority of blastocysts were still enclosed in the zona pellucida.

CBA-T6T6 morulae recovered after 72 h were composed of 34 cells on average (range 24–50 cells); the four available CBA-T6T6 blastocysts flushed from the oviducts after 96 h were composed respectively of 38, 49, 52, and 84 cells. Swiss albino embryos were inspected only in the living state and re-transplanted to another recipient in order to evaluate their developmental potential after implantation; the results of these experiments will be described later.

DISCUSSION

In order to produce diploid gynogenetic embryos and adults it is necessary to eliminate the sperm nucleus from the fertilized egg and to restore diploidy. The first step has been achieved either by using for fertilization irradiated homologous sperm or non-irradiated heterologous sperm (amphibia: Volpe & Dasgupta, 1962; Volpe, 1970; Trottier & Armstrong, 1976; Tompkins, 1978; Gillespie & Armstrong, 1979) or by microsurgical removal of the male pronucleus (mammals: Markert & Petters, 1977; Hoppe & Illmensee, 1977; Modliński, 1980; this paper). The second step, i.e. diploidization, can be achieved either by suppression of the 2nd meiotic division (amphibia: papers referred to above; mammals – this paper) or first or second cleavage division (mammals: papers referred to above).

The genetic implications of diploidization at mitosis and meiosis are not, however, identical. While suppression of a cleavage division in haploid eggs leads to a complete homozygosity, suppression of the 2nd polar body in eggs originating from the heterozygous mother results in organisms which may be either heterozygous or homozygous depending on whether or not the locus in question has undergone crossing-over in the first meiotic division. By determining the frequency of homozygotes and heterozygotes one can map the distance of particular gene locus from the centromere. This method of gene mapping has been used with success in gynogenetic leopard frog (Volpe, 1970), and recently in ovarian teratomas of parthenogenetic origin in the mouse (Eicher, 1978). However, since diploid parthenogenetic embryos in the mouse do not survive till birth in utero, and in the ovary they give rise to teratomas (or teratocarcinomas) rather than to normal foetuses, the method cannot be applied to mapping genes which are expressed in postnatal life and/or affect morphological characters. The only way to overcome these limitations is to obtain viable gynogenones of the genetic constitution similar to that of parthenogenones. The experiments described in this report represent the first step toward achieving this goal.
The method which we have developed to obtain diploid gynogenones has proved satisfactory: 75% of eggs fertilized in vitro and subsequently subjected to CB were tri-pronucleate monospermics, i.e. suitable for producing diploid gynogenetic embryos. About 70% of the operated eggs survived removal of the male pronucleus. Nearly 30% of the operated eggs transplanted to the oviduct developed normally, i.e. formed compacted morulae after 72 h and medium or advanced blastocysts after 96 h. As far as the cell number was concerned, the development of these gynogenones was normal or only slightly delayed, and was definitely superior to the development of gynogenetic diploids produced by suppression of the cleavage division (Modliński, 1980). This was probably due to the fact that while suppression of one cleavage halves the number of cells, suppression of the 2nd polar body has no such effect. The number of cells accumulated in the embryo by the time of cavitation is of great importance for the structure of the blastocyst and its developmental potential. It has been shown that when the embryo starts to cavitate on the basis of a subnormal number of cells it is the size of the inner cell mass that is affected in the first instance; in extreme cases this structure – and consequently the embryo proper which develops from it – does not form at all (Tarkowski, 1959b; Tarkowski & Wróblewska, 1967; Snow, 1973).

We are now studying the postimplantation development of diploid gynogenones. In view of the promising results described in this study and taking into account that fertilized eggs first haploidized microsurgically and subsequently diploidized at the 1st cleavage division can give rise to adult animals (Hoppe & Illmensee, 1977), it is conceivable that the development of gynogenetic diploid embryos produced by this technique should be superior to that of parthenogenones. If this turns out to be the case, then the type of genetic analysis described by Eicher (1978) could be extended to gene loci which are expressed in the postnatal life.

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REFERENCES


