Preimplantation development of microsurgically obtained haploid and homozygous diploid mouse embryos and effects of pretreatment with Cytochalasin B on enucleated eggs

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SUMMARY

Haploid embryos were obtained by microsurgical removal of one pronucleus, followed by doubling of the haploid chromosome set with Cytochalasin B (CB), either at the first or second mitosis. This procedure provides a source of fully homozygous diploid embryos, which were grown in vitro or in vivo. The effect of CB treatment before and during operation on the course of enucleation and further development of embryos was studied. Out of 81 eggs made diploid at 2-cell stage and transplanted into the oviducts of immature or pseudopregnant recipients 27 morulae and blastocysts were recovered, but not a single case of implantation occurred by the eighth or ninth day of development. After 72–80 h of in vitro culture, most of the homozygous embryos were morulae but after an additional 24 h the majority of them transformed into blastocysts. The rate of development of homozygotes was markedly better than that of haploids, which progressed beyond morula stage. The immediate survival rate of operated eggs was dependent on whether or not the eggs were pre-incubated and the enucleation was performed in the presence of CB. In the former case the immediate survival rate was nearly twice as high as in the absence of CB, but more of the treated eggs underwent fragmentation and early developmental arrest.

INTRODUCTION

Homozygous embryos represent very interesting material from the point of view of genetics as well as embryology because heterozygosity often makes genetic studies cumbersome. Inbred strains of mice derived by many generations of brother–sister mating have achieved a high degree of homozygosity but are unlikely ever to be homozygous at every locus. Diploid parthenogenetic embryos formed by suppression of second polar body formation are also not fully homozygous, since some gene segregation occurs at the second meiotic division. However, the failure of diploid parthenogenetic embryos to develop

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to term is often blamed on their high degree of homozygosity uncovering residual recessive lethal mutations.

Modliński (1976), Markert & Petters (1977) and Hoppe & Illmensee (1977) showed that it was possible to obtain fully homozygous embryos, some of which survived to full term and gave adult homozygous females (Hoppe & Illmensee, 1977). At present the only way to obtain such embryos is by removal of one of the pronuclei and then diploidization of the haploid chromosome set by treatment with Cytochalasin B. It is desirable to test different ways of increasing the efficiency with which such homozygotes can be obtained. The object of this paper was to examine and to compare the development of homozygous diploid embryos obtained either from 1-cell or 2-cell eggs, treated at the time of manipulation in different ways, grown in vitro or in vivo, and also to compare their development with microsurgically obtained haploid embryos.

**MATERIALS AND METHODS**

**Series I**

Eggs originated from hormonally induced ovulation in females of 129/terSv strain mated with CBA-T6T6 males. Females with copulation plug were killed between 18–21 h after injection of HCG. Eggs collected at this time contained small but already clearly visible pronuclei. After one of the two pronuclei had been removed (Modliński, 1975), eggs were transplanted into the oviducts of immature females. About 35–38 h later the eggs were flushed from the oviduct and 2-cell embryos in which the second cleavage division had already begun, recognized by the absence of a visible nucleus in one or both blastomeres, were placed for 5–7 h in Whitten’s medium containing 10 μg/ml Cytochalasin B to suppress cytokinesis (Snow, 1973; Tarkowski, Witkowska & Opas, 1977). For further experiments only those embryos were selected in which cytokinesis of the second cleavage had been blocked in both blastomeres, so that two nuclei were clearly visible in each blastomere. These embryos were transferred for 3 h to Whitten’s medium without Cytochalasin B to determine whether cytokinesis previously blocked by Cytochalasin B occurred after removal of the block. Embryos which remained at the 2-cell stage were transplanted either to the oviduct of immature females or to females on the first day of pseudopregnancy. In the first case oviducts were flushed 2, 3 or 4 days after transplantation (the age of embryos was thus 4, 5 or 6 days) and karyological preparations were made from the recovered embryos (Tarkowski, 1966). In the second case females were killed on the eighth or ninth day of development to detect implantations.

**Series II**

Spontaneously ovulating females from C57BL inbred and CR randomly bred strains were used. The mice were killed between 10 a.m. and noon on the first day (i.e. the day of plug). The eggs recovered were in a similar pronuclear
Haploid and homozygous diploid mouse embryos

stage to those in Series I. The four experimental groups were based on different treatment with Cytochalasin B.

Group A. Eggs were enucleated in medium 16 (Whittingham, 1971) without preincubation in Cytochalasin B. After operation the ova were kept in medium 16 and developed as haploids.

Group B. Eggs were enucleated in medium 16 without preincubation in Cytochalasin B and were then cultured in the same medium for 8 to 12 h. Between 10 p.m. and 12 midnight, the eggs were placed in medium 16 with Cytochalasin B and cultured overnight. Those eggs which remained at the one-cell stage and were diploidized, i.e. contained two visible nuclei, were washed carefully five times in Cytochalasin B-free medium and cultured to morula or blastocyst stage.

Group C. Before enucleation eggs were cultured for 0.5–1.5 h in medium 16 with Cytochalasin B, and the enucleation was performed in the presence of Cytochalasin B (10 μg/ml). After operation the eggs were washed carefully in Cytochalasin B-free medium and cultured as in Group A.

Group D. Preincubation and enucleation as in Group C, followed by overnight culture with Cytochalasin B as in Group B.

Embryos in all groups were cultured for 72 to 96 h, then placed for approximately 3–5 h in medium with Colcemid (about 0.2 μg/ml) and karyological preparations were made.

Two groups of control experiments were carried out, without removal of pronuclei. In the first group 11 eggs were punctured, by micropipette without any treatment with Cytochalasin B. In the second group 14 eggs were preincubated and also punctured by micropipette in the presence of Cytochalasin B. Eggs from both groups were then cultured in plain medium: seven blastocysts, two morulae and two 3-cell eggs were obtained from the first group, five blastocysts, three morulae, one 4-cell and five fragmented eggs from the second group.

RESULTS

Series I

Of 385 eggs from which one pronucleus was removed, 160 (41.5%) survived the operation; 126 2-cell embryos were recovered after transplantation. In most of these, the interphase nuclei were not visible in one or both blastomeres, suggesting that the second cleavage division was about to begin. After 5–7 h of culture in medium containing Cytochalasin B, followed by 3 h culture in Cytochalasin B-free medium, 45 embryos were rejected because they had degenerated (three embryos), because karyokinesis had failed to occur in one or both blastomeres (five and twenty-eight embryos respectively), or because the second cleavage division had been resumed (nine embryos). The remaining 81 embryos, all of which had two nuclei in each blastomere, were transplanted to recipient females (Table 1). None were recovered from pseudopregnant
Table 1. **Transplantation of 2-cell diploid homozygous embryos to the oviduct**

<table>
<thead>
<tr>
<th>Recipient females</th>
<th>Time of development \textit{in vivo} (days)</th>
<th>No. of transplanted/embryos</th>
<th>% Recovery*</th>
<th>Stage of recovered embryos</th>
<th>No. of cells, mean (range) (morulae and blastocysts only)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature</td>
<td>2 + 2 (4)</td>
<td>20</td>
<td>55.0</td>
<td>1 × 7 cells</td>
<td>13.6 (8–25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9 morulae</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 blastocyst</td>
<td></td>
</tr>
<tr>
<td>Immature</td>
<td>2 + 3 (5)</td>
<td>17</td>
<td>58.6</td>
<td>1 × 6; 1 × 7 cells</td>
<td>36.7 (23–50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2 morulae</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6 blastocysts</td>
<td></td>
</tr>
<tr>
<td>Immature</td>
<td>2 + 4 (6)</td>
<td>16</td>
<td>56.2</td>
<td>1 morula</td>
<td>55.0 (31–74)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8 blastocysts</td>
<td></td>
</tr>
<tr>
<td>Pseudo-pregnant</td>
<td>8 – 9†</td>
<td>28</td>
<td>0.0</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

* Embryos which were composed of cell number less than 6 are not included.
† Time of development only in pseudopregnant recipients.
Table 2. Development in vitro after different treatments with Cytochalasin B, of haploid and homozygous diploid embryos

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of eggs</th>
<th>Eggs survived No. (%)</th>
<th>Eggs diploidized No. (%)</th>
<th>Fragmentation No. (%)</th>
<th>Cleavage arrested at 2-4 stage No. (%)</th>
<th>72-80 hrs of culture</th>
<th>80-96 hrs of culture</th>
<th>Embryos (morulae and blastocysts) as % of eggs enucleated</th>
<th>Blastocysts as % of cleaving embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>95</td>
<td>34 (35.8)</td>
<td>—</td>
<td>0</td>
<td>11 (32.3)</td>
<td>9</td>
<td>1</td>
<td>10 3 (24.2)</td>
<td>11.7</td>
</tr>
<tr>
<td>B</td>
<td>86</td>
<td>40 (46.5)</td>
<td>29 (72.5)</td>
<td>2 (5.0)</td>
<td>6 (15.0)</td>
<td>—</td>
<td>—</td>
<td>10 11 (24.1)</td>
<td>40.7</td>
</tr>
<tr>
<td>C</td>
<td>76</td>
<td>57 (75.0)</td>
<td>—</td>
<td>17 (29.8)</td>
<td>9 (15.8)</td>
<td>18</td>
<td>2</td>
<td>9 2 (40.8)</td>
<td>10.0</td>
</tr>
<tr>
<td>D</td>
<td>100</td>
<td>85 (85.0)</td>
<td>60 (70.0)</td>
<td>14 (16.5)</td>
<td>17 (20.0)</td>
<td>13</td>
<td>4</td>
<td>4 8 (29.0)</td>
<td>26.0</td>
</tr>
</tbody>
</table>
Table 3. Mean cell number of haploid and homozygous diploid embryos obtained after 72–96 hours in vitro culture*

<table>
<thead>
<tr>
<th>Group</th>
<th>72–80 h</th>
<th>80–96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>Bl.</td>
</tr>
<tr>
<td>A</td>
<td>18.6</td>
<td>36</td>
</tr>
<tr>
<td>B</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>34</td>
</tr>
<tr>
<td>D</td>
<td>12</td>
<td>19.0</td>
</tr>
</tbody>
</table>

* These counts were taken from all morulae and blastocysts shown in Table 2.

recipients on the eighth and ninth day, but from immature females over 50% were recovered 2–4 days after transplantation. The mean cell number of the embryos recovered increased from 13.6 after 2 days to 55.0 after 4 days (Table 1). Metaphase plates were seen in 11 morulae and blastocysts. All were diploid, and although their poor quality did not allow accurate karyological analysis, T6 marker chromosomes were observed in three embryos, indicating that their chromosomes had originated from the male pronucleus.

Series II

The results for the four groups A to D, in which one pronucleus was removed from a total of 357 eggs, are given in Tables 2 and 3. The immediate survival rate of the operated eggs was strikingly dependent on whether or not the eggs were preincubated and the pronucleus removed in the presence of Cytochalasin B. In Groups C and D, the overall immediate survival rate was nearly twice as high as in groups A and B. However, the proportion of fragmenting eggs was greater in groups C and D, so that the superiority in terms of survival to morula and blastocyst stage, though still significant for the haploid embryos in Group C, was no longer so evident for the homozygous diploids of Group D.

**DISCUSSION**

Embryos made haploid by removal of one pronucleus showed markedly delayed development. On the 4th day of in vivo development (Modliński, 1975) or after 72–80 h of in vitro culture (the present paper) the embryos had a low cell number, usually less than 20, and rarely progressed beyond morula stage. During subsequent 24 h, however, in some embryos the cell number increased considerably (above 40 cells) and some (but not all) transformed into blastocysts.

Diploidization of haploid embryos either at the first or second cleavage increases the number of embryos developing to blastocyst stage, although treatment with Cytochalasin B halves cell number. Indeed the number of cells
of tetraploid embryos made by Cytochalasin B treatment is reduced by more than 50 % (Snow, 1973, 1975; Smith & McLaren, 1977; Tarkowski et al. 1977) and the formation of blastocysts occurs around 16 cells (Smith & McLaren, 1977; Tarkowski et al. 1977). Homozygous diploid embryos obtained in my experiments after 72–80 h \textit{in vitro} or on the fourth day \textit{in vivo} also had a low cell number (less than 16 on average) and a few of them had transformed into blastocysts. By the next day, however, the number of cells had increased markedly (Table 3) and majority of embryos transformed into blastocysts. The slight discrepancy between mean cell number reported for tetraploid blastocysts by Snow (1973) and Smith and McLaren (1977) and homozygous diploid embryos probably reflects differences in their ages, or in treatment and culture conditions, although it is conceivable that their ploidy could also be involved.

It seems that the differences between the developmental potency of haploid and homozygous diploid embryos are not connected with their cell number. Although the mean cell number in homozygous diploid blastocysts is more than 40 % lower than that in haploids, the former ones are formed much more frequently. Indeed Table 2 shows that 74 cleaving haploid embryos produced only 8 blastocysts (Groups \(A + C \) combined) while 73 cleaving diploidized embryos produced 23 blastocysts (Groups \(B + D \) combined).

Thus the rate of development of homozygous diploid embryos is similar to tetraploid ones and markedly better than that of haploids. Similarly parthenogenetic diploid embryos develop better than haploid ones (Witkowska, 1973; Kaufman & Gardner, 1974; Kaufman & Sachs, 1976).

No difference in the development of embryos diploidized either at one or two-cell stage could be detected.

Homozygous embryos develop much better \textit{in vivo} than under \textit{in vitro} conditions. The mean cell number in embryos developing for 96 h \textit{in vitro} was 22.5 in comparison to 36.7 (Table 1) of those developing \textit{in vivo}.

Interesting is the fact that in the present work some androgenetic diploid homozygotes developed to the morula and blastocyst stage while haploids obtained by removal of the female pronucleus (Modliński, 1975) never developed beyond four to six cells. The successful diploid embryos were of course XX androgenes, since haploid embryos carrying Y chromosome die during early cleavage (Tarkowski, 1977).

When the eggs were preincubated in medium with Cytochalasin B, the immediate survival rate was significantly increased, in agreement with the findings of Hoppe & Illmensee (1977). Indeed, for many types of microsurgery on mouse eggs the presence of Cytochalasin B in culture medium before and during the operation is very advantageous (my unpublished observations). Probably changes occur in the egg membrane under the influence of Cytochalasin B that render it more resistant to mechanical injuries. Preincubation for 40–60 min is long enough to cause such changes, but 20–30 mins seems to be not sufficient. In those eggs that degenerated in spite of Cytochalasin B
treatment before and during micromanipulation, the process of degeneration proceeded much more rapidly than in the absence of the drug.

There is no doubt that in producing haploid embryos by removal of one pronucleus the treatment of embryos with Cytochalasin B before and during manipulation is advisable: the percentage of embryos obtained as the percentage of eggs enucleated increased from 24.2 (enucleation without CB) up to 40.8 if the operation is performed in the presence of the drug. On the other hand, the treatment with CB, particularly on two occasions, increased the number of eggs fragmenting or arrested in early stages of development. The advantages of the higher immediate survival are counteracted both by this increased early developmental failure, and by the fact that only about 70% of enucleated eggs undergo diploidization.

The attempt to obtain postimplantation development of homozygotes in series I gave negative results: not a single case of implantation occurred. These results are consistent with those of Opas (1977), who found no implantation after elimination of one pronucleus and suppression of the second polar body. However, in the light of the results obtained by Hoppe & Illmensee (1977), the failure of implantations in such embryos is unlikely to have a genetic basis, but could perhaps result from the embryo being damaged during successive operations.

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REFERENCES

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