Complete preimplantation development in culture of parthenogenetic mouse embryos

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SUMMARY

The present experiments were undertaken to determine whether, in parthenogenesis, heterozygous embryos develop better than homozygous embryos. Such experiments may provide an approach to elucidating whether fertilized embryos develop better than parthenogenetic ones because of heterozygosity, or if the sperm provides another contribution necessary for complete embryonic development. The parthenogenetic embryos studied included uniform haploids after extrusion of the second polar body, mosaic haploids in which each blastomere contained a genetically different haploid nucleus, and heterozygous diploid mouse embryos. Eggs were activated and cultured in a chemically defined medium. About three times as many mosaic haploid or heterozygous diploid eggs developed beyond the 4-cell stage after 98–100 h and to the blastocyst stage after 120 h in culture, than uniform haploid eggs. This indicates that the development of parthenogenetic embryos is probably under genetic control and that there was a better development of the heterozygous embryos. Mosaic haploid embryos showed the same high frequency of development as heterozygous diploids. The results therefore indicate that heterozygosity provided a developmental advantage even when distributed between two genetically different clones of cells in the same embryo.

INTRODUCTION

Fertilization, in addition to introducing the sperm, results in heterozygosity. This raises the question whether the better development of fertilized compared to parthenogenetic embryos is due only to heterozygosity, or whether the sperm provides another contribution necessary for complete embryonic development (Tarkowski, 1971; Graham, 1974). One approach to answering this question is to study whether, in parthenogenesis, heterozygous embryos have a better development than homozygous embryos.

The mouse was chosen for the present study because its eggs can be efficiently activated in vitro (Graham, 1970; Kaufman, 1973a, b) and conditions for the culture of preimplantation fertilized embryos have been established (Whitten, 1956; Brinster, 1963; Biggers, Whittingham & Donahue, 1967; Whittingham & Biggers, 1967; Whitten & Biggers, 1968). Parthenogenetic eggs of various

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types, with different genetic constitutions, may be obtained by controlling the post-ovulatory age of oocytes at activation and the culture conditions during the first few hours after activation (Graham, 1972; Kaufman, 1973a; Graham & Deussen, 1974; Kaufman & Surani, 1974). As a result of crossing-over, both products of meiosis I are genetically dissimilar and three classes of parthenogenetic embryos can be obtained, depending on whether the second polar body is expelled or not (Tarkowski, 1971; Graham, 1974). Under normal circumstances one set of chromosomes is discarded with the second polar body, leaving the egg with a single haploid set of chromosomes (uniform haploid). The embryos which result from this type of development would be expected to be homozygous at all genetic loci. When, however, second polar body extrusion is inhibited, the egg may either contain one heterozygous diploid or two genetically different haploid pronuclei (Fig. 1). The third class of parthenogenetic development has been termed ‘immediate cleavage’ (Braden & Austin, 1954). In this case the egg divides into two equally sized blastomeres instead of forming a second polar body, so that each blastomere contains a genetically different pronucleus. When eggs undergo ‘immediate cleavage’ or ‘delayed cleavage’ (Fig. 1: the ‘delayed immediate cleavage’ of Graham, 1971) both blastomeres give rise to separate clones of cells within the embryo. Since these clones are genetically dissimilar these embryos will be referred to as ‘mosaic haploids’.

We have examined in the present experiments the preimplantation development of uniform haploid, mosaic haploid and heterozygous diploid parthenogenetic embryos. Eggs which developed a single diploid pronucleus (Fig. 1) were not observed. The development of genetically different parthenogenetic embryos was also compared with the development of fertilized embryos. Oocytes were obtained from (C57BL x CBA-LAC)F1 hybrid females as these had previously been successfully employed (Graham, 1970; Kaufman & Sachs, 1975) in in vitro activation studies. All eggs were cultured in a simple chemically defined medium (Whittingham, 1971).

**MATERIALS AND METHODS**

(a) *Parthenogenetic activation of eggs*

Eggs were isolated from the ampullar region of the oviduct of 8- to 12-week-old (C57BL x CBA-LAC)F1 female mice (hereafter referred to as F1LAC) at 21-21.5 h after human chorionic gonadotrophin (HCG) injection for superovulation. Oocytes were liberated from the ampullae into a modified Krebs-Ringer bicarbonate culture medium containing 4 mg/ml bovine serum albumin (Whittingham, 1971) and 100 i.u./ml hyaluronidase (Koch-Light, ovine testes) between 08.30 and 09.30 h, and incubated at 37 °C in 5% CO2 in air. After 10–15 min the eggs were isolated from this medium, transferred to hyaluronidase-free medium and culture continued for a further 4–5 h. All culture
Parthenogenetic development in culture

was carried out under light paraffin oil in 60 × 15 mm plastic Petri dishes (Falcon plastics No. 3002). Eggs were examined under the 50 × magnification of a Wild dissecting microscope to determine the overall activation frequency and types of parthenogenetic eggs induced.

Fig. 1. Pathways of development and genetic constitution of parthenogenetically activated eggs.

Activated eggs of three types were observed at this time, namely those which developed a single pronucleus following extrusion of the second polar body, eggs which developed two pronuclei in the absence of second polar body extrusion, and eggs which underwent immediate cleavage. Activated eggs of each type were isolated and transferred, generally in batches of 10 or 15, to 30–50 µl drops of culture medium under oil in separate Petri dishes. The activated eggs were retained in culture for a further 94–96 h. Eggs and embryos were then classified according to whether they had fragmented or degenerated, or were at the 2-, 3- or 4-cell stage, or were more advanced in development. Only embryos which were more advanced than the 4-cell stage were examined by air-drying (Tarkowski, 1966). In a few experiments embryos were cultured in medium containing 0.5 µg/ml Colcemid for approximately 2 h prior to air-drying, to accumulate an increased number of cells in mitosis.
A maximum of three embryos were carefully placed on each microslide, and preparations were stained with 4% Giemsa. The air-drying technique allowed the number of blastomere nuclei present to be recorded, and the ploidy of the cells in mitosis to be determined.

Parthenogenetic embryos were also cultured for a total of 120 h to determine the proportion which were capable of developing to the blastocyst stage.

(b) Recovery of fertilized 1-cell eggs

Eight- to twelve-week-old spontaneously ovulating F1LAC females were examined by vaginal inspection (Champlin, Dorr & Gates, 1973) at approximately 20.00 h, and those in oestrus were mated to fertile F1LAC males. A further group of 8- to 12-week-old F1LAC females were treated with an intraperitoneal injection of 5 i.u. pregnant mares' serum gonadotrophin followed 48 hours later by 5 i.u. of HCG. The gonadotrophins were injected at 12.00 h, and females were placed with fertile males shortly after the HCG injection. Both groups of females were checked at approximately 08.00 h the following morning for evidence of mating. Those with vaginal plugs were killed 1-2 h later and the fertilized eggs released into medium containing hyaluronidase. After 10-15 min the cumulus-free eggs were transferred to hyaluronidase-free medium, and retained in culture for a further 98-100 h. Eggs and embryos were then classified according to their stage of development and those more advanced than the 4-cell stage were examined by air-drying. The preparations were stained and analysed as described in section (a).

RESULTS

(a) Pathways of development of parthenogenetic eggs

4-5 h after in vitro activation

As cytoplasmic cleavage took place about 6–8 h after activation in most of those eggs with two pronuclei that subsequently underwent delayed cleavage, all eggs were examined 4–5 h after hyaluronidase treatment. This allowed the immediate-cleavage eggs to be distinguished from the eggs that developed two pronuclei (see Fig. 1). The overall activation frequency in the present series of experiments was 70% (Table 1), and the incidence of eggs which fragmented was 0.5%.

(b) Development beyond the 4-cell stage of parthenogenetically activated and fertilized 1-cell eggs after 98–100 h in culture

Twenty-one per cent of the total 1-cell parthenogenetically activated eggs with a single pronucleus that were retained in culture for 98–100 h progressed beyond the 4-cell stage, compared to 66% of the 1-cell eggs with two pronuclei, and 65% of the eggs that underwent immediate cleavage. In the two fertilized series, 96% and 72% of the 1-cell eggs isolated from the spontaneously
ovulating and gonadotrophin-induced females, respectively, developed beyond the 4-cell stage during a similar period in culture (Table 2). Seventy-four per cent and 67% of the spontaneously ovulating and gonadotrophin-induced embryos, respectively, that developed beyond the 4-cell stage had become blastocysts, while the remaining embryos were all morulae.

Table 1. The pathways of development of parthenogenetic eggs at 4–5 h after in vitro activation

<table>
<thead>
<tr>
<th>Total number of eggs examined</th>
<th>Activated eggs</th>
<th>Overall activation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 pronucleus + 2nd polar body</td>
<td>2 pronuclei without 2nd polar body</td>
</tr>
<tr>
<td>2756</td>
<td>1725 (68.9)*</td>
<td>113 (5.8)</td>
</tr>
</tbody>
</table>

* The percentage of the total number of eggs activated is given in parentheses.

(c) The cell number and ploidy of embryos which developed beyond the 4-cell stage after 98–100 h in culture

The cell numbers of the parthenogenetic and fertilized embryos which developed beyond the 4-cell stage after 98–100 h in culture are presented in Table 2. The mean cell number of the single pronuclear parthenogenetic embryos was significantly lower than that of the 2-pronuclear and immediate-cleavage embryos, while the mean cell numbers of the two groups of fertilized embryos were approximately double those of the 2-pronuclear and immediate-cleavage embryos. As the initiation of development in the fertilized eggs probably took place 5–8 h before activation was induced in the parthenogenetic eggs, this would only partially account for the greater mean cell numbers of the fertilized embryos. This suggests that the cleavage rate of the fertilized eggs was on average faster than that of the parthenogenetic embryos when all groups were cultured under similar conditions.

The ploidy of the embryos with cells in mitosis at the time of analysis is presented in Table 3. The mitoses were scored as ‘haploid’, ‘diploid’ or ‘tetraploid’ without necessarily making an unequivocal count of the chromosome number, though accurate counts could be made in a high proportion of the cells examined. Seventy-two per cent of the single pronuclear eggs had only haploid mitoses present, having the expected ploidy for this type of embryo. Sixteen per cent and 76%, respectively, of the eggs which developed two pronuclei appeared to be either uniform haploids or uniform diploids. This suggests that a higher proportion developed as diploids than underwent ‘delayed cleavage’ (Fig. 1). Eighty-one per cent of the immediate cleavage embryos had only haploid mitoses present, having the expected ploidy for this type of embryo. All (11/16) of the immediate cleavage embryos in which unequivocal counts could be made had either 20 or 40 chromosomes.
Table 2. The cell number of parthenogenetic and fertilized embryos which developed beyond the 4-cell stage after 98-100 h in culture

<table>
<thead>
<tr>
<th>Parthenogenetic or fertilized group</th>
<th>Type</th>
<th>Total number of activated eggs cultured</th>
<th>Total number of embryos which developed beyond the 4-cell stage</th>
<th>Cell number</th>
<th>Mean cell number of embryos with more than 4 cells ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parthenogenetic</td>
<td>One pronucleus + second polar body</td>
<td>1053</td>
<td>93 85 35 6 1</td>
<td>12.3 ± 0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Two pronuclei without second polar body</td>
<td>65</td>
<td>7 12 17 6 1</td>
<td>21.9 ± 2.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immediate cleavage</td>
<td>71</td>
<td>15 15 9 6 1</td>
<td>17.9 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Fertilized</td>
<td>Spontaneous ovulation</td>
<td>68</td>
<td>2 8 12 25 18</td>
<td>48.2 ± 3.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gonadotrophin-induced ovulation</td>
<td>58</td>
<td>2 4 13 19 4</td>
<td>38.3 ± 3.4</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. The ploidy of parthenogenetic and fertilized embryos which developed beyond the 4-cell stage after 98–100 h in culture

<table>
<thead>
<tr>
<th>Parthenogenetic or fertilized group</th>
<th>Type</th>
<th>Total number of embryos examined</th>
<th>Total number of embryos with cells in mitosis*</th>
<th>Ploidy of embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Haploid</td>
</tr>
<tr>
<td>Parthenogenetic</td>
<td>One pronucleus + second polar body</td>
<td>220</td>
<td>87 (39.6)</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>Two pronuclei without second polar body</td>
<td>43</td>
<td>25 (58.1)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Immediate cleavage</td>
<td>46</td>
<td>16 (34.8)</td>
<td>13</td>
</tr>
<tr>
<td>Fertilized</td>
<td>Spontaneous ovulation</td>
<td>65</td>
<td>49 (75.4)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Gonadotrophin-induced ovulation</td>
<td>42</td>
<td>28 (66.7)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diploid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Haploid/ diploid mosaic</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diploid/ tetraploid mosaic</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Tetraploid</td>
</tr>
</tbody>
</table>

* The ploidy of these embryos was scored without necessarily making an unequivocal count of the chromosome number.
† The percentage of the total number of embryos examined is given in parentheses.
In the two fertilized series 92% and 93% of the spontaneously ovulating and gonadotrophin-induced groups, respectively, had only diploid mitoses present. One embryo in the gonadotrophin-induced group was a diploid-tetraploid mosaic, while the remaining five embryos in which the ploidy could be determined had only tetraploid mitoses present.

Table 4. Development of parthenogenetic eggs to the blastocyst stage after 120 h in culture

<table>
<thead>
<tr>
<th>Type</th>
<th>Total number of activated eggs cultured</th>
<th>Total number of eggs which developed to the blastocyst stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>One pronucleus + second polar body</td>
<td>188</td>
<td>40</td>
</tr>
<tr>
<td>Two pronuclei without second polar body</td>
<td>33</td>
<td>19</td>
</tr>
<tr>
<td>Immediate cleavage</td>
<td>29</td>
<td>16</td>
</tr>
</tbody>
</table>

(d) Development of parthenogenetic eggs to the blastocyst stage after 120 h in culture

When the 2-pronuclear and immediate cleavage eggs were retained in culture for 120 h, 58% and 55% of these eggs, respectively, developed to the blastocyst stage compared to 21% blastocysts from the eggs with one pronucleus (Table 4).

DISCUSSION

In the present experiments, we have examined the preimplantation development in culture of three types of parthenogenetic embryos with different genetic constitutions, namely uniform haploids, mosaic haploids and heterozygous diploid embryos. These three out of the four possible types of pronuclear eggs (Fig. 1) were found 4–5 h after activation according to whether second polar body extrusion occurred or not, whether the egg underwent immediate cleavage, and depending on whether the egg contained one or two pronuclei. The development of these three types of parthenogenetic eggs was compared with fertilized eggs.

The results show that about three times as many parthenogenetic eggs developed beyond the 4-cell stage in the eggs with two haploid pronuclei or after immediate cleavage, compared with eggs that developed a single haploid pronucleus following extrusion of the second polar body. Similar results were obtained when eggs were examined after 98–100 h in culture, or when the rate of development to the blastocyst stage was determined after 120 h in culture.
In order to substantiate the hypothesis that preimplantation parthenogenetic development is under genetic control, other possible explanations for the poorer development of the uniform haploids must be considered and excluded. Three obvious differences exist between the uniform haploids and the other two classes of embryos. Only in the uniform haploids is a certain volume of cytoplasm lost following second polar body extrusion. The actual volume of cytoplasm involved is extremely small, and similar to that lost following fertilization. Here, second polar body extrusion is both a normal and necessary process with no apparent detrimental effect on the resulting embryo. Thus the loss of this volume of cytoplasm in the uniform haploids is unlikely to account for the poorer development of these embryos. It is more difficult to evaluate the importance of the different nuclear-cytoplasmic ratios of the uniform haploid compared to the immediate cleavage and 2-pronuclear embryos. It has generally been assumed that the ratio of $2N:1$ observed in fertilized embryos must be optimal, and that other ratios are in some way sub-optimal. Thus the ratios observed in the 2-pronuclear and immediate cleavage eggs of $2N:1$ and $N:0.5$, respectively, might be optimal, whereas the ratio of $N:1$ observed in the uniform haploids might be sub-optimal. Evidence which tends to suggest that this general hypothesis may be an oversimplification has recently been published by Modlinski (1975). This author demonstrated that the preimplantation development potential of fertilized mouse eggs from which one of the two pronuclei had been withdrawn depended on whether the remaining pronucleus originated from the male or female gamete. In this case the resulting nuclear-cytoplasmic ratios were similar, but the development potential was clearly not so. These findings must shed some doubt on the importance which should be assigned to the concept of an optimal nuclear-cytoplasmic ratio controlling early embryonic development, and on this explanation for the present findings.

These three classes of parthenogenetic embryos are also genetically dissimilar. The eggs with two haploid pronuclei developed either as mosaic haploids or heterozygous diploids, the immediate cleavage eggs as mosaic haploids, and the eggs with a single haploid pronucleus as uniform haploids. The mosaic haploid embryos contain two genetically different clones of cells. Since these mosaic embryos showed the same high frequency of development as heterozygous diploids, this presumably indicates that heterozygosity in an embryo, even when distributed between two clones of cells, provides a developmental advantage. Products produced by the two clones of cells may diffuse from one to the other, so as to compensate for missing products or balance the effect of deleterious genes. It remains to be seen whether the heterozygous diploids have a developmental advantage over mosaic haploids beyond the preimplantation period. The higher implantation rate of the heterozygous diploids over the homozygous haploids and mosaic haploids (Kaufman & Gardner, 1974) suggests that this cooperation may not be as efficient at later
stages of development. The fact that 21% of the uniform haploids were able to achieve a comparable stage of development to the mosaic haploids, suggests that the actual number of deleterious factors that might inhibit preimplantation development are relatively few in inbred strains (Kaufman, Huberman & Sachs, 1975).

All of the immediate cleavage embryos in which unequivocal counts could be made had either 20 or 40 chromosomes. This suggests that normal segregation of chromosomes occurred at the second meiotic division when embryos of this type were induced to develop under the present culture conditions. Previously Graham & Deussen (1974) had demonstrated that over 60% of immediate cleavage embryos had abnormal karyotypes. The present findings suggest that these abnormal karyotypes may have resulted from differences in the culture conditions after activation.

No homozygous diploid embryos were obtained with certainty in the present experiments. Diploid eggs of this type would show complete genetic homozygosity at all loci, and thus may have no developmental advantage over uniform haploid embryos. It should be possible to produce homozygous diploid embryos from uniform haploid eggs by inhibition of their first cleavage division with, for example, Cytochalasin B (Snow, 1973), and it would be interesting to compare their development with uniform haploid and heterozygous diploid embryos.

A higher proportion of the fertilized embryos developed beyond the 4-cell stage after 98-100 h in culture, and their cleavage rate was on average faster than that of all three types of parthenogenetic embryos. Whether their better development was due to a greater degree of heterozygosity in the fertilized compared to the parthenogenetic embryos, or was due to the presence of some other factor contributed by the sperm remains to be determined. If a factor from the sperm is necessary for advanced embryonic development, the production of chimaeric embryos by the aggregation of one parthenogenetic with one fertilized embryo (see Graham, 1970) may be the only means of obtaining complete parthenogenetic development. If, however, the degree of heterozygosity is the critical factor, more advanced development could possibly be obtained by aggregating two or more parthenogenetic embryos from different genetic backgrounds. A parallel can be drawn to the situation in tetraparental mice (Tarkowski, 1961; Mintz, 1962), especially where these have been used for the survival of embryos with lethal genes (Mintz, 1962; Eicher & Hoppe, 1973).

$F_1$ hybrid mice were used in the present experiments because it had previously been shown that a high proportion of their fertilized 1-cell eggs developed to the blastocyst stage in simple chemically defined medium (Whitten & Biggers, 1968). One-cell eggs from inbred and random-bred animals have so far generally failed to develop beyond the 2-cell stage in culture (Biggers, Whitten & Whittingham, 1971). However, if an inbred strain rather than an $F_1$ hybrid
Parthenogenetic development in culture 189 could be found whose preimplantation fertilized and parthenogenetic eggs developed completely in vitro, this might provide additional information on the genetics of early development without the possible complicating factor of using F₁ hybrids.

The present results demonstrate that in vitro activated eggs may be routinely cultured from the 1-cell stage to the blastocyst in chemically defined medium, and that the development of parthenogenetic eggs is probably under genetic control. This supplements the previous findings on the genetic control of haploid parthenogenetic development, which suggested that haploid embryos had a greater developmental potential if they were derived from inbred rather than from random-bred animals (Kaufman, Huberman & Sachs, 1975).

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REFERENCES


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