Interactions of blastomeres suggest changes in cell surface adhesiveness during the formation of inner cell mass and trophectoderm in the preimplantation mouse embryo

By SUSAN J. KIMBER¹, M. AZIM H. SURANI² 
AND SHEILA C. BARTON²

From the A.R.C. Institute of Animal Physiology, 
Animal Research Station, Cambridge

SUMMARY

Whole 8-cell morulae can be aggregated with isolated inner cell masses from blastocysts. On examining semithin light microscope sections of such aggregates we found that cells of the morula changed shape and spread over the surface of the ICM, thus translocating it to the inside of the aggregate. Using single cells from 8-cell embryos in combination with single cells from other stage embryos or isolated ICMs we show that 1/8 blastomeres spread over other cells providing a suitably adhesive surface. The incidence of spreading is high with inner cells from 16-cell embryos (56%) and 32-cell embryos (62%) and isolated inner cell masses (64%). In contrast, the incidence of spreading of 1/8 blastomeres is low over outer cells from 16-cell embryos (26%) and 32-cell embryos (13%). Blastomeres from 8-cell embryos do not spread over unfertilized 1-cell eggs, 1/2 or 1/4 cells or trophectoderm cells contaminating isolated ICMs. When 1/8 cells are aggregated in pairs they flatten on one another (equal spreading) as occurs at compaction in whole 8-cell embryos. However, if 1/8 is allowed to divide to 2/16 in culture one of the cells engulfs the other (51–62% pairs). Based on the ideas of Holtfreter (1943) and Steinberg (1964, 1978) these results are interpreted to indicate an increase in adhesiveness at the 8-cell stage as well as cytoskeletal mobilization. Following the 8-cell stage there is an increase in adhesiveness of inside cells while the outside cells decrease in adhesiveness. The difference in adhesiveness between inside and outside cells in late morulae is probably central to the divergent differentiation of (inner) ICM and (outer) trophectoderm cell populations.

INTRODUCTION

Cell adhesiveness is central to the differentiation and morphogenesis of developing systems (Holtfreter, 1943; Steinberg, 1964, 1978). Since differences in cell surface adhesiveness have been correlated with cell position it has been

¹ Author’s present address: M.R.C. Laboratory, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.
² Author’s address: A.R.C. Institute of Animal Physiology, 307 Huntingdon Road, Cambridge, CB3 0JQ, U.K.
suggested that such differences are responsible for the arrangement of cells in the embryo. For instance, cells which are more adhesive than other cells by independent criteria tend to segregate towards the middle in a mixed aggregate of cells (Steinberg, 1978). Recent evidence for differences between apical and lateral surfaces of the superficial cells of amphibian embryos (Roberson, Armstrong & Armstrong, 1980) may relate to regional differences in adhesive-ness of these cells (Holtfreter, 1943; Roberson et al. 1980).

At the 8-cell stage in the mouse embryo all blastomeres have their apices exposed to the outside and are comparable to the superficial cells of amphibian embryos. Indeed, there is now a considerable body of literature showing differences between cell surfaces facing other cells and those exposed to the outside in 8-cell mouse embryos (Ducibella & Anderson, 1975; Handyside, 1980; Reeve & Ziomek, 1981; Ziomek & Johnson, 1981). Another important property of 8-cell blastomeres is their ability to undergo a calcium-dependent change in shape (Lewis & Wright, 1935; Ducibella & Anderson, 1975, 1979; Lehtonen, 1980) spreading on one another to increase the area of intercellular contact. During this process, known as compaction, there is a reorganization of cytoskeletal elements (Ducibella & Anderson, 1975, 1979; Ducibella, Ukena, Karnovsky & Anderson, 1977) and a redistribution of microvilli with the extension of cell processes (Calarco & Epstein, 1973; Lehtonen, 1980). Compaction is prevented by inhibitors affecting the cytoskeleton (Ducibella & Anderson, 1975; Surani, Barton & Burling, 1980; Kimber & Surani, 1981a; Pratt, Chakraborty & Surani, 1981), protein glycosylation (Atienza-Samols, Pine & Sherman, 1981; Surani, 1979; Surani, Kimber & Handyside, 1981; Surani, Osborn & Kimber, unpublished) and by antisera to embryonal carcinoma cells (e.g. Johnson et al. 1979; Hyafil, Morello, Babinet & Jacob, 1980).

Our understanding of the divergent cellular differentiation into inner cell mass and trophectoderm of the blastocyst depends on our knowledge of the regulation of cell allocation to inside and outside positions during earlier development. The outer cells develop as trophectoderm and the inner cells as ICM, presumably under the influence of the cellular microenvironment (Mintz, 1964; Tarkowski & Wroblewska, 1967; Hillman, Sherman & Graham, 1972; Kelly, 1979). It is at the 8- to 16-cell stage that a group of cells which are completely enclosed within the embryo are first detected (Barlow, Owen & Graham, 1972; Kelly, Mulnard & Graham, 1978; Handyside, 1981). Although extensive cell movements do not occur during development (Garner & McLaren, 1974; Kelly, 1979) restriction of the potential of cells by segregation of cytoplasmic components (Dalcq, 1957; Mulnard, 1965) seems unlikely since isolated groups of either inside or outside cells from late morulae yield viable blastocysts (Handyside, 1978; Rossant & Vijh, 1980). Furthermore, cell interactions and relative cell movements do occur and appear to be responsible for the cellular arrangement of the 16-cell embryo (Graham & Lehtonen, 1979).

With this information in mind we wished to test experimentally our proposal
that cell adhesiveness influences the position of blastomeres in the mouse embryo. It is known that ICMs can readily be aggregated with one another (Gardner, 1971) or with 8- to 16-cell morulae (Rossant, 1975). In the latter case they probably translocate to the inside of the host embryo since they contribute to the ICM of the chimera. Early morulae will also aggregate together, but 8–9 h before blastocoel formation intact embryos lose their ability to aggregate (Burgoyne & Ducibella, 1977) although the inner cells may retain the capacity to aggregate. We therefore first examined the process of aggregation of ICMs with 8-cell morulae. We also used combinations of single cells with cells from different stages of development or with ICMs to examine cell surface changes. Blastomeres from 8-cell embryos were ideal test cells because of their ability to spread on appropriately adhesive surfaces. Using this assay of cell adhesiveness we show in this study that inner cells of late morulae and ICMs are substantially more adhesive than peripheral cells or cells from earlier embryos. These findings support our proposal that the most adhesive blastomeres occupy the inner position in the embryo and therefore are most likely to give rise to the inner cell mass while the outer less adhesive cells form the trophoderm (Kimber & Surani, 1981; Surani, Kimber & Barton, 1981).

**MATERIALS AND METHODS**

Embryos were obtained from females of an outbred strain of albino MF1 mice (bred at ARC Animal Research Station from MF1 parents obtained from OLAC). Five-week-old females were superovulated using 5 i.u. pregnant mare’s serum (PMS) followed 44–48 h later by 5 i.u. human chorionic gonadotrophin (HCG) (Intervet, Milton, U.K.). Each female was caged with a C57 BL × CBA,F1 male for mating and checked the following morning for a vaginal plug to confirm mating. The day of the vaginal plug was counted as day 1 of pregnancy.

Embryos were flushed from the oviduct at the 2-cell stage between 2 p.m. and 4 p.m. on day 2 of pregnancy approximately 45–47 h post HCG, and cultured *in vitro* for later stages up to 32-cell stage in Brinster’s medium (Brinster, 1970) to which 4 mg/ml bovine serum albumin (BSA) (Sigma, Poole, U.K.) were added. For inner cell masses blastocysts were flushed from the uterus on day 4 of pregnancy. The rate of cleavage and development was established as being identical to that observed *in vivo*.

**Isolation of cells**

2- to 8-cell blastomeres. To obtain single cells from the 2- to 8-cell stage the zona pellucida was removed with acid tyrode (pH 2) (Nicolson, Yanagimachi & Yanagimachi, 1975). Single 8-cell blastomeres were always obtained from embryos in an early stage of compaction. The embryos were cultured in Ca²⁺- and Mg²⁺-free medium with 4 mg/ml BSA at 37 °C in 5% CO₂ in air for up to
20 min. The cells were then separated by pipetting through finely drawn Pasteur pipettes. Separated cells were washed nine times in BMOC-3 and returned to the incubator and utilised within 1–2 h after isolation.

16-cell outer and inner blastomeres. Zona-pellucida-denuded embryos were decompacted by incubating for 10 min at 37 °C in tyrode medium containing 0.2 mM-EDTA and 0.125% trypsin (Rossant & Vijh, 1980) or Ca²⁺- and Mg²⁺-free culture medium for 20 min as described above. The embryos were washed eight times in phosphate-buffered saline with 10% heat-inactivated foetal calf serum (FCS) (Gibco, Paisley, Scotland). They were then allowed to recover for 2 h in BMOC-3 containing 10% FCS. Up to three of the outermost cells from each embryo were plucked from the surface of the decompacted morulae using a Leitz micromanipulator (Rossant & Vijh, 1980). The remainder of the embryo (and additional embryos) was gently pipetted to release the bulk of the cells, usually leaving behind a clump of about three cells (see below for further details).

An alternative procedure was also used to yield both the inner and outer populations partly based on studies by others (Handyside, 1980; Johnson & Ziomek, 1981). The 16-cell embryos were incubated for 20 min in Ca²⁺- and Mg²⁺-free culture medium at 37 °C. After decompaction, the embryos were gently pipetted through flame-polished Leitz glass capillaries. A variable number of couplets were obtained from each embryo which in some cases had one large and one small cell. Only these couplets were used and the two cells were separated to yield a large and a small cell. The larger cells corresponded to the outer cells and the smaller cells to the inner cells in the morula (see below for further details). The results using cells separated by the two procedures were combined since they were similar.

32-cell outer and inner blastomeres. Morulae were decompacted as described above (Rossant & Vijh, 1980) and the outermost cells (maximum of four) were plucked with the aid of the micromanipulator. For the inner cells, the morulae were repeatedly pipetted to yield a clump of eight to ten cells. This clump was repeatedly pipetted to yield the individual inner cells.

Isolation of the inner cell mass

Inner cell masses were either isolated by microsurgery (Gardner, 1971) or by using the ionophore A23187 (Surani, Torchiana & Barton, 1978) from day-4 or day-5 blastocysts. Day-4 ICMs were cultured overnight before aggregating with 1/8 blastomeres.

Validation for the inner and outer cell types

During the course of these studies, we carried out extensive preliminary investigations to assess the origin of cells used from the 16- and 32-cell morulae. For this purpose embryos were incubated in FITC-Concanavalin A (FITC-Con A) (Miles, Slough, U.K.) at 1 mg/ml in PBI medium with 4 mg/ml BSA
Interaction of cells from different stage mouse embryos

and 0.02% (w/v) sodium azide for 15 min at room temperature prior to the separation of cells. This procedure predominantly labels the outer cells (Handyside, 1980). After isolation of inner and outer cells populations by the methods described above, these groups of cells were examined by fluorescence microscopy. In the majority of the cases (> 80%), the identification was found to be valid with the outer cells being fluorescent and the inner cells being non-fluorescent. This procedure has been repeated periodically since cells typed in this way are being used in other studies in progress and the results will be published elsewhere.

Fluorescence microscopy

Groups of 10–15 cells (designated as outer or inner) were placed in a microdrop of PBI + BSA with azide, under paraffin oil (BDH, Poole, U.K.) on cytotoxicity slides (Baird & Tatlock, Romford, U.K.) and examined under a Zeiss microscope with epifluorescence attachments, incident source HB200 with excitation filter BP450-490 and barrier filter LP520.

Criteria for cell viability

During the course of these and other experiments, some isolated typed cells were not utilized. This was in many cases because of uncertainty about their origin. Cells of uncertain origin and those which had been typed were cultured either individually or in groups of 10–30 cells. In the case of isolated cells between 70–80% went through one cleavage division by 12–14 h in culture (324 cells observed). Larger groups of typed as well as undefined cells were cultured for varying periods of time depending on the stage of development and monitored concurrently with intact embryos up to the blastocyst stage. In the majority of cases, the isolated cells formed aggregates and subsequently blastocysts. These criteria allowed us to assess our culture conditions and demonstrated that the cells were viable, capable of division, cell interactions and morphogenesis up to the blastocyst stage.

Aggregation and culture of blastomeres

The cells used in the aggregation studies were separated using the criteria established above but concanavelin A was not used in order not to influence cell surface properties and hence influence cell interactions. The procedure was, however, checked periodically to ensure correct typing of cells.

The cells to be aggregated were placed in a microdrop of BMOC-3 medium with BSA under paraffin oil in a Sterilin petri dish. Foetal calf serum (10%) was used instead of BSA when ICMs or 32-cell blastomeres were being used. The dishes were placed in an incubator for 15–30 min at 37 °C in 5% CO₂ in air. The cells were then aggregated by rolling them over each other until they were not dislodged by shaking the dish. This procedure has several advantages over the use of PHA (Ziomek & Johnson, 1981) to induce aggregation. Firstly,
this procedure may ensure that cells aggregate with regions of other cells which are naturally most adhesive. In the presence of PHA cells may attach randomly or by regions which are not naturally adhesive to other cells. Secondly, different amounts of PHA may bind to different cell types and this could lead to interactions between cells which do not reflect their natural behaviour. Some cells persistently failed to aggregate even after a second attempt to encourage adhesion following 1 h in culture. One of the cells in these instances did not survive, or failed to divide, and hence this eliminated aggregates where one of the cells was perhaps damaged. This procedure further ensured that those aggregates we monitored consisted of healthy cells.

Aggregates were observed and photographed after 6 h and 8–9 h in culture using a Leitz Diavert inverted-phase microscope. Some aggregates were fixed for light and electron microscope after this time as described below. We continued to culture most aggregates after these observations up to 22 h, especially where spreading of one cell stage on another did not occur, to verify our results. However, the results included in this paper are those obtained after 6–9 h unless otherwise stated.

Preparation for light and electron microscopy

Aggregates were fixed in 2.5% glutaraldehyde 1% paraformaldehyde in 0.075 M sodium cacodylate buffer (pH 7.5–7.6) containing 2 mM calcium. In some cases 0.1% potassium ferricyanide was added. Aggregates were post fixed in 1% osmium tetroxide, stained overnight in 1% uranyl acetate and embedded in Epon. Thick, 0.5 μm, and thin, 50–100 nm, sections were cut on an LKB ultramicrotome. Thick sections were stained with 1% toluidine blue and viewed and photographed using a Zeiss microscope with camera attachment. Thin sections were stained with a saturated solution of uranyl acetate in 50% ethanol followed by lead citrate (Reynolds, 1963) and examined in an AEI 801 electron microscope.

RESULTS

The behaviour of various combinations of cells from preimplantation mouse embryos of different stages is recorded in Table 1. The table shows that cells from 8- and 16-cell morulae can spread over those from certain other stages.

Aggregation of 8-cell embryos with ICMs and 1/8 blastomeres with ICMs

The process by which ICMs become incorporated into whole morulae was examined using light microscopy on semithin sections of aggregates. Figures 1 and 2 show two stages in the engulfment of ICMs by blastomeres of morulae. The blastomeres adjacent to the ICM send out cytoplasmic extensions over the ICM surface. These give the appearance of drawing the ICM into the centre of the morula so that it is finally surrounded by blastomeres.

Individual blastomeres from 8-cell embryos (1/8) spread over ICMs in 64%
Interaction of cells from different stage mouse embryos

Table 1. Interaction between cells from different stages of preimplantation mouse embryos in culture following 6–9 h of adhesion

<table>
<thead>
<tr>
<th>(a)</th>
<th>(b)</th>
<th>No. of experiments</th>
<th>Total no. of aggregates</th>
<th>Incidence of spreading of cell stage (a) over cell stage (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/8 + 1ICM</td>
<td></td>
<td>6</td>
<td>148</td>
<td>95 %</td>
</tr>
<tr>
<td>1/8 + 1/32 unselected</td>
<td></td>
<td>3</td>
<td>126</td>
<td>61 %</td>
</tr>
<tr>
<td>1/8 + 1/32 outside</td>
<td></td>
<td>3</td>
<td>52</td>
<td>7 %</td>
</tr>
<tr>
<td>1/8 + 1/32 inside</td>
<td></td>
<td>1</td>
<td>13</td>
<td>8 %</td>
</tr>
<tr>
<td>1/8 + 1/16 outside</td>
<td></td>
<td>4</td>
<td>72</td>
<td>19 %</td>
</tr>
<tr>
<td>1/8 + 1/16 inside</td>
<td></td>
<td>3</td>
<td>48</td>
<td>27 %</td>
</tr>
<tr>
<td>1/8 → 1/16 + 1/16</td>
<td></td>
<td>4</td>
<td>277</td>
<td>140 %</td>
</tr>
<tr>
<td>1/8 + 1/8</td>
<td></td>
<td>4</td>
<td>84</td>
<td>— *</td>
</tr>
<tr>
<td>1/8 + 1/4</td>
<td></td>
<td>2</td>
<td>46</td>
<td>1 %</td>
</tr>
<tr>
<td>1/8 + 1/2</td>
<td></td>
<td>2</td>
<td>116</td>
<td>2 %</td>
</tr>
<tr>
<td>1/8 + 1-cell unfertilized</td>
<td></td>
<td>1</td>
<td>20</td>
<td>—</td>
</tr>
<tr>
<td>1/4 + 1ICM</td>
<td></td>
<td>2</td>
<td>17</td>
<td>—</td>
</tr>
<tr>
<td>1/4 + 1/4</td>
<td></td>
<td>1</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>1/4 + 1/2</td>
<td></td>
<td>1</td>
<td>14</td>
<td>—</td>
</tr>
</tbody>
</table>

* Spreading of 1/16 over its sibling occurred after division (see Results).
† See also Table 2.

of 1/8 ICM aggregates. There was never any indication of ICM cells spreading over 1/8 cells. When 1/8 blastomeres spread over ICMs they put out similar cytoplasmic processes to those seen when ICMs were aggregated with whole morulae (Figs. 4 and 5). In combinations of 1/8 blastomeres with ICMs the 1/8 cell rarely covers more than two thirds of the surface of the ICM. This is perhaps because day-5 ICMs are larger than 1/8 blastomeres. Alternatively, endoderm is being formed on the outside of the ICM at this time and may prevent the 1/8-cell spreading all round. In 5 out of the 15 1/8-ICM combinations fixed after 8 h adhesion the 1/8-cell had divided to 2/16 and these were spreading over the surface of the ICM. In three of these aggregates the division plane of the 1/8-cell had been perpendicular to the surface of the ICM while in the other two cases the division plane was parallel to the surface of the ICM. In the first three aggregates both cells spread over the ICM while in the other two aggregates only the 1/16 cell in contact with the ICM was spreading. Only 33% of 1/8-cells divided in combination with ICMs, in contrast to 78% of isolated 1/8-cells (Table 2).

The electron micrographs in Figs. 5 and 6 reveal more details about the interaction between 1/8 blastomeres and ICMs. The processes of the 1/8 cell are 1–4 μm thick and make close contact with the cells of the ICM (Figs. 6 and 8). Lipid droplets are frequently found in the distal region of the extending processes and there are also large numbers of mitochondria within the processes
Fig. 1. Light micrograph of a section through a whole morula (M) aggregated with an ICM (I) for 6 h. Two blastomeres are beginning to spread over the surface of the ICM (arrows). Bar = 20 μm.

Fig. 2. Light micrograph of a section through a morula aggregated with an ICM (I) for 9 h. The ICM has been almost entirely engulfed by the blastomeres of the morula. Bar = 20 μm.

Fig. 3. Light micrograph of a section through a 1/8 blastomere aggregated with an ICM (I) for 6 h. The 1/8 blastomere has compacted onto the ICM. Bar = 10 μm.

Fig. 4. Light micrograph of a section through a 1/8 blastomere aggregated with an ICM (I) for 9 h. The blastomere has put out processes (arrows) spreading over the ICM surface. Bar = 10 μm.
Fig. 5. Electron micrograph showing 1/8 blastomere (1/8) spreading over the surface of an ICM (i) 9 h following initial adhesion. Note the close membrane approximation. Mitochondria, m; lipid droplets, li. Bar = 2 μm.

(Figs. 5 and 7). Microtubules are abundant beneath the plasma membrane of the 1/8 blastomeres (Fig. 6).

A small number of the isolated ICMs were contaminated by large cells with the appearance of trophoderm (Figs. 7 and 8) and in these cases 1/8 blastomeres were found to spread over the surface of the ICM until they contacted the
Interaction of cells from different stage mouse embryos
trophectoderm-like cells. They never spread over the surface of these cells
although they did make fairly intimate contact with them. The only specialized
cell contacts found between 1/8 blastomeres and the cells they spread over were
focal contacts (Fig. 8) which appear similar to the ‘cell feet’ of cells adhering
to a substratum in culture (Abercrombie, Heaysman & Pegrum, 1971; Brunk,
Ericsson, Ponten & Westermark, 1971).

Aggregation of 1/8 blastomeres with 1/32 blastomeres

1/8 blastomeres spread over 1/32 blastomeres in 48% of pairs (Figs. 9 and
10). However, in further experiments blastomeres were obtained from the outside
of 32-cell embryos as described in Materials and Methods. When 1/8 blasto-
meres were combined with these they spread over them in only 13% of pairs.
However, 1/8 cells spread over inside cells from 32-cell morulae in 62% of
pairs. When spreading of 1/8 over 1/32 occurs the 1/32 cell may be almost
completely engulfed (Fig. 10) apart from a small bleb of cytoplasm remaining
in contact with the outside in some instances.

Division of 1/8 cells in combination with 1/32 cells was reduced to approx-
imately 30% with inner 1/32 cells and approximately 40% with outer 1/32
cells after 14 h.

Aggregation of 1/8 blastomeres with 1/16 blastomeres

When 1/8 blastomeres were combined with blastomeres from the outside of
16-cell embryos, spreading of the 1/8 cell over the second cell occurred in only
26% of pairs. However, with inside cells from 16-cell morulae spreading of
1/8 over 1/16 occurred in 56% of cases (Fig. 11). Again cell division was
suppressed, occurring in 28% of 1/8 cells with inner 1/16 cells and in 42% of
1/8 cells in combination with outer 1/16 cells after 12-14 h.

Aggregation of 1/8 blastomeres with 1/8 blastomeres

When 1/8 cells were paired with other 1/8 cells they compacted together,
flattening on one another within 2–3 h. Unequal spreading of 1/8 cell on
the other was never seen. Seventy-five per cent of 1/8 cells had divided within
12 h in this combination and one cell of the 2/16 arising was observed in several
sectional aggregates to have spread over its sibling (see below). In several other
aggregates a 1/8 cell spread over only one of the 2/16 cells derived from the other 1/8 cell. Insufficient aggregates were sectioned to quantify this behaviour, analysis of which was complicated by the number of cells present.

**Behaviour of 2/16 blastomeres derived from 1/8**

When isolated 1/8 cells were allowed to divide in culture to 2/16 one cell spread over and engulfed the second (Figs. 12 and 13) in 51% of cases (range 30–65%).

An additional experiment was subsequently carried out in which all the blastomeres from a single 8-cell morula were examined for the incidence of engulfment (see Table 2). A total of 20 embryos were examined in this way (total 160 cells). As shown, in the majority of cases, six or more isolated cells from each embryo divided after 9 h culture with an overall frequency of cell division of 78%. Of the cells that divided, in 62% of cases one of the cells totally, or almost totally, engulfed the other whilst in the remainder (38%), the cells compacted in a manner similar to that observed when two 1/8 cells

<table>
<thead>
<tr>
<th>Embryo no.</th>
<th>No. cells from each 8-cell embryo which divided after isolation (%)</th>
<th>Unequal spreading (%)</th>
<th>Compacted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7 (87-5)</td>
<td>5 (71)</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>6 (75)</td>
<td>3 (50)</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>6 (75)</td>
<td>3 (50)</td>
<td>3</td>
</tr>
<tr>
<td>*4</td>
<td>8 (100)</td>
<td>4 (50)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>5</td>
<td>7 (87-5)</td>
<td>5 (71)</td>
<td>2</td>
</tr>
<tr>
<td>*6</td>
<td>8 (100)</td>
<td>6 (75)</td>
<td>2 (25)</td>
</tr>
<tr>
<td>7</td>
<td>4 (50)</td>
<td>4 (50)</td>
<td>0</td>
</tr>
<tr>
<td>*8</td>
<td>8 (100)</td>
<td>7 (87-5)</td>
<td>1 (12-5)</td>
</tr>
<tr>
<td>9</td>
<td>3 (37-5)</td>
<td>0 (0)</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>5 (62-5)</td>
<td>4 (80)</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>5 (62-5)</td>
<td>2 (40)</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>7 (87-5)</td>
<td>7 (100)</td>
<td>0</td>
</tr>
<tr>
<td>*13</td>
<td>8 (100)</td>
<td>3 (37-5)</td>
<td>5 (62-5)</td>
</tr>
<tr>
<td>14</td>
<td>7 (87-5)</td>
<td>7 (100)</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>7 (87-5)</td>
<td>1 (17)</td>
<td>6</td>
</tr>
<tr>
<td>16</td>
<td>5 (62-5)</td>
<td>0 (0)</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>6 (75)</td>
<td>6 (100)</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>7 (87-5)</td>
<td>3 (43)</td>
<td>4</td>
</tr>
<tr>
<td>19</td>
<td>7 (87-5)</td>
<td>6 (86)</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>3 (37-5)</td>
<td>1 (33)</td>
<td>2</td>
</tr>
<tr>
<td>Total (%)</td>
<td>124 (77-5)</td>
<td>77 (48-1)</td>
<td>47 (29-4)</td>
</tr>
</tbody>
</table>

% of those which divided 62-1 37-9

* All the 1/8-cells from each embryo divided.
were combined. However, it is also noteworthy that of the embryos in which all the cells divided (Embryo nos. 4, 6, 8 and 13), the incidence of engulfment varied substantially from 38 to 88%.

**Other combinations of cells**

Blastomeres from 8-cell embryos adhere to 1/4 cells but do not spread on them. This is also true for 1/8 blastomeres with 1/2 cells or unfertilized 1-cell eggs. Blastomeres from 4-cell embryos will not spread on 1/8 cells, 1/2 cells, other 1/4 cells or ICMs. However, after 1/4 has divided to 2/8 cells, these will spread on ICMs. Seventy per cent of 1/8 cells in combination with 1/4 cells and 1/2 cells divided to 2/16 while 75–100% of 1/4 cells in combination with 1/8 cells, 1/2 cells, other 1/4 cells and ICMs had divided (some more than once) after 12–14 h.

**DISCUSSION**

When inner cell masses are aggregated with morulae they contribute to the ICM of the combined embryo (Rossant, 1975). ICMs (this study) or embryonal carcinoma cells (Stewart, 1980; Stewart & Kimber, submitted) are translocated to the inside of the morula by the active spreading of blastomeres over these cells using processes similar to the lamellipodia or filopodia characteristic of moving cells (Trinkhaus, 1976) rather than these cells burrowing into the morula. Similarly, filopodia-like processes have been observed extending from one cell over another during compaction of 8-cell morulae (Calarco & Epstein, 1973).
If more adhesive cells tend, on thermodynamic grounds, to maximise their cell
contacts and segregate to the inside of a mixed aggregate of cell (Steinberg,
1964, 1978) an ICM may be more adhesive than the outer surface of an 8-cell
morula. This study also shows that 1/8 blastomeres spread over as much as
two thirds of the surface of an isolated ICM in 64% of aggregates. Although the
process of spreading over day-5 ICMs may have been restricted by the different-
iation of endoderm, this seems unlikely since ICMs are effectively translocated
to the inside of whole morulae at this stage.

There are several interesting features of 8-cell blastomeres which are probably
essential for the organization of the embryo and, after subsequent division, for
the allocation of cells to the inner and outer positions in the embryo. At the
8-cell stage in the mouse embryo all cells are equivalent with apices exposed to
the outside and can contribute to formation of both ICM and trophectoderm
(Kelly, 1975, 1977). The 8-cell blastomere is a superficial or peripheral cell as
defined by Holtfreter (1943) who suggested that 'one of the earliest indications
of an existing polarity is a difference in adhesiveness between the exterior and
the interior walls of the peripheral cells. This lack of adhesiveness is character-
istic of all peripheral regions of the egg and the embryo'. Regionalization of the
membrane domains in the 8-cell mouse embryo is also well established on the
basis of the distribution of various cell surface antigens (Handyside, 1980;
Wiley & Eglitis, 1981), localization of alkaline phosphatase (Mulnard &
Huygens, 1978; Izquierdo, Lopez & Marticorena, 1980), distribution of
microvilli (Ducibella et al. 1977; Reeve & Ziomek, 1981; Wiley & Eglitis,
1981) and adhesiveness (Burgoyne & Ducibella, 1977; Surani et al. 1981). It is
also well known that 8-cell blastomeres are capable of undergoing a change in
cell shape presumably as a result of mobilization of the cytoskeleton (Lewis &
Wright, 1935; Ducibella & Anderson, 1975; Lehtonen, 1980; Kimber & Surani,
1981a).

When two 1/8 blastomeres are aggregated they flatten against each other
in a way which is reminiscent of compaction (Lehtonen, 1980). This behaviour,
in contrast to engulfment indicates the equivalence of the two cells especially
perhaps with respect to their adhesiveness (Kimber & Surani, 1981a; Surani
et al. 1981). The 1/8 blastomeres show no evidence of spreading over earlier
blastomeres which may lack adhesive properties as well as the ability to undergo
a change in shape themselves.

---

Fig. 11. Blastomere from an 8-cell embryo spreading over the surface of an inside
cell from a 16-cell embryo 9 h following initial adhesion. Bar = 10 μm.

Fig. 12. Isolated 1/8 cell divided to 2/16-cells in culture. One cell is spreading over
the surface of the second, 9 h following initial adhesion. Bar = 10 μm.

Fig. 13. Light Micrograph showing 2/16 blastomeres derived from a 1/8-blastomere
30 h following initial adhesion. Bar = 10 μm.
In marked contrast to the results above the 1/8 blastomeres behave in a different manner towards cells from later morulae depending on whether these are derived from the inner or outer cell populations. When combined with an inner cell a 1/8 blastomere almost entirely engulfs it (56–62%) whilst in combination with outer cells this occurs in a much reduced percentage of aggregates (13–26%). Comparing these two groups as a whole, the inner cells are substantially more adhesive than the outer cells assuming that 1/8 blastomeres can only spread over sufficiently adhesive cells.

There are several explanations for the failure of the remainder of the inner cells to be engulfed while a small proportion of the outer cells were engulfed. A small proportion of the typed cells may have been wrongly assigned to inner cell or outer cell groups. It is also possible that some cells were damaged or their surface properties altered by the separation procedure although damaged cells do not adhere in our experience (in the absence of PHA) and would therefore be eliminated. In some cases 1/8 blastomeres may have been attached to the cells by their non-adhesive outer microvillous pole (Reeve & Ziomek, 1981) but this seems less likely in the absence of PHA. Furthermore, ICMs placed on the outside of whole morulae are translocated to the inside so that the region of initial attachment may not necessarily influence the process of spreading. A more likely explanation is that the inner cells when aggregated with 1/8 blastomeres may undergo a change in properties in cases where engulfment is delayed since they are now exposed to outer conditions and to cell contact over only a small region of their surfaces. There is some evidence for cell contact inducing regional differences in the cell surface (polarity) such as is normally seen in outer cells (Ziomek & Johnson, 1980, 1981). Even if these factors are not allowed for there is clearly a distinction between the behaviour of 1/8 cells towards the inner group of cells and the outer group of cells.

Division of 1/8 blastomeres was depressed compared to isolated control 1/8 cells (70–80%) when these cells were placed with ICMs, 1/32 cells and 1/16 cells (28–38%). Division of 1/8 in combination with other 1/8, 1/4 or 1/2 cells was similar to the controls (75%). It is noteworthy that the division rate was lower when 1/8 combined with inner 1/16 cells (28%) than with outer 1/16 cells (42%). We observed a similar suppression of cell division when 1/8 cells spread on lectin-coated agarose beads (Kimber & Surani, 1982). The extreme spreading seen when 1/8 blastomeres are combined with the more adhesive inner cells is probably incompatible with the rounding up of cells prerequisite for cell division.

Since the 1/8 blastomere is a typical superficial cell and probably regionalised with respect to the adhesive membrane domains, two groups of inner (adhesive) and outer (less adhesive) cells may arise when 1/8 cells divide to 2/16 cells. Indeed after division of 1/8 blastomeres one of the progeny cells was engulfed by the other with a frequency of 51–62% (Kimber & Surani, 1981b; Surani et al. 1981; this study) although in another study this proportion was found to
Interaction of cells from different stage mouse embryos

be 76% (Ziomek & Johnson, 1981). In a further experiment (Table 2) the average incidence of engulfment of 1/16 cell by its sibling was 62% but the variation between individual embryos in which all cells divided was marked (38–88%). Based on our results (Table 2) we estimated the number of adhesive inner cells at the 16-cell stage to vary from three to seven (mean 5) compared with three to eight inner cells (mean 6) (Handyside, 1981). There is evidence to show that after the division of 1/8 blastomeres two morphologically distinct cell types are formed depending on the extent to which the outer microvillous area is segregated to one of the progeny cells (Johnson & Ziomek, 1981). This is in accord with the idea that 1/16 sibling cells inherit disproportionate amounts of inner (adhesive) and outer less adhesive membrane (Surani et al. 1981). The generation of two distinct populations of cells at the division of 1/8 blastomeres has been proposed by others (Johnson, Pratt & Handyside, 1981).

We previously encountered the phenomenon of engulfment after release of 2-cell embryos from cleavage arrest in cytochalasin D (Kimber & Surani, 1981a). Embryos continue to differentiate during cleavage arrest as judged by several criteria including synthesis of all the stage-specific polypeptides (Pratt et al. 1981). One would have assumed that after arrest both cells would behave as peripheral cells and undergo compaction as in the 1/8 + 1/8 combination. Perhaps earlier dividing cells are relatively more adhesive since they contribute disproportionately more cells to the ICM (Graham & Deussen, 1978). In support of this, it is the more advanced cell which is engulfed after release from cleavage arrest (Kimber & Surani, 1981a). It is necessary therefore to check if internal location of adhesive earlier dividing cells, in addition to the mechanism discussed above, could account for the populations of adhesive and non-adhesive cells in embryos. However, there is no evidence at present to suggest that any particular 1/8 blastomere can contribute exclusively to the ICM (Kelly, 1977; Kelly et al. 1978).

This study supports our proposal that the generation of adhesive and non-adhesive cells during cleavage occurs after the 8-cell stage so that the more adhesive cells are localized on the inside and likely to give rise to the ICM whilst the outer less adhesive cells remain peripheral and give rise to trophectoderm (Surani et al. 1981). The nature of the adhesive components of the cells surface is at present unknown. Their identification could help our understanding of the organization of the embryo and the divergence of cell differentiation. Either quantitative or qualitative differences in membrane components could explain both regional differences in cell surface adhesiveness and the arrangement of cells. Quantitative differences in the distribution of a particular group of molecules would be sufficient for the allocation of cells to inner or outer positions prior to the gradual acquisition of the specific properties of ICM and trophectoderm in the blastocyst.
We thank Dr D. Szollosi for advice about preparation for electron microscopy, Dr A. Handyside for help in identifying inside and outside blastomeres using fluorescent labelling and Mrs J. Constable for typing the manuscript. S.J.K. is in receipt of a postdoctoral fellowship from the Medical Research Council.

REFERENCES


Interaction of cells from different stage mouse embryos 151


(Received 12 October 1981, revised 1 March 1982)