Investigation of the determinative state of
the mouse inner cell mass

II. The fate of isolated inner cell masses transferred to the oviduct

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SUMMARY

Inner cell masses (ICMs) were dissected from 3½- and 4½-day mouse blastocysts and inserted into empty zonae before transfer to the oviducts of pseudopregnant mice. The ICMs survived in the oviduct for at least 2 days with little evidence of reduction in cell number. After 1 day, isolated 3½-day ICMs were still viable, since they could form chimaeras when injected into 3½-day blastocysts. However, no evidence of trophoblast formation could be detected in any of the isolated ICMs, despite exposure of some of their cells to ‘outside’ conditions. This is further evidence that ICM cells, although not overtly differentiated, are determined by 3½ days. Although no trophoblast was formed, 3½-day ICMs formed an outer endoderm layer after 1 day in the oviduct, as judged by light and electron microscopical evidence. It is suggested that cell position may be important in endoderm differentiation.

INTRODUCTION

In the preceding paper (Rossant, 1975) it has been shown that exposing mouse inner cell mass (ICM) cells to ‘outside’ conditions by aggregating them with morulae cannot induce them to form trophoblastic derivatives. This suggests that ICM cells, although not overtly differentiated, are determined by 3½ days. However, it is possible that the ICM cells in such aggregates were not exposed to the outside for long enough to induce them to form trophoblast. An alternative method of exposing ICM cells to ‘outside’ conditions, described in this paper, is to study the further development of isolated ICMs, where some cells will be exposed to the outside for the entire period chosen for study. However, this method poses considerable problems. Although post-implantation development has been achieved in vitro (Jenkinson & Wilson, 1970; Hsu, 1971; Hsu, Baskar, Stevens & Rash, 1974), survival of ICM cells under simple culture conditions is generally poor, both in blastocyst outgrowths (Cole & Paul, 1965) and in isolated ICMs (Rossant, unpublished observations). Isolated ICMs also do not seem to survive long when transferred to the uterus (Gardner, 1972). Thus it was considered that the oviduct might provide a more suitable environment for the isolated ICMs. However, zona-free cleavage stages

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show poor survival in the oviduct (Bronson & McLaren, 1970; Modlinski, 1970), possibly because the zona normally keeps blastomeres together and prevents them sticking to the oviduct wall. Since ICMs resemble cleavage stages in several ways, it was thought unlikely that they would survive in the oviduct without zonae. A technique was therefore devised for inserting ICMs into empty zonae before transfer to the oviduct. The presence of the zona was also important for correct identification of recovered ICMs.

Three criteria were used to judge whether trophoblast was formed in the isolated ICMs:

1. Formation of highly cavitated cells (Gardner, 1972).
2. Induction of the decidual response (Gardner, 1972).
3. Fate of ICMs recovered after one day in the oviduct when injected into 3½-day blastocysts.

MATERIALS AND METHODS

Recovery of embryos from donor females

The mice used in these experiments were from the two strains listed previously (Rossant, 1975) which were homozygous for different electrophoretic variants of glucose phosphate isomerase (GPI). Blastocysts were recovered, after superovulation or natural mating, by flushing the uteri between 12.00 and 15.00 on the 4th day after mating (3½-day blastocysts) and between 10.00 and 12.00 on the 5th day after mating (4½-day blastocysts). Fertilized one-cell eggs for the preparation of empty zonae were obtained from the ampullary region of the oviduct on the morning after mating. The cumulus cell mass was removed by incubation in hyaluronidase solution (100 i.u./ml phosphate buffered saline). Eggs and blastocysts were recovered, stored, manipulated and transferred in PBI medium (Whittingham & Wales, 1969), containing 10% foetal calf serum.

Microsurgery

All microsurgical procedures were carried out using a Leitz micromanipulator assembly. Insertion of isolated ICMs into empty zonae involved the following three microsurgical steps:

(a) Preparation of empty zonae

Fertilized eggs were held by a suction pipette and pierced with a siliconized glass needle, which passed through the egg into the opening of the pipette. Removal of the needle, followed by an increase in the suction pressure of the holding pipette, caused the contents of the egg to be sucked into it. Thus, the empty zona was intact except for two small holes on opposite sides.

(b) Dissection of inner cell masses

ICMs from 3½-day and 4½-day blastocysts were dissected free of trophoblast as described by Gardner (1972); 4½-day ICMs were left singly but 3½-day ICMs
were pushed together in pairs with a glass needle and placed in an incubator at 37 °C. These fragments were usually firmly aggregated after 1 or 2 h. Any pairs that did not aggregate were discarded because they were probably contaminated with trophoblast cells (Gardner, 1972).

(c) Insertion of inner cell masses into empty zonae

Single 4½-day ICMs and aggregated pairs of 3½-day ICMs were inserted into empty zonae using essentially the same technique as described by Gardner for injecting ICMs into 3½-day blastocysts (Gardner, 1971), treating the cavity inside the zona as though it were the blastocoel. A single 4½-day ICM and two aggregated 3½-day ICMs were approximately equal in size and about half the size of a normal 3½-day blastocyst (Fig. 1).

Transfer of ICMs to pseudopregnant recipients

ICMs in zonae were transferred to the right oviducts of recipient females on the 1st day of pseudopregnancy.

Examination of ICM development

The oviducts or uteri of recipients were flushed 1, 2 or 3 days after transfer and the flushings examined for ICMs in zonae. ICMs which were large enough were fixed for 10 min in Dalton's fluid (Dalton, 1955), dehydrated and embedded in Araldite resin. The resin was hardened overnight at 60 °C. Blocks containing ICMs were sectioned at 2–3 μm on a Huxley ultramicrotome. The
sections were then stained with a 50:50 mixture of 1% methylene blue in 1% borax and 1% Azur II. The stained sections were mounted in Araldite, which prevented excessive fading of the stain. A few 4½-day and aggregated pairs of 3½-day ICMs were fixed in this way without transfer to the oviduct.

Some recipients were killed on the 6th day of pseudopregnancy and their uteri were examined for implantation sites.

Electron microscopy

Two aggregated pairs of 3½-day ICMs in zonae, recovered after 1 day in the oviduct, were embedded in Araldite as described previously. Ultrathin sections (ca. 70 nm) were cut on an LKB Ultratome II, using a diamond knife. The sections were then stained for 30 min in a saturated solution of uranyl acetate in 50% alcohol, followed by 30 min in lead citrate (Reynolds, 1963). Stained sections were examined using an AEI Electron Microscope 6B at 60 kV. Thick sections for light microscopical examination were cut from the remaining parts of the blocks, as previously described.

A few unoperated 3½-day and 4½-day blastocysts were also examined in the electron microscope.

Injection of recovered ICMs into 3½-day blastocysts

Several aggregated pairs of 3½-day ICMs, which were from Gpi-l<sup>b</sup>/Gpi-l<sup>b</sup> mice, were flushed from the oviduct 1 day after transfer. Their zonae were removed physically or by a 0.5% solution of Pronase (Calbiochem, grade B) in phosphate-buffered saline (Mintz, 1962). Denuded ICMs were injected into the blastocoel of 3½-day Gpi-l<sup>a</sup>/Gpi-l<sup>a</sup> blastocysts, which were then cultured for 1–2 h before transfer to the uterus of recipients on the 3rd or 4th day of pseudopregnancy.

Recipient females were killed on the 10th day after mating and any decidua were removed. Conceptuses were dissected into embryonic, extra-embryonic (amnion, chorion, allantois and yolk sac) and trophoblastic (ectoplacental cone and trophoblastic giant cells) fractions. Clean separation of trophoblastic and extra-embryonic fractions was possible in all conceptuses. The dissected samples were treated and analysed electrophoretically for GPI as described by Chapman (Chapman, Whitten & Ruddle, 1971).

RESULTS

Survival of isolated ICMs in the oviduct

Isolated ICMs inside their zonae could be recovered after 1, 2 or 3 days in the oviduct (Table 1). The size of the ICMs varied considerably, but most of those recovered after 1 or 2 days seemed to be roughly similar in size to the original transferred ICMs. The cell number of those recovered after 3 days was apparently reduced. In only three ICMs from one recipient was there any sign of invasion
of maternal cells inside the zonae. This mouse was probably not pseudopregnant, since no unfertilized eggs were recovered and no corpora lutea were present in the ovaries. The low rate of recovery in general was probably due to loss of ICMs from the zonae, particularly when the dissected ICMs were small.

Although vesicles of various sizes were seen in the cells of the recovered ICMs, no highly cavitated cells were seen, such as occur when ICMs are contaminated with trophoblast (see Gardner, 1972, fig. 12). When five recipients which had received 19 aggregated pairs of 3\( \frac{1}{2} \)-day ICMs on the 1st day of pseudopregnancy were killed 5 days later, no implantation sites were found. Similarly, 31 4\( \frac{1}{2} \)-day ICMs transferred to six recipients did not induce any decidual swellings.

Structure of recovered ICMs

(a) Light microscopy

The number of ICMs examined histologically was limited due to the difficulties of fixing such small fragments and subsequently locating them in the Araldite blocks. However, five aggregated pairs of 3\( \frac{1}{2} \)-day ICMs and two 4\( \frac{1}{2} \)-day ICMs were recovered after 1 day in the oviduct, sectioned and examined in the light microscope.

Both 4\( \frac{1}{2} \)-day ICMs were bilaminar in structure, consisting of an outer monolayer of rather darkly staining cells, surrounding a compact group of inner cells (Fig. 2A). By 4\( \frac{1}{2} \) days, endoderm has delaminated in the mouse blastocyst and isolated 4\( \frac{1}{2} \)-day ICMs include this endoderm layer. It seemed likely that the outer layer of cells in the recovered ICMs consisted of endoderm since it was histologically similar to endoderm cells in whole 4\( \frac{1}{2} \)-day blastocysts and isolated 4\( \frac{1}{2} \)-day ICMs not transferred to the oviduct.

When the aggregated pairs of 3\( \frac{1}{2} \)-day ICMs were examined, all five showed a bilaminar appearance similar to the 4\( \frac{1}{2} \)-day ICMs just described (Fig. 2B). The close resemblance between the outer layer of 3\( \frac{1}{2} \)- and 4\( \frac{1}{2} \)-day ICMs recovered from the oviduct suggested that the outer cells in the 3\( \frac{1}{2} \)-day ICMs might also be endoderm, although ICMs in the intact 3\( \frac{1}{2} \)-day blastocyst or after isolation showed no such morphological differentiation into two cell layers. However, precise identification of endoderm cells is not possible in the light microscope since there are no definite cytological features distinguishing them from other blastocyst cells at this level of resolution.
Fig. 2. Light micrographs of inner cell masses recovered after one day in the oviduct. (A) One 4½-day ICM. (B) Two aggregated 3½-day ICMs.

(b) Electron microscopy

At the electron microscopical level it has been reported that endoderm cells can be distinguished from both trophoblast and other ICM cells by the presence of large amounts of rough endoplasmic reticulum (ER) (Enders & Schalfke, 1965; Enders, 1971). It was also observed that junctional complexes occur between endoderm cells, similar to those seen between trophoblast cells, but that no such junctions occur between other ICM cells. These results were confirmed in the present study in electron micrographs of 3½- and 4½-day mouse blastocysts.

Two aggregated pairs of 3½-day ICMs recovered after 1 day in the oviduct, which showed bilaminar structure in the light microscope, were also examined in the electron microscope (Fig. 3). In both cases the outer layer of cells showed features typical of endoderm, i.e. large amounts of rough ER and intercellular junctional complexes. The inner cells showed no such features. The junctional complexes found between the outer cells are also typical of trophoblast but the presence of rough ER makes it very unlikely that the outer cells are trophoblastic.

Developmental potential of isolated 3½-day ICMs after 1 day in the oviduct

Seven conceptuses developed from blastocysts into which aggregated pairs of 3½-day ICMs, recovered after 1 day in the oviduct, had been injected. Six were chimaeric by GPI analysis (Table 2, Fig. 4). In four chimaeras the injected ICM only colonized the extra-embryonic membranes, while in a fifth chimaera, C2, the exact localization of the donor ICM cell progeny could not be deter-
mined, since the embryo was resorbing. In the sixth chimaera, C₅, the injected ICM had apparently given rise to the whole embryo, since no host-type isozyme was detectable. However, there was no contribution of donor-ICM-type isozyme to the ectoplacental cone and trophoblastic giant cells in any of the conceptuses.

**DISCUSSION**

ICMs inserted into zonae and transferred to the oviducts of pseudopregnant mice can survive for at least 2 days with little evidence of reduction in cell number, although survival does seem to be adversely affected after 3 days. After 1 day in the oviduct, 3½-day ICM cells are still viable, as shown by the
Fig. 4. Electrophoretograms of conceptuses derived from blastocysts injected with ICMs recovered after one day in the oviduct. E, Embryonic fraction. EX, extra-embryonic fraction. T, trophoblastic fraction. (A) Gpi-1a/Gpi-1a mouse standard; (B) Gpi-1a/Gpi-1b mouse standard. Numbers correspond to conceptus code numbers in Table 2.

Table 2. Distribution of GPI isozymes in chimaeric foetuses derived from blastocysts injected with ICMs recovered after 1 day in the oviduct

<table>
<thead>
<tr>
<th>Conceptus code no.</th>
<th>Somite no.</th>
<th>Embryo</th>
<th>Extra-embryonic membranes</th>
<th>Ectoplacental cone and giant cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁</td>
<td>20 + s</td>
<td>A</td>
<td>A + B 10:1</td>
<td>A</td>
</tr>
<tr>
<td>C₂*</td>
<td>Resorption</td>
<td>A + B 10:1</td>
<td>—</td>
<td>A</td>
</tr>
<tr>
<td>C₃</td>
<td>20 + s</td>
<td>A</td>
<td>A + B 1:1</td>
<td>A</td>
</tr>
<tr>
<td>C₄</td>
<td>20 + s</td>
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<tr>
<td>C₆</td>
<td>ICS</td>
<td>A</td>
<td>A + B 2:1</td>
<td>A</td>
</tr>
</tbody>
</table>

The ratios next to the enzyme analyses indicate rough estimates of the proportion of A:B isozyme.

* C₂ had just begun to resorb so that division into embryonic and trophoblastic fractions was still possible.

A high rate of chimaerism (6/7) among conceptuses derived from blastocysts which had been injected with such ICMs (Table 2, Fig. 4). In one case, C₅, the injected ICM apparently gave rise to the whole embryo and its membranes, showing that 3½-day ICMs can retain their full potentiality in the oviduct environment.

However, although isolated 4½- and 3½-day ICMs can survive in the oviduct, they apparently do not form any trophoblast cells. This is indicated by the absence of typical trophoblastic properties, such as accumulation of fluid within cells and induction of the decidual cell reaction (Gardner, 1972). More critical evidence for the lack of trophoblast formation is provided by the experiment in which pairs of aggregated 3½-day ICMs, recovered after 1 day in the oviduct,
were injected into blastocysts. In all six chimaeras obtained thus, there was never any contribution of injected ICM cell progeny to the trophoblastic fraction (Table 2, Fig. 4).

Thus, no evidence has been found that exposing ICMs to 'outside' conditions in the oviduct environment can induce them to form trophoblast cells. This agrees with the results of aggregating ICMs and morulae, when no contribution of donor ICM cell progeny could be detected in the ectoplacental cone and trophoblastic giant cells of chimaeric foetuses (Rossant, 1975). Thus, although not overtly differentiated, ICM cells appear to be determined by 3½ days, since they do not form trophoblast either after isolation or transplantation.

Although isolated ICMs cannot 'reverse' their development in the oviduct environment, 3½-day ICMs can proceed to form an endoderm layer, as judged by histological and electron-micrographical evidence. The endoderm layer normally appears by 4½ days as a monolayer on the blastocoelic surface of the ICM, and spreads out over the inside of the trophoblast cells. Its origin has been in some doubt and indeed Dalcq has suggested on histochemical evidence that rat endoderm originates from the trophoblast (see Dalcq, 1957, fig. 43k). The present evidence argues that, in the mouse embryo, endoderm is derived from the ICM and that the presence of trophoblast is not needed, even indirectly, for this initial differentiation. Since no development beyond apparent endoderm formation has been observed in the isolated ICMs, a role for trophoblast in promoting later egg-cylinder development cannot be ruled out. Indeed, by using reconstituted blastocysts, Gardner has shown that there is a cellular contribution from the trophoblast to the egg-cylinder (Gardner, Papaioannou & Barton, 1973).

In isolated 3½-day ICMs recovered after 1 day in the oviduct, the endoderm cells always form a complete monolayer on the outer surface of the ICM, despite considerable size variation in the ICMs caused by damage during dissection. The endoderm layer in the intact 4½-day blastocyst lies in a comparable position on the surface of the ICM exposed to the blastocoel, and is also a complete monolayer. These similarities suggest that endoderm differentiation may depend on the position of the cells in the ICM. In intact blastocysts and isolated ICMs, it is possible that cells with surfaces exposed to the blastocoelic or oviducal fluid respectively may be induced to form endoderm. An alternative explanation is that predetermined but cytologically undifferentiated endoderm cells are present at 3½ days and later migrate to the outside. However, this seems unlikely since endoderm cells always form a complete monolayer around isolated 3½-day ICMs in the oviduct whatever the size of the ICM. The possible importance of position in endoderm formation has also been suggested by experiments in which rat ICMs were injected into mouse blastocysts (Gardner & Johnson, 1975). Endodermal chimaerism was predominant in the rat/mouse chimaeras and it was observed that the rat cells spread as a monolayer over the blastocoelic surface of the mouse ICM, i.e. in the region where endoderm is
formed. The fact that the rat ICMs occasionally formed complete egg-cylinders argued that this was a true position effect and not just enhanced survival of rat endoderm cells. Thus, it seems that cell position may play an important role in post-blastocyst development in the mouse. It has already been suggested as being of importance in pre-blastocyst development in the so-called ‘inside–outside’ hypothesis of ICM and trophoblast differentiation (Tarkowskis & Wroblewska, 1967; Graham, 1971).

Finally, the survival of zona-enclosed ICMs, despite damage to the zonae during microsurgery, supports the hypothesis that the main role of the zona in the mouse is physical protection of the egg in the oviduct (Bronson & McLaren, 1970; Modlinski, 1970). This may not apply to all other species. In the rabbit, for example, it was shown that single blastomeres injected into empty zonae did not develop well and leucocytic invasion was apparent in several cases (Moore, Adams & Rowson, 1968). In the present experiments, the only case of possible maternal cell invasion of the zonae occurred when the recipient was apparently not pseudopregnant.

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REFERENCES


Determination in the mouse inner cell mass. II


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