Time of commitment of inside cells isolated from preimplantation mouse embryos

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SUMMARY

Groups of inside cells (ICs) and inner cell masses (ICMs) were isolated from individual mouse embryos between the late morula and 3½-day expanded blastocyst stages using a modified immunosurgical procedure, and their purity and developmental potential were assessed in vitro.

Several different techniques failed to detect the presence of viable contaminating outside cells on ICs isolated from any of the stages studied. The numbers of inside cells isolated from the earlier stages, counted in air-dried preparations, were considerably higher than previous estimates from serial sections; whereas the numbers isolated from expanded blastocysts were in reasonable agreement. Thus the proportion of inside cells recovered by immunosurgery decreases over this period of development. In view of the evidence that inside cells divide at a faster rate than outside cells at these stages, it is argued that there may be an outward movement of inside cells capable of forming trophectoderm, during expansion of the blastocyst.

ICs and ICMs in vitro were observed to develop in one of two distinct ways according to the stage at which they were isolated. ICs from late morulae and some early cavitating blastocysts formed blastocyst-like vesicles over a period of 24–36 h in culture. The presence of trophectoderm cells in these vesicles was confirmed by the persistence of giant cells after ectopic transfer. In contrast ICs from a minority of early cavitating blastocysts, and all ICMs from 3½-day expanded blastocysts did not form vesicles, but proliferated endoderm-like cells. Thus at least some inside cells do not appear to lose the capacity to form trophectoderm and do not become committed to an ICM fate until after the initial formation of the blastocoel cavity.

INTRODUCTION

The earliest stage during mouse embryogenesis at which it has been possible to demonstrate cell commitment is the 3½-day blastocyst. At this stage the embryo consists of a hollow, spherical monolayer of trophectoderm cells surrounding the blastocoel cavity, and enclosing at one pole a second, less numerous, group of cells - the inner cell mass (ICM). Both of these groups of cells have been demonstrated to be committed to restricted developmental fates after their microsurgical (Rossant, 1975; Gardner & Johnson, 1972, 1973, 1975; Gardner & Papaioannou, 1975; Gardner & Rossant, 1976), or immunosurgical (Handyside & Barton, 1977) isolation and subsequent culture in vivo or in vitro.

In contrast, individual blastomeres isolated from the earlier (2½-day) eight-cell
stage do not appear to show restricted potential but may develop into blastocyst-like forms \textit{in vitro} (Tarkowski & Wroblewska, 1967). Furthermore, it has been demonstrated that after transfer to pseudopregnant recipients, individual blastomeres aggregated with several carrier blastomeres of a different genotype contribute to both ICM and trophectoderm derivatives in postimplantation embryos (Kelly, 1975, 1977). Thus, it appears that individual cells isolated from the eight-cell stage are totipotent, and that commitment to the ICM or trophectoderm of the 3½-day blastocyst must occur at some time during the intermediate morula stage (Johnson, Handyside & Braude, 1977).

There are two main hypotheses which attempt to explain how cells become committed to one of the alternative cell lineages. The first hypothesis proposes that commitment to a particular developmental line results from the presence of maternally-derived cytoplasmic factors which are unequally distributed between blastomeres during cleavage (Dalcq, 1957; Mulnard, 1965). Although the totipotency of all the blastomeres from a single eight-cell embryo has not yet been demonstrated, presumably for technical reasons, no convincing evidence has yet been found to support the segregation of cytoplasmic or nuclear factors during cleavage (see Kelly, 1975, 1977; Gardner & Rossant, 1976).

The alternative hypothesis, which is currently favoured (Tarkowski & Wroblewska, 1967), suggests that commitment results from the exposure of cells to different microenvironments. According to this hypothesis, cells that remain on the outside during cleavage are thereby committed to form trophectoderm while those that become wholly enclosed are committed to form ICM cells. Several experiments involving the rearrangement of genetically or physically marked blastomeres followed by an assessment of their subsequent fate, have tended to support this epigenetic hypothesis (Hillman, Sherman & Graham, 1972; Wilson, Bolton & Cuttler, 1972). These experiments have also been taken to suggest that commitment may occur late in the morula stage (Johnson \textit{et al.}, 1977).

It has now proved possible by use of a modification of the immunosurgical method to isolate a group of inside cells (IC) from individual embryos at various stages in the transition between morula and blastocyst (Solter & Knowles, 1975; Handyside & Barton, 1977). This has allowed the developmental capacity of ICs to be assessed directly \textit{in vitro}. The technique involves sequential incubation of embryos first in a rabbit antiserum to mouse species antigens, secondly in washing medium, and finally in complement. This protocol results in the selective lysis of the exposed outside cells. The ICs are then recovered as a compact ball of cells by drawing the embryo through a fine pipette.

The ICs recovered in this way were analysed for (1) contamination with outside cells and (2) their developmental potential \textit{in vitro}. 

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METHODS AND MATERIALS

Supply of embryos

CFLP female mice were superovulated and caged with males. Mating was detected the following day (day ½) by the presence of a copulation plug. Embryos were flushed from the oviducts or uteri at various times post-fertilization into Phosphate Buffered Medium 1, supplemented with 10% foetal calf serum (PBl + 10% FCS) (Whittingham & Wales, 1969). The zona pellucidae were removed by a 15–30 sec incubation in acid tyrode solution (pH 2.5 + 0.04% polyvinylpyrolidone) at 36 °C. The embryos were then allowed to recover for 1 h in PBl + 10% FCS at 36 °C to restore any loss of junctional adherence.

Staging

Seventy-two hours after fertilization (84 h after HCG injection) embryos were flushed either from the uterus or oviducts of individual mice and were generally found to be a mixture of late morulae and early cavitating blastocysts. Following zona removal and recovery in culture, embryos were sorted into three groups according to their morphology: late morulae; late morulae with some fluid accumulating outside cells; and early cavitating blastocysts. Embryos representative of these stages are shown in Fig. 1A–C. Furthermore the numbers of cells in a representative sample of embryos from each group were determined (Table 1).

Immunosurgery

The modified immunosurgical procedure (adopted after testing a number of schedules) consisted of a 2–4 min incubation in heat-inactivated rabbit anti-mouse antiserum (diluted 1:10 with PBl) at 36 °C, extensive washing in PBl + 10 % FCS, and finally a 15–30 min incubation in guinea pig complement (diluted 1:10 with PBl) at 36 °C, followed again by extensive washing. Reagents were as described previously (Handyside & Barton, 1976) and both incubations were carried out in 1 ml volumes. The lysed outside cells were removed by drawing the embryos through a finely drawn-out pasteur pipette with an internal diameter of approximately 30–50 μm. ICs were transferred through a final wash, to 25 μl droplets of PBl + 10% FCS or RPMI 1640 + 10% FCS under oil, in Falcon plastic Petri dishes. They were incubated at 36 °C in the presence of appropriate gas mixtures. The medium was changed every 2 days.

Assessment of purity

Various techniques were devised to detect the presence of any intact contaminant outside cells after immunosurgery.
Fig. 1. Embryos representative of the three developmental stages recovered 72 h post-fertilization (after zonae removed). (A) Late morulae (some outside cells appear to be decompacted). (B) Late morulae with some outside cells beginning to accumulate fluid (one zona intact; not all fluid accumulating cells in focus). (C) Early cavitating blastocysts. (Phase contrast.)
Table 1. Numbers of inside cells,* recovered by immunosurgery, from various embryonic stages

<table>
<thead>
<tr>
<th>Embryonic stage</th>
<th>Mean total number of cells per embryo ± S.D.</th>
<th>Mean number of inside cells ± S.D.</th>
<th>Ratio of inside/outside cells</th>
<th>Mean number of inside cells after 2 days in culture ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Late morula</td>
<td>30-24 ± 4-37 (25)†</td>
<td>12-93 ± 2-79 (14)</td>
<td>0-7470</td>
<td>12-50 ± 8-04 (6)</td>
</tr>
<tr>
<td>Morula with fluid accumulating cells</td>
<td>28-76 ± 5-12 (17)</td>
<td>13-90 ± 3-78 (10)</td>
<td>0-9354</td>
<td>10-00 ± 1-79 (6)</td>
</tr>
<tr>
<td>Early cavitating blastocyst</td>
<td>36-92 ± 8-33 (12)</td>
<td>17-56 ± 5-63 (16)</td>
<td>0-9070</td>
<td>N.C.</td>
</tr>
<tr>
<td>Expanded 3½-day blastocyst</td>
<td>82-50 ± 7-78 (2)</td>
<td>24-00 ± 4-36 (3)</td>
<td>0-4103</td>
<td>N.C.</td>
</tr>
<tr>
<td>4½-day blastocyst</td>
<td>141-25 ± 11-30 (4)</td>
<td>36-20 ± 10-33 (5)</td>
<td>0-3446</td>
<td>N.C.</td>
</tr>
</tbody>
</table>

* Cells counted in Giemsa stained, air-dried preparations (Tarkowski, 1966).
N.C. Not counted.
† Number of embryos studied.
(1) Incubation in melanin granules

Embryos were incubated for 2 h in a suspension of melanin granules in PB1 + 10% FCS at 36 °C before immunosurgery. The suspension was prepared by scraping fragments of melanin from the pigmented layer of the eye of C57Bl mice in a little medium, and decanting to eliminate large fragments (Gardner, 1975). Control embryos, embryos after incubation with melanin granules and subjected to 'mock' immunosurgery with heat inactivated complement, and ICs isolated from embryos that had been incubated with granules, were examined under phase contrast for cells with adherent granules.

(2) Immunofluorescence

Various fluorescein-conjugated antisera were used to detect the presence of outside cells which had bound the rabbit antibody but which had not been lysed in complement.

(a) Detection of rabbit antibodies

Direct. A fluorescein-conjugated globulin fraction of the rabbit anti-mouse antiserum was prepared and used for immunosurgery. Control embryos incubated in the fluorescent antiserum alone and ICs isolated from similarly treated embryos by subsequent incubation in complement were examined for fluorescence.

 Indirect. Control untreated embryos, embryos incubated in the unconjugated rabbit antiserum alone, and ICs were incubated for 15 min at room temperature in a fluorescein-conjugated goat antiserum to rabbit IgG (Miles Labs) diluted 1:10 with PB1.

(b) Use of fluorescent Concanavalin A as a marker for outside cells

Embryos were preincubated in fluorescein-conjugated Concanavalin A (Con A) diluted 1:10 with PB1 for 20 min at room temperature before immunosurgery. Control embryos incubated in fluorescent Con A alone, and ICs isolated from similarly treated embryos were examined.

The embryos were examined for fluorescence using a Zeiss epifluorescence microscope (incident fluorescent source HBO 200 with excitation filter system 427902 and barrier filter system 427903).

Cell counting

The numbers of cells in embryos were determined using an air-drying technique (Tarkowski, 1966). Embryos were incubated in 1% sodium citrate for 2–30 min at room temperature, until the cells swelled visibly, and were then transferred to a slide with minimal fluid. Drops of a 3:1 mixture of absolute ethanol and acetic acid were applied directly from above to fix the embryos and the slide was air-dried to obtain a spread of nuclei. The embryos were then
stained with drops of 2–5% Giemsa for 1–2 min, and the nuclei were counted under a microscope. If the lysed outside cells were not removed from ICs, the nuclei from these cells were also stained. However, these nuclei were easily distinguished as small darkly-stained discs and were only rarely detected in air-dried preparations of ICs freed of lysed outside cell debris.

Ectopic transfer of embryos

Control blastocysts and blastocysts derived from ICs in culture were separately transferred under the kidney capsules of anaesthetized male mice. Seven days later the mice were sacrificed and any resulting haemorrhagic nodules excised and fixed for 36 h in 50% Bouins. After alcohol dehydration and embedding in paraffin wax, the material was sectioned and stained with haematoxylin and eosin.

RESULTS

Purity of ICs

(1) Melanin granules

In three separate experiments control late morulae and early cavitating blastocysts subjected to mock immunosurgery were found to have adherent granules on all their outside cells under phase contrast. During complement lysis, a halo of lysing outside cells with adherent granules, surrounding the IC group was visible. After immunosurgery no granules could be detected in or on the 50 ICs isolated from these embryos (Fig. 2A).

(2) Immunofluorescence

(a) In a series of eight experiments, both the direct and indirect methods used to detect the presence of rabbit antibodies bound to the outside cells gave similar results. All of the outside cells of intact embryos incubated in rabbit antiserum, either directly fluoresceinated, or after incubation in fluorescein-conjugated goat anti-rabbit IgG were stained, when examined by varying the focal plane. This confirmed that all the outside cells had bound the rabbit antibodies after the 2–4 min incubation. Untreated whole embryos and all 39 ICs isolated from early cavitating blastocysts showed complete absence of fluorescence. Sixty-six ICs from morulae were often weakly stained but this was never as intense as that observed with the intact embryos incubated in the rabbit antiserum. This staining was most likely due to the immaturity of the junctions between the outside cells at this earlier stage which might allow detectable amounts of antibody to reach the inside cells but not sufficient for complement lysis during immunosurgery. Also, any debris from lysed outside cells adhering to the ICs and visible under phase contrast was intensely stained. Furthermore, when viable contaminating outside cells were deliberately left on ICMs crudely separated mechanically in the presence of pronase from 3½-day
Fig. 2. ICs representative of the various forms of development after 48 h in culture. (A) ICs isolated immunosurgically from early cavitating blastocysts preincubated in melanin granules. (B) IC, isolated from a late morula, with a number of trophectoderm-like fluid accumulating cells. An example of the 'non-integrated' forms that developed. (C) Two vesicles developed from late morula ICs, with a small number of inner cell mass cells visible. An example of the 'integrated' forms that developed. (D) Solitary outgrowing cells and fluid accumulating cells typical of endodermal-type development from an immunosurgically isolated 3½-day ICM. (Phase contrast.)
expanded blastocysts preincubated in fluorescent antisera, the cells were clearly and positively identified.

(b) The outer surfaces of intact embryos incubated in fluorescent Concana-valin A were also completely stained. However, treatment of embryos with Con A impaired efficient lysis and removal of labelled outside cell debris, and in some cases it was obvious during dissection that some of the outside cells had not been lysed.

In one experiment, when ten ICs isolated from early cavitating blastocysts pretreated in fluorescent Con A, were examined for fluorescence, four were found to be contaminated with fluorescent outside cells; two ICs were contaminated with lysed cell debris and the remaining four ICs showed no evidence of contamination. The detection of contaminating outside cells (never detected at this stage with other reagents when embryos were not pretreated with Con A) offered the opportunity to compare the behaviour of contaminated and uncontaminated ICs directly under the same culture conditions. Nine out of the ten ICs formed trophoblastic vesicles in culture. However, the contaminated ICs all showed evidence of fluid accumulation at least 12 h in advance of the uncontaminated ICs or those with lysed cell contamination. The one uncontaminated IC which did not show trophoblastic vesicle formation, showed evidence of considerable endodermal proliferation.

Numbers of inside cells recovered by immunosurgery

The numbers of cells in intact embryos, ICs immediately after isolation and ICs after 48 h in culture, are presented in Table 1. There was no significant difference in the numbers of cells counted in intact embryos from the various morphologically defined categories at 72 h post-fertilization. The numbers of inside cells recovered from these stages was just less than half the total number counted in control embryos. There did not appear to be an increase in the number of IC cells after they had been in culture long enough to develop vesicles (see below).

Development of ICs in vitro

Table 2 summarizes the behaviour of (226) ICs and ICMs isolated from various embryonic stages. Development in PB1 + 10% FCS and RPMI 1640 + 10% FCS were similar, so the observations for both conditions were pooled. ICs isolated from stages between the late morula and early cavitating blastocyst developed in one of two ways. The majority developed trophectoderm-like cells in culture, as indicated by their ability to accumulate fluid and form vesicles. After 16–24 h in culture ICs from each of these stages developed fluid accumulating cells (Fig. 2B), and by 36 h most of these had formed small blastocyst-like vesicles generally containing a few ICM cells (Fig. 2C).

A minority of ICs from early expanding blastocysts and all ICMs isolated immunosurgically from expanded 3½-day blastocysts developed an outer layer
Table 2. Development of immunosurgically-isolated ICs, after 48 h in vitro

<table>
<thead>
<tr>
<th>Percentage ICs isolated from:</th>
<th>Late morulae (incl. those with accumulating cells)</th>
<th>Early cavitating blastocysts</th>
<th>Expanded 3½-day blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trophoderm-like development only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Integrated forms*: Miniature blastocysts or trophodermal vesicles</td>
<td>70.97</td>
<td>55.63</td>
<td>0</td>
</tr>
<tr>
<td>(2) Non-integrated forms*: Loosely associated groups of fluid-accumulating cells</td>
<td>13.53</td>
<td>9.15</td>
<td>0</td>
</tr>
<tr>
<td>No morphological development</td>
<td>15.49</td>
<td>9.86</td>
<td>11.11</td>
</tr>
<tr>
<td>Endoderm-like development only</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3) Proliferation of outermost cells and outgrowth of solitary cells</td>
<td>0</td>
<td>19.01</td>
<td>61.11</td>
</tr>
<tr>
<td>(4) Proliferation of outermost cells with occasional fluid accumulating cell</td>
<td>0</td>
<td>2.82</td>
<td>22.22</td>
</tr>
<tr>
<td>(5) Development of both solitary outgrowing cells and endoderm-like fluid-accumulating cells</td>
<td>0</td>
<td>3.52</td>
<td>5.56</td>
</tr>
<tr>
<td>No. of ICs studied</td>
<td>66</td>
<td>142</td>
<td>18</td>
</tr>
</tbody>
</table>

* As defined in Tarkowski & Wroblewska, 1967.

Table 3. Morphological characteristics of trophoderm- and endoderm-type development of immunosurgically isolated ICs and ICMs, in vitro

<table>
<thead>
<tr>
<th>Trophoderm-type development (Arising from late morula-early cavitating blastocyst ICs)</th>
<th>Endoderm-type development (Arising from early to expanded blastocyst ICMs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Intracellular fluid accumulation after 18 h.</td>
<td>(1) Proliferation of outermost layer of cells; some intracellular fluid accumulation after 24 h.</td>
</tr>
<tr>
<td>(2) Intercellular fluid accumulation and formation of blastocyst-like vesicles, after 24–36 h.</td>
<td>(2) Formation of very large vesicles, involving large numbers of cells after 7–10 days in culture.</td>
</tr>
<tr>
<td>(3) Outgrowth of confluent monolayer of large cells with prominent nucleoli after 4 days in culture.</td>
<td>(3) Development of solitary outgrowing cells after 18 h. Confluent outgrowths sometimes seen after 10 days.</td>
</tr>
</tbody>
</table>
Fig. 3. (A) Trophoblast outgrowth from late morula IC-derived blastocyst after 4 days in culture. (Phase contrast.) (B) Trophoblast giant cells derived from blastocysts formed after groups of ICs were aggregated in culture and transferred under the kidney capsule 7 days previously. Stained with haematoxylin and eosin.
of endoderm-like cells characteristic of the 4½-day ICM. However, they also developed solitary outgrowing cells and small fluid-accumulating cells which were probably endodermal in origin (Fig. 2D). The morphological differences between these cells and those that developed from ICs isolated from earlier stages, and especially the timing of their appearance, were sufficient to allow them to be distinguished (Table 3) in the absence of more reliable criteria for endoderm.

After 4–6 days in culture blastocysts derived from ICs isolated from late morulae or early cavitating blastocysts attached and developed typical trophoblastic outgrowths (Fig. 3A). Under the same culture conditions control 3½-day blastocysts attached and outgrew after 3 days in culture.

**Ectopic transfer of ICs**

Seven days after ectopic transfer of IC-derived blastocysts, typical trophoblast giant cells were detected in the resulting haemorrhagic nodule (Fig. 3B).

**DISCUSSION**

The published procedures for the immunosurgical isolation of ICMs from expanded 3½-day blastocysts involve relatively long incubations (30 min) in both antiserum and complement (Solter & Knowles, 1975; Handyside & Barton, 1977). Under these conditions the recovery of viable inside cells depends on the continuing exclusion of antibody molecules by the tight junctions between the surrounding outside cells. Since these junctional complexes are not completely impermeable at the late morula stage (Ducibella & Anderson, 1975), prolonged incubation of morulae in antiserum allows sufficient binding to the inside cells to cause them to be lysed on the addition of complement (A. H. Handyside, unpublished; McLaren & Smith, 1977). However, by reducing the incubation period in antiserum, thereby reducing the amount of antibody binding to the inside cells of late morulae, it was possible to recover these cells in a viable condition in most cases.

Prior to the analysis of the developmental capacity of ICs, it was essential to establish that this modified immunosurgical procedure lysed all of the outside cells. Both the direct evidence presented here and several other observations have confirmed that ICs isolated from embryos between the late morula and early cavitating blastocyst stages were pure, and that the development observed in culture was not due simply to contamination with outside cells.

(1) After immunosurgery ICs appeared, under phase contrast, to be completely surrounded by lysed cells, whereas with embryos preincubated in fluorescent Con A it was obvious in some cases during dissection that lysis of outside cells was incomplete.

(2) Embryos incubated in a suspension of melanin granules and then subjected to 'mock' immunosurgery using heat-inactivated complement had considerable
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numbers of adherent granules over their entire surface. These granules could not be detected after similar embryos were subjected to normal immunosurgery.

(3) The presence of contaminating outside cells could not be detected with either fluorescent rabbit anti-mouse, or fluorescent goat anti-rabbit IgG antisera. Although ICs from late morulae were weakly stained with these reagents, probably due to the penetration of rabbit antibody, ICs isolated from early cavitating blastocysts which showed no staining nevertheless developed trophectoderm-like cells in the majority of cases (64-78%). Furthermore, ICs could be isolated in a viable state from early cavitating blastocysts with 15 min incubations in rabbit antibody, without affecting their ability to develop trophectoderm-like cells.

(4) ICs isolated from early cavitating blastocysts pretreated with fluorescent Con A (which noticeably impaired the efficiency of immunosurgery) could be clearly divided into contaminated and pure ICs. The development observed with these two groups of ICs showed that:

(a) the appearance of fluid accumulation and vesicle formation occurred 12 h earlier in contaminated ICs; and

(b) pure ICs did have the ability to form trophectoderm-like vesicles.

ICs routinely recovered immunosurgically from both morulae and early blastocysts do not show signs of fluid accumulation until 16 h after their recovery and do not form expanded vesicles until 24-36 h after recovery. Control morulae were fully expanded within 12 h and hatched from their zonae by 24 h.

(5) Analysis of the polypeptides synthesized by ICs immediately after isolation failed to detect any of the trophectoderm-specific polypeptides synthesized by intact embryos at these stages (Handyside & Johnson, 1977). However, the morphological development of trophectoderm-like vesicles in culture is paralleled by the initiation of the synthesis of all the polypeptides specific for trophectoderm isolated from 3½-day blastocysts (A. H. Handyside & M. H. Johnson, unpublished).

(6) When ten ICs isolated from early cavitating blastocysts were immediately subjected to a second immunosurgery, the small number of cells recovered also accumulated fluid and formed small trophectoderm-like vesicles after 36 h in culture (A. H. Handyside, unpublished).

Thus, by six independent criteria, the ICs recovered by immunosurgery regularly show no evidence of contamination. Furthermore, when trophectodermal cells do remain, as after control protocols or when Con A impairs efficiency of lysis, the behaviour of the contaminant cells clearly differs from that of ICs.

The numbers of cells in ICs recovered by immunosurgery from late morulae and early cavitating blastocysts were considerably greater than those previously counted in serial sections of intact embryos (Barlow, Owen & Graham, 1972). These authors counted a mean number of 10-86 inside cells in embryos with a total of 33–64 cells. More recent data from sections of 24 embryos with total
cell numbers in the range 25–37 cells (mean 30.83 cells), indicated the presence of 6.79 inside cells (Dr C. Graham, personal communication). The mean number of inside cells recovered by immunosurgery ranged from 12.93 for late morulae with a mean total number of 30.24 cells, to 17.56 inside cells for early cavitating blastocysts with a mean total number of 36.92 cells. The numbers of inside cells recovered by immunosurgery from late morulae are, however, in close agreement with the number of non-fluorescent cells detected after enzymic disaggregation of intact embryos incubated in fluorescent antisera (A. H. Handyside, unpublished). There are several possible explanations for the discrepancy between estimates based on serial section, and those resulting from incubation of embryos in antisera.

Firstly, immunosurgery allows the recovery of all functionally enclosed cells from the living embryo, whereas in serial sections shrinkage during fixation, or the presence of thin tongues of outside cell cytoplasm overlying inside cells might result in low estimates for inside cell numbers and inflation of outside cell estimates. Secondly, the numbers of inside cells recovered by immunosurgery can only be compared with the numbers of cells in control intact embryos, whereas in serial sections both populations can be counted for each individual embryo. Thirdly, there may be a genuine difference in cell numbers in the different mouse strains used.

In contrast to the data on morulae and early cavitating blastocysts, the numbers of ICM cells isolated immunosurgically from expanded blastocysts are in reasonable agreement with those previously published. This means that the ratio of inside/outside cells (as determined by immunosurgery) actually decreases over this period of development (Table 1). However, it has been suggested that inside cells divide at a faster rate than outside cells (Barlow et al. 1972). In that study, intact embryos were incubated in radioactive thymidine for varying periods, and it was shown that the proportion of labelled inside cell nuclei increased faster than those of outside cells as ascertained by autoradiography of serial sections. Since this conclusion was based on the rate of increase of the proportion of labelled inside cells, it is only strengthened by the possibility that some inside cells may have been erroneously identified as outside cells. Thus, if the proportion of inside cells recovered by immunosurgery is decreasing, despite the fact that they are dividing at a faster rate, an outward movement of inside cells during the expansion of the blastocyst would seem to be implied.

Evidence for such a rearrangement of cells already exists from experiments in which individual blastomeres or embryos were physically marked and reaggregated in inside or outside positions (Hillman et al. 1972; Wilson et al. 1972). In all of these experiments, cells or markers placed on the outside retained their position, whereas a significant proportion of cells placed internally ended up on the outside of the embryo. Furthermore, false blastocysts arising from individual blastomeres in culture have been described in which the ICM
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cells appear to be migrating into the trophectoderm and connect with the surface of the embryo (Tarkowski & Wroblewska, 1967). Also, the organization of the rabbit blastocyst into an embryonic disc (ICM) confluent with the trophoblast might be explained as a more extreme example of this outward movement. However, since there may be considerable strain differences in the division rate of cells at various stages, more direct evidence would be needed to establish this conclusion unequivocally.

ICs and ICMs immunosurgically isolated from embryos at each of the stages studied, developed in one of two distinct ways (Table 3). The first was characterized by a progression from the appearance of intracellular fluid accumulation after 18 h to the formation of blastocyst-like vesicles, over a period of 24–36 h. Since these vesicles gave rise to trophoblast giant cells after ectopic transfer, both these and the intermediate non-aggregated forms were considered to be due to the development of trophectoderm cells. Development of the second type was characterized by the appearance of solitary outgrowing cells after 18 h and the proliferation of the outermost cells after 24–36 h. These cells occasionally showed intracellular fluid accumulation after 24 h, but never formed blastocyst-like vesicles. Since this form of development closely resembled the behaviour of ICMs isolated from 3½-day expanded blastocysts in culture, these cells were considered to be various manifestations of endodermal type development. However, independent markers for endodermal cells are needed.

ICs isolated from 3-day late morulae (including those with intracellular fluid accumulation), always showed trophectoderm type development, whereas although most ICs isolated from 3-day early cavitating blastocysts developed in this way, a significant proportion developed only endoderm-like cells (Table 2). When the numbers of cells in embryos classified as early cavitating blastocysts were counted, the majority contained 28–34 cells, but a few contained 37–54 cells. The proportion of ICs giving rise to trophectoderm after isolation from embryos as this stage was the same in two groups segregated on the basis of size at a criterion of developmental age.

The development of trophectodermal vesicles from immunosurgically isolated ICs generally occurred earlier than the proliferation of endoderm, so that it is possible that some of the inside cells had the potential to form endoderm but this was obscured by the precocious development of trophectoderm. Thus the fact that no ICs were observed to develop both endoderm and trophectoderm cell types simultaneously at any stage does not necessarily imply that there is an abrupt changeover in developmental potential (Johnson et al. 1977). However, the overt capacity of inside cell groups to develop trophectoderm appears to be lost at some time during the expansion of the blastocyst, i.e. between the 32–64 cell stage (3·2 days post-fertilization). This is considerably later than the time at which inside and outside cells can be differentiated from each other by various criteria (Johnson et al. 1977; Handyside & Johnson, 1977).
The majority of trophectodermal vesicles which developed from ICs isolated from the earliest stages contained visible clusters of ICM cells. The existence of these cells raises the possibilities either that the inside cells from these embryos may have been heterogeneous in their state of commitment, or that all of the inside cells may have been capable of forming trophectoderm, but only those exposed on the outside of the compacted IC groups were stimulated to express this potential. In order to distinguish between these two possibilities, it is essential to assess the developmental potential of individual inside cells, and this study is at present underway.

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