Compaction of the mouse embryo: an analysis of its components

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SUMMARY

A variety of agents (viz.: cytochalasin D, colcemid, cytochalasin D + colcemid, Ca2+-free medium, 7-ketocholesterol, cholesterol, concanavalin A, anti-embryonal carcinoma antiserum and tunicamycin) which modify the cell membrane and/or cytoskeleton were used to investigate the molecular and cellular basis of the intercellular and intracellular components of compaction and analyse the relationships between them. It was found that the individual components could be selectively dissociated from one another. Cell flattening and close intercellular apposition were the most sensitive features and affected by the majority of agents. Tight junctions did not form in the absence of intercellular apposition, however an apparently normal degree of intercellular apposition did not necessarily lead to the assembly of these junctions. Polarization of individual blastomeres, as assessed by the reorganization of the cell surface, was the component most resistant to experimental intervention since it occurred in the presence of all agents used, though it was modified by some of them. The results are discussed in terms of the molecular and cellular events underlying polarization, intercellular apposition and tight junction formation as well as the significance of these events for normal blastocyst formation.

INTRODUCTION

Compaction of the 8-cell mouse embryo marks the beginning of processes leading to blastocyst formation. Compaction may be described in terms of four types of morphological change, one of which involves reorganization within a cell, whilst the other three involve intercellular reorganization. The intracellular component consists of the polarization of each blastomere within the 8-cell embryo. Surface microvilli become restricted to a few basal sites and to an externally facing (apical) pole which also exhibits an increased ligand-binding capacity (Ducibella, Ukena, Karnovsky & Anderson, 1977; Handyside, 1980; Ziomek & Johnson, 1980; Reeve & Ziomek, 1981). Actin-containing microfilaments appear to concentrate beneath the apical surface (Lehtonen & Badley, 1980), microtubules align with mitochondria parallel to the basolateral membranes (Ducibella & Anderson, 1975) and a column of endocytotic vesicles comes to lie between the apical pole and the more basally located nucleus.

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An analysis of compaction of the mouse embryo (Reeve, 1981a, b). The three main intercellular features of compaction on the other hand involve changes in the relationships among cells of the embryo. Prior to compaction the blastomeres are spherical and lack specialized intercellular junctions. During compaction the cells flatten against one another, thus maximizing intercellular contact and obscuring intercellular boundaries (Lewis & Wright, 1935). Focal tight junctions, which subsequently become zonular and apical, assemble in areas of membrane contact, and gap junctional complexes form low-resistance channels between the basolateral membranes (Ducibella & Anderson, 1975; Magnuson, Demsey & Stackpole, 1977; Lo & Gilula, 1979).

It is believed that the events of compaction have an important influence on the processes involved in blastocyst formation, namely (i) the initiation of inner cell mass (ICM) and trophectoderm differentiation, (ii) the loss of developmental totipotency as these tissues become committed to restricted developmental fates, and (iii) the morphogenesis of the blastocyst with its outer rim of trophectoderm cells which surround the blastocoel and eccentrically placed ICM. The problem is to dissect out the various components of compaction and establish their roles in the process of blastocyst formation (Johnson, 1979, 1981a). In this paper we describe the effects of a variety of agents on the events which generate a compacted morula, and demonstrate that the different components of the process can be dissociated from one another.

MATERIALS AND METHODS

Collection and culture of embryos

HC-CFLP mice (Hacking and Churchill Ltd, Alconbury) were superovulated with 5 i.u. PMS followed after 44–48 h by 5 i.u. of hCG. All times are expressed as h post-hCG. Embryos were flushed from the oviducts with phosphate-buffered medium containing 4 mg/ml bovine serum albumin (BSA) (PB1 + BSA) (Whittingham & Wales, 1969) and cultured in medium 16 containing 4 mg/ml BSA (M16 + BSA) (Whittingham, 1971) at 37 °C in 5% CO₂ in air.

Analysis of compaction

(i) Cell flattening and tight junction formation. Cell flattening was assessed visually (over the period 68–97 h post-hCG, see Table 1) under the dissecting microscope or Wild inverted phase microscope. Embryos within the population were assessed as showing complete cell outlines (non-compacted; Fig. 1f), or showing some cell outlines (compacting; Fig. 1h) or showing complete absence of distinguishable cell outlines (compacted; Fig. 1a). Results are expressed as

Fig. 1. Morphology (at 72 h post hCG) of embryos cultured in (a) control medium; (b) colcemid; (c) 7-ketocholesterol; (d) Con A; (e) CCD; (f) Ca²⁺-free medium; (g) anti-embryonal carcinoma serum; (h) tunicamycin (details in Materials and Methods). (Bar in (h) represents 50 μm.)
percentage of embryos in each state. The fine structure of the intercellular contacts and the development of tight junctions was analysed by incubating the embryos until 90–96 h post-hCG and then processing them for transmission electron microscopy (TEM). Embryos were washed briefly in protein-free medium, fixed for 1 h in 2·5 % glutaraldehyde (Sigma U.K.) in 0·1 M cacodylate buffer (pH 7·4), post-fixed for 45 min in 1 % OsO₄, washed in the cacodylate buffer, dehydrated in ethanol and embedded in TAAB (TAAB Labs. Reading, U.K.). Thick sections (1 μm) were stained with methylene blue. Thin sections (30–40 nm) were stained with uranyl acetate and lead citrate and viewed on a Philips EM 300.

(ii) Cell polarization. Cell polarization was scored in three ways. Some intact embryos were fixed for TEM (as above) and the distribution of microvilli was examined. Other embryos were disaggregated partially or completely in Ca²⁺-free medium 16 containing 6 mg/ml BSA (Ca²⁺-free M16 + BSA) or in medium 16 + BSA containing either cytochalasin D (1 μg/ml), or in trypsin-EDTA (0·5 % trypsin, 0·2 % EDTA) as described previously (Handyside, 1980). These embryos were prepared for scanning electron microscopy (SEM) and examined for a pole of microvilli as described previously (Reeve & Ziomek, 1981). Other embryos were disaggregated to a single cell suspension in one of the disaggregating media and the cells examined for the polarized binding of fluorescein-conjugated concanavalin A (FITC-Con A) or rabbit anti-mouse species antiserum (RAMS) (Handyside, 1980; Ziomek & Johnson, 1980). In brief, the method involves incubating the cells in FITC-Con A (Miles Labs. U.K.) (usually at 0·7 mg/ml) in PB1 + BSA containing 0·02 % sodium azide (PB1 + BSA + azide) at room temperature for 10–15 min, washing them through three changes of PB1 + BSA + azide and then mounting the cells under oil in microdrops of this medium on tissue-typing slides (Baird & Tatlock, U.K.). The cells were examined using a Zeiss epifluorescence microscope – further details in Ziomek & Johnson (1980). Indirect immunofluorescence was conducted by incubating the cells for 10–15 min in heat-inactivated RAMS (Handyside, 1980) diluted 1 : 10 with PB1, three washes in PB1 + BSA + azide, a second incubation in fluorescein-conjugated goat anti-rabbit IgG (FITC-GAR Miles Labs.) diluted 1:10–1:15 with PB1 + azide and finally, extensive washing (for details see Handyside, 1981).

Agents used to modify compaction

(1) Cytochalasin D (CCD). Late 2-, early 4- and early 8-cell embryos were flushed from oviducts at approximately 43–46, 50–52 and 56–59 h post-hCG respectively, and cultured for varying periods of time in either M16 + BSA or M16 + BSA + 0·5 μg/ml CCD (Sigma U.K.) (1:2000 dilution of CCD stock solution at 1 mg/ml in dimethyl sulphoxide (DMSO)). DMSO alone at this concentration had no effect on development. The zonae pellucidae of control and CCD-treated embryos were removed with acid Tyrode's medium (Nicolson,
Table 1. Effect of agents on cell division and cell flattening

<table>
<thead>
<tr>
<th>Agent*</th>
<th>At start</th>
<th>At analysis</th>
<th>Degree of cell flattening: % (no. embryos)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time after hCC (h)</td>
<td>No. cells/embryo (no. embryos)</td>
<td>Time after hCG (h)</td>
</tr>
<tr>
<td>(1) None</td>
<td>46-48</td>
<td>2 (255)</td>
<td>74</td>
</tr>
<tr>
<td>(2) CCD</td>
<td>43-46</td>
<td>2 (172)</td>
<td>68-75</td>
</tr>
<tr>
<td>(3) Colcemid (0-5 μg/ml)</td>
<td>44-46</td>
<td>2 (146)</td>
<td>70-97</td>
</tr>
<tr>
<td>(4) Colcemid + CCD</td>
<td>45-46</td>
<td>2 (96)</td>
<td>73-97</td>
</tr>
<tr>
<td>(5) Ca++-free</td>
<td>45</td>
<td>2 (40)</td>
<td>75</td>
</tr>
<tr>
<td>(6) 7-ketocholesterol</td>
<td>46</td>
<td>2 (32)</td>
<td>72</td>
</tr>
<tr>
<td>(7) Cholesterol</td>
<td>46</td>
<td>2 (12)</td>
<td>72</td>
</tr>
<tr>
<td>(8) Anti-EC</td>
<td>46-48</td>
<td>2 (355)</td>
<td>74</td>
</tr>
<tr>
<td>(9) Con A</td>
<td>46</td>
<td>2 (109)</td>
<td>96</td>
</tr>
<tr>
<td>(10) Tunicamycin</td>
<td>46</td>
<td>2 (40)</td>
<td>74</td>
</tr>
</tbody>
</table>

* For details of incubation conditions see Materials and Methods.
† Approximately 10% of embryos containing 8 or more cells contained a minority of cells without visible cell outlines.
Table 2. Effect of agents on the development of polarized FITC-Con A binding patterns and polarized microvillous distribution assayed on disaggregated cells

<table>
<thead>
<tr>
<th>Agent*</th>
<th>Time after hCG at analysis (h)</th>
<th>FITC-Con A binding pattern†</th>
<th>Distribution of microvilli‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NP</td>
<td>P</td>
</tr>
<tr>
<td>(1) None</td>
<td>72–75</td>
<td>98</td>
<td>297</td>
</tr>
<tr>
<td>(2) CCD</td>
<td>68–72</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>(3) Colcemid (0.5 µg.ml)</td>
<td>74–75</td>
<td>28</td>
<td>72</td>
</tr>
<tr>
<td>(4) CCD + Colcemid</td>
<td>75</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>(5) Ca²⁺-free</td>
<td>74</td>
<td>9</td>
<td>26</td>
</tr>
<tr>
<td>(6) 7-ketocholesterol</td>
<td>72</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>(7) Cholesterol</td>
<td>72</td>
<td>18</td>
<td>33</td>
</tr>
<tr>
<td>(8) Anti-EC</td>
<td>72</td>
<td>16</td>
<td>56</td>
</tr>
<tr>
<td>(9) Con A§</td>
<td>75 [1/8] 38</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>82 [1/8] 13</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>82 [1/16] 24</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>(10) Tunicamycin</td>
<td>74</td>
<td>36</td>
<td>9 (25)</td>
</tr>
</tbody>
</table>

* For details of incubation conditions see Materials and Methods.
† Number of disaggregated cells showing FITC-Con A binding pattern. NP = non polar, P = polar, NS = not scorable. A cell was designated as polar if FITC-Con A fluorescence was restricted to 75% or less of the cell surface (see Reeve & Ziomek, 1980). Numbers in parentheses represent additional cells in which there was a clear heterogeneous distribution of surface stain but it was not organized into a coherent pole. % Polarized includes all cases of non-homogeneous surface staining.
‡ Disaggregated cells were classified as non-polar (NP), polar (P) or not scorable (NS) by assessing the distribution of surface microvilli (as described by Reeve & Ziomek, 1981). Cells which exhibited a heterogeneity of microvillous distribution are designated as polar and indicated in parentheses. % Polarized includes all cells in which heterogeneous distribution of surface microvilli was observed.
§ Approximately 50% of Con-A treated cells lysed during disaggregation in either trypsin or Ca²⁺-free media. Polarization was assessed by indirect immunofluorescence using rabbit anti-mouse species antibody and FITC-goat anti-rabbit IgG (Handyside, 1980).
¶ '1/8' denotes 1 blastomere of an 8-cell embryo. '1/16' denotes 1 blastomere of a 16-cell embryo.
|| Percentage polarization of intact embryos. Since disaggregated cells from Con-A treated embryos were so susceptible to lysis, whole embryos were assessed for polarization of microvilli by SEM at 96 h post hCG. Embryos with 25% or more of their cells with a heterogeneous distribution of microvilli were scored as polarized. Controls had formed blastocysts by this time.
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Yanagimachi & Yanagimachi, 1975) containing 0.5 μg/ml CCD and the embryos were disaggregated in M16 + BSA + 0.5 μg/ml CCD as previously described (Handyside, 1980; Reeve & Ziomek, 1981). Both disaggregated controls and disaggregated CCD-treated embryos were analysed for polarity by SEM and the fluorescent-ligand-binding assay. FITC-Con A labelling and all washes were done in the presence of 0.5 μg/ml CCD and 0.02% sodium azide.

(2) Colcemid. Embryos were cultured from the late 2-cell stage (44–46 h post-hCG) in M16 + BSA or M16 + BSA + 0.5 or 5.0 μg/ml colcemid (Sigma U.K.) (1:2000 or 1:200 dilution of 1 mg/ml stock solution). The zona pellucidae were removed from treated and control embryos with acid Tyrode’s solution and disaggregation was in Ca²⁺-free medium M16 + BSA (Handyside, 1980) + 0.5 or 5.0 μg/ml colcemid. Disaggregated control and treated blastomeres were analysed for the polarized binding of FITC-Con A in the presence of 0.5 or 5.0 μg/ml colcemid and 0.02% sodium azide. All PB1 + BSA washes also contained colcemid and azide.

(3) Colcemid + CCD. Late 2-cell embryos (44–46 h post-hCG) were cultured in M16 + BSA or M16 + BSA + 0.5 μg/ml colcemid + 0.5 μg/ml CCD for varying periods of time. Zona removal from both control and treated embryos was with acid Tyrode’s solution and disaggregation was done in Ca²⁺-free M16 + BSA + 0.5 μg/ml CCD + 0.5 μg/ml colcemid. Disaggregated blastomeres were assayed for the polarized binding of FITC-Con A in the presence of CCD and colcemid.

(4) Ca²⁺-free medium. The Ca²⁺ salt in M16 was replaced by the isotonic equivalent amount of NaCl, which entailed increasing the NaCl concentration from 94 mM to 97 mM. The BSA concentration was raised to 6 mg/ml to reduce cell lysis. Embryos with intact zonae were cultured from the late 2-cell stage (44–46 h post-hCG) in Ca²⁺-free M16 + BSA. The zonae were removed with acid Tyrode’s solution and the embryos were disaggregated to single cells in Ca²⁺-free M16 + BSA.

(5) 7-ketocholesterol and cholesterol. Media containing 7-ketocholesterol or cholesterol were made up as described previously (Pratt, Keith & Chakraborty, 1980). Stock solutions of sterols at 50 mg/ml benzene were diluted into 5% BSA in 0.14 M-NaCl, pH 7.0, vortexed and cleared by centrifugation to give a sterol solution of 500 μg/ml. This solution was then diluted into M16 + 2% foetal calf serum (M16 + FCS) to give the appropriate sterol concentration for embryo culture (50 μg/ml). Embryos developed normally in this final dilution of benzene in the absence of sterol. Embryos were cultured from the 4-cell (approx. 52 h post-hCG) or 8-cell (60–66 h post-hCG) stages in this medium (in the absence of liquid paraffin due to tendency of the sterols to partition into the oil) for 4–5 h and then transferred to normal M16 + FCS under liquid paraffin oil. Zonae were removed using acid Tyrode’s solution and the embryos were disaggregated in Ca²⁺-free M16 + BSA.

(6) Rabbit antiserum to mouse embryonal carcinoma cells (LS 5770). This
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Antiserum has been described previously (Johnson et al. 1979). In the experiments reported here, 2-cell embryos were flushed from the oviducts at 46–48 h post-hCG, placed in culture with intact zonae in either M16 + BSA or M16 + BSA + antiserum diluted 1 in 40 after several hours of dialysis against M16 (see Johnson et al. 1979 for details). At 74 h post-hCG, control and experimental embryos were placed in acid Tyrode’s solution to remove the zonae pellucidae, rinsed in PB1 + BSA and then prepared for analysis. All embryos were at the late 8-cell stage at this time. The antiserum causes adjacent blastomeres to stick to each other, and Ca\(^{2+}\)-free medium was not effective in disaggregating them to single cells. The embryos were therefore exposed to trypsin-EDTA in Ca\(^{2+}\)-free medium for 10 min at 37 °C. This treatment has been shown previously not to affect the polar organization of the cell surface (Handyside, 1980; Reeve & Ziomek, 1981).

(7) Concanavalin A. Embryos were flushed from oviducts as either 2-cells (46 h post-hCG) or 4-cells (53 h post-hCG). Zonae pellucidae were removed with acid Tyrode’s solution and the embryos were cultured individually in drops of 20 μg/ml Con A (Sigma) in M16 + BSA under oil in glass culture dishes which had been siliconized with Repelcote (Hopkin and Williams, U.K.). Embryos cultured in Con A from 53 h post-hCG were prepared for analysis at 75 and 82 h post-hCG. Disaggregation was easier in trypsin-EDTA in Ca\(^{2+}\)-free medium than in Ca\(^{2+}\)-free medium 16 + BSA. The polar organization of the cell surface was assessed by indirect immunofluorescence. Con A-treated embryos were prone to lysis following disaggregation, therefore 2-cell embryos were cultured intact until 96 h post-hCG (when they showed a wide range of developmental stages (Table 1)) and fixed in situ in 6% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h. Embryos (which had stuck to the glass) were then dislodged and processed for SEM.

(8) Tunicamycin. Two-cell embryos (46 h post-hCG) were incubated in M16 + BSA containing 1 μg/ml tunicamycin (a gift from Dr M. A. H. Surani, A.R.C. Institute of Animal Physiology, Cambridge, U.K.) until 74 h post-hCG when they were disaggregated to single cells in Ca\(^{2+}\)-free M16 + BSA and processed for the FITC-Con A binding assay, SEM and TEM.

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Fig. 2. Microvillous distribution and FITC-Con A binding pattern (insert) (at 74 h post hCG) of (a) control 1/8 blastomere (insert ×400). (b) and (c) 1/2 blastomeres disaggregated from 2-cell embryos incubated in CCD and analysed by SEM at 72 h post hCG (×300). Upper insert in (c) shows FITC-Con A binding patterns of intact 4-cell embryo cultured in CCD and analysed at 70 h post hCG (note ‘patchy poles’) (×225). (d) 1/2 blastomere from 2-cell embryo incubated in colcemid (0.5 μg/ml) and CCD (0.5 μg/ml) and analysed by SEM at 72 h post hCG. Insert shows FITC-Con A binding pattern (×300). (e) 1/2 blastomere from 2-cell embryo incubated in colcemid (0.5 μg/ml) and analysed by SEM at 72 h post hCG. Insert shows FITC-Con A binding pattern (×300). Bar represents 10 μm.
Table 3: FITC-Con A binding patterns of isolated blastomeres from 2-cell, 4-cell and 8-cell embryos following CCD treatment

<table>
<thead>
<tr>
<th>Time in CCD (h)*</th>
<th>At analysis</th>
<th>FITC-Con A binding pattern</th>
<th>(Total no. of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ring</td>
<td>Pole</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>100</td>
<td>(31)</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
<td>0</td>
<td>(26)</td>
</tr>
<tr>
<td>8</td>
<td>57</td>
<td>0</td>
<td>(52)</td>
</tr>
<tr>
<td>16</td>
<td>57</td>
<td>0</td>
<td>(52)</td>
</tr>
<tr>
<td>20+</td>
<td>70</td>
<td>0</td>
<td>(48)</td>
</tr>
<tr>
<td>40</td>
<td>70</td>
<td>0</td>
<td>(48)</td>
</tr>
<tr>
<td>55</td>
<td>70</td>
<td>0</td>
<td>(83)</td>
</tr>
<tr>
<td>70</td>
<td>70</td>
<td>0</td>
<td>(46)</td>
</tr>
</tbody>
</table>

Cell no. Age (h post hCG) Nonhomogeneous Ring Pole Patchy pole Not scorable

(31) (26) (52) (30) (48) (83) (46)

- CCD was present throughout the disaggregation and FITC-Con A labelling procedures and the duration of CCD treatment includes this period (1-2 h).
- Percentage of blastomeres showing pattern indicated. 'Patchy pole' includes all cases where there was a clear heterogeneous distribution of surface stain which was restricted to 75% or less of the cell surface but was not organized into a coherent pole. 'Non-homogeneous surface' indicates that staining was distributed over the entire cell surface.
RESULTS

(1) Cytochalasin D (CCD)

Cell division was irreversibly inhibited when 2-, 4- or early 8-cell embryos were cultured for 24 h or more in CCD (0.5 µg/ml). Control embryos were fully compacted at 68–75 h post-hCG as judged by complete loss of cell outlines, whereas the 2- and 4-cell CCD-treated embryos showed no signs of cell flattening over the period 68–85 h post-hCG when assessed by light microscopy or SEM (Table 1, Fig. 1e) and no evidence of increased intercellular contact when observed by TEM. Embryos grown in CCD from the 2- or 4-cell stage, and labelled with FITC-Con A as either intact embryos or disaggregated blastomeres at the equivalent of the late 8-cell stage, showed a similar incidence of heterogeneous ligand binding to control 8-cell stage embryos (Table 2). However, whereas control 8-cell embryos and 1/8 blastomeres exhibited single discrete poles of FITC-Con A binding, many CCD-treated cells had one or more poles with irregular boundaries and the remainder had highly disorganized heterogeneous patterns (Table 2, Fig. 2b and c inserts). The microvilli of CCD-treated embryos appeared to be longer than those of control embryos (Fig. 2b and c, and unpublished TEM observations) and contained assemblies of intact core microfilaments. Formation of tight junctions was totally suppressed by continuous treatment with CCD (Pratt, Chakraborty & Surani, 1981).

The developmental age of the embryos and the duration of exposure to CCD influenced the type of FITC-Con A binding patterns observed (Table 3). Control 2-cell embryos bound the ligand in a homogeneous ring pattern (Table 3, line 1) which was transformed to a patchy distribution all over the cell surface within 6 h of CCD treatment (Table 3, line 2, Fig. 2b and c). This non-homogeneous binding pattern remained unchanged if CCD treatment was prolonged until 70 h post-hCG (Table 3, lines 3 and 4) at which time control 1/8 blastomeres already showed a high incidence of the normal polarized FITC Con A binding pattern (Table 3, line 7).

In contrast, a mixed population of 4-cell embryos cultured for 6 h in CCD contained blastomeres which showed a non-homogeneous labelling pattern (Table 3, line 5), but after 16 h in culture 44% of the blastomeres were not only non-homogeneous but also showed clear evidence of a discrete pole of Con A binding (Table 3, line 6, Fig. 2c upper insert). The disposition of ligand within these poles was, however, more patchy than that observed in 8-cell blastomeres. A similar ‘patchy’ pole was observed in 35% of blastomeres from early 8-cell embryos cultured for 6 h in CCD (Table 3, line 8). The form and incidence of polarity (as assessed by FITC-Con A binding) in late (i.e. fully polarized) 1/8 blastomeres was unaffected by CCD treatment (Handyside, 1980).
(2) Colcemid

Late 2-cell embryos (44–46 h post-hCG) did not divide any further when cultured for 24–50 h in medium containing 0.5 or 5.0 μg/ml colcemid, and the inhibition of cytokinesis was found to be irreversible after 28 h exposure to the drug. At 70–73 h post-hCG, when cell flattening in control embryos was complete, colcemid-treated embryos remained as non-compacted 2-cells (Table 1, Fig. 1b), though a few small areas of intercellular contact were observed by TEM at 90 h post-hCG. Blastomeres disaggregated from embryos treated with either 0.5 or 5.0 μg/ml colcemid showed a similar incidence of polarized FITC-Con A binding patterns and microvillous distribution to controls (Table 2, data for 5.0 μg/ml not shown). Colcemid was included in all the fluorescent labelling reagents since if it was omitted cells began to elongate during labelling and the FITC-Con A became localized predominantly to the cleavage furrow. At both concentrations of colcemid the fluorescent and microvillous poles were spread over larger areas of the cell surface (‘broad poles’) than in control embryos (Fig. 2a, e). There was no evidence for the presence of tight junctions at 90–96 h post-hCG. If pre-compact 8-cell embryos (65 h post-hCG) were treated with 5 μg/ml colcemid the degree of cell flattening achieved depended upon the length of exposure to the drug. After 6 h treatment experimental embryos were indistinguishable from controls (ca. 50% showing cell flattening) whereas more prolonged treatment tended to decompact these embryos and led to a reduction in cell flattening (36% at 10 h and 19% at 20 h) as compared with 90% and 95% in controls. This inhibition of cell flattening became irreversible by 10 h of exposure to the drug.

(3) Colcemid + CCD

Embryos grown from the 2-cell stage in medium containing 0.5 μg/ml CCD + 0.5 μg/ml colcemid had similar properties to CCD-treated embryos. Cell division was inhibited, the embryos showed no sign of cell flattening over the period 73–97 h post-hCG (Table 1) and there was no evidence of any increased intercellular contact at the TEM level. When disaggregated, these embryos exhibited irregular or disorganized poles of fluorescent ligand binding identical to embryos treated with CCD alone, rather than the unified ‘broad’ poles seen in colcemid-treated embryos (Table 2, Fig. 2d insert). A similar disorganized arrangement of microvilli was observed by SEM (Table 2, Fig. 2d). Tight junctions were not present at 90–96 h post-hCG.

(4) Ca²⁺-free medium

Cell division proceeded in 2-cell embryos cultured in Ca²⁺-free medium (Table 1). However, at 75 h post-hCG when control embryos were compacted, as judged by cell flattening, the cell outlines of Ca²⁺-free treated embryos were still clearly visible (Table 1, Fig. 1f) and there was no evidence of any increased
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intercellular apposition by TEM at 90 h post-hCG. The dissociated cells had poles of FITC-Con A binding and microvilli similar in organization and frequency to controls (Table 2). No tight junctions were observed at 90–96 h post-hCG.

(5) 7-ketocholesterol and cholesterol

Two-cell and early 8-cell embryos progressed through 1–2 cycles of cell division when exposed to 50 μg/ml 7-ketocholesterol for 4–5 h, though the rate of cell proliferation was low compared with cholesterol-treated embryos, which continued to divide at normal rates (Pratt et al. 1980 and Table 1). The cell flattening aspect of compaction (Table 1, Fig. 1c) and the incidence of blastocyst formation (Pratt et al. 1980) were substantially reduced in 7-ketocholesterol but normal in cholesterol-containing medium. Both types of 8-cell embryos showed polarized FITC-Con A binding patterns when analysed as single cells, and the poles were of similar frequency and organization to controls (Table 2). Microvilli also had a polarized distribution similar to controls (Table 2) and these were localized to the externally facing apical membranes in intact embryos (Pratt et al. 1980). Despite the fact that the 7-ketocholesterol-treated embryos appeared relatively unflattened at 72 h post-hCG when observed under the light microscope, investigation at the TEM level demonstrated substantial areas of intercellular apposition which were less extensive than in cholesterol-treated or untreated control embryos (Pratt et al. 1980). Apical tight junctions formed in both types of embryos, though individual junctional complexes were less well developed and the blastocoel was frequently absent or very small in 7-ketocholesterol-treated embryos (Pratt et al. 1980).

(6) Rabbit antiserum to embryonal carcinoma cells (LS 5770)

Cleavage up to the 32-cell stage was not affected by the antiserum (Johnson et al. 1979). At 74 h post-hCG, control embryos were fully compacted as assessed by cell flattening, whereas the majority of experimental embryos still retained visible cell outlines (Table 1, Fig. 1g) even though extensive areas of intercellular contact were observed by TEM (Johnson et al. 1979). Analysis of individual cells demonstrated a similar incidence of polarized ligand binding and microvillous distribution in both control and experimental groups (Table 2). The organization of fluorescent and microvillous poles did not differ from that of controls. As reported previously, tight junctions were not observed (Johnson et al. 1979).

(7) Concanavalin A (Con A)

Zona-free 2-cell or 4-cell embryos cultured in 20 μg/ml Con A usually progressed through 1–2 cycles of cell division, frequently at a slower rate than controls, though this varied among embryos (Table 1, Fig. 1d, Reeve, 1982). Cells of slowly dividing embryos did not flatten upon one another (Table 1,
Table 4. Summary of effects of agents on various aspects of compaction

<table>
<thead>
<tr>
<th>Agent</th>
<th>Cell division</th>
<th>Cell* flattening</th>
<th>Polarized FITC-Con A binding</th>
<th>Polarized distribution of microvilli</th>
<th>Intercellular† apposition</th>
<th>Presence† of tight junctions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CCD</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Col</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Col + CCD</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ca²⁺-free 7-ketocholesterol</td>
<td>Retarded</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>Large areas</td>
<td>±</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anti-EC</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>Large areas</td>
<td>−</td>
</tr>
<tr>
<td>Con A</td>
<td>Retarded and</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>Extensive in some embryos</td>
<td>−</td>
</tr>
<tr>
<td>variable</td>
<td>(Develops late)</td>
<td>(Develops late)</td>
<td>(Develops late)</td>
<td>(Develops late)</td>
<td>Extensive, develops late</td>
<td></td>
</tr>
<tr>
<td>Tunicamycin</td>
<td>Retarded</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>Extensive, develops late</td>
<td></td>
</tr>
</tbody>
</table>

+ indicates equivalence to control embryos. − indicates absence. ± indicates present but abnormal (see text for details).

* For time of analysis see Table 1. † Assayed at 96 h post hCG.
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Fig. 1d); however, some embryos with eight or more blastomeres contained some cells without clearly visible cell outlines (Table 1). Upon examination by TEM these embryos displayed extensive areas of intercellular contact and the cell membranes were covered by an external fuzzy coat. When cultured from the 4-cell stage and examined at 75 or 82 h post-hCG, disaggregated 1/8 blastomeres from Con A-treated embryos showed less polarity (assessed by indirect immunofluorescence) than controls. However, the incidence of polarity was increased amongst cells which had progressed through to the 16-cell stage (Table 2). A high level of lysis occurred when Con A-treated embryos were dissociated for immunofluorescent labelling, which introduces the possibility that subpopulations of cells were selectively eliminated. To overcome this problem, polarization of microvilli as assessed by SEM was examined in intact embryos (Table 2). When examined between 75 and 82 h post-hCG, 76% of intact embryos had 25% or more of their cells polarized (Table 2). No tight junctions were identified in these embryos at 96 h post-hCG.

(8) Tunicamycin

Tunicamycin at 1 µg/ml permitted a variable degree of cell division and the degree of flattening increased with increased cell number (Table 1, Fig. 1h). Examination at the TEM level demonstrated extensive areas of close intercellular apposition in these embryos. The number of cells showing polarized FITC-Con A binding and microvillous distribution was substantially reduced when assayed at 74 h post-hCG in comparison with controls. The majority of fluorescent poles tended to be patchy and extended but the poles of microvilli appeared to be more discrete. When tunicamycin-treated embryos were assayed at the 16-cell stage (results not shown) the proportion of polarized cells (assayed by SEM and FITC-Con A binding) had increased to the same level (54%) as seen in control embryos (Johnson & Ziomek, 1981a). Apical tight junctions were not detected at 96 h post-hCG.

DISCUSSION

The use of a variety of agents that modify the cell membrane and/or cytoskeleton has demonstrated an apparent hierarchy of events during the process of compaction (Table 4). Polarization of individual cells as assessed by reorganization of the cell surface occurs in the presence of any of the agents examined, although the normal topography of the membrane is modified by some of them. Cell flattening, and the close intercellular apposition of membranes that develops subsequently, is affected by most of the agents, but not by all. In cases where the development of close intercellular apposition is inhibited, tight junction formation is not detected. However, in other cases, where extensive intercellular apposition does occur this does not necessarily lead to the development of tight junctional complexes. These results will be discussed first
in terms of the events of polarization, intercellular apposition and junction formation themselves, and second in terms of the consequences of these events for normal blastocyst formation.

The polarization of individual blastomeres that occurs at the 8-cell stage is a consequence of the asymmetry of contacts between the cells at this stage (Ziomek & Johnson, 1980; Johnson & Ziomek, 1981b). The ability to induce polarity develops in the blastomere membranes during cleavage (Johnson & Ziomek, 1981b) and once polarization has been induced, the axis of polarity is stable throughout the life of the blastomere (Johnson & Ziomek, 1981b) and during the ensuing two rounds of division to the 16- and 32-cell stages (Johnson & Ziomek, 1981a; Ziomek & Johnson, 1982). Disturbances of polarization could result from a failure of the inducing cues, of the polarizing response to these cues, or from a combination of both. In experiments, such as those described here, in which intact embryos are treated with modifying agents, it may prove difficult to discriminate between these alternatives since all cells will be affected by the agents, and thus both inducing and responding capacities may be altered. None the less, these experiments provide important clues to the mechanism underlying polarization.

Reorganization of the cell surface, which results in a recognizable polarized morphology in the majority of cases, occurs in the presence of all the agents tested. These agents differ in their effects on cell division and intercellular apposition. However, one agent, Ca\(^{2+}\)-depleted medium, abolishes normal cell flattening and intercellular apposition while allowing cell division to continue on schedule. The development of normal polarity in these Ca\(^{2+}\)-depleted embryos provides the clearest evidence that the inductive cue is not related simply to the extent of intimate cell contact. Further support for this suggestion comes from the experiments using anti-embryonal carcinoma serum, 7-ketocholesterol, concanavalin A and tunicamycin, all of which permit normal polarization despite their variable effects on cell division, cell flattening and the degree of intercellular contact. Additionally, although the peculiar surface morphologies that develop as a consequence of colcemid or CCD treatment are difficult to interpret (discussed below), the results from use of these drugs also support the idea that normal cell flattening is not required for transmission of the induction cue for polarization. However, the anomalous surface patterns that develop in the presence of these drugs might suggest an alternative explanation, namely that continuing cytokinesis is essential for normal polarization. We consider this unlikely because molecular and morphological maturation can occur in the absence of cytokinesis induced by CCD (Pratt et al. 1981). Furthermore, 4-cell blastomeres do occasionally polarize naturally (Johnson & Ziomek, 1981b), or if division to the 8-cell stage is arrested by prior application of \(\alpha\)-amanitin or Mitomycin C (Pratt, unpublished) or in the face of variable degrees of inhibition of cell division (e.g. after treatment with 7-ketocholesterol, Concanavalin A or tunicamycin, Table 4; or following culture-induced cleavage...
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'block' at the 2-cell stage (Goddard & Pratt, in preparation). We therefore suggest that the ability to transmit or respond to the cue to polarize is not directly related to either cell division or the extent of intimate cell contact.

Furthermore, since gap junctions can only form between areas of intimate cell contact the results suggest, but do not prove, that the inductive signal is unlikely to be transmitted between cells via gap junctions. Results from other experiments in which cells can induce polarization of 8-cell blastomeres under circumstances where no gap junction-mediated transfer can be detected between them (Johnson & Ziomek, 1981b; Goodall & Johnson, 1982) strengthen this possibility. The retarded development of polarity in the presence of either tunicamycin or Concanavalin A, both of which interfere with surface glycoconjugates but neither of which affects all these residues completely (Surani, 1979; Jacob, 1979), hints at a role for glycosylated molecules in either the induction of polarity or the recognition of the inducing signal.

The considerable reorganization of the cell that occurs during the polarization process might be expected to involve cytoskeletal elements (Wessells et al. 1971; Siracusa, Whittingham & de Felici, 1980). It is therefore interesting that surface reorganization of any kind can occur in the presence of either colcemid, CCD or a combination of both. Colcemid-treated blastomeres apparently undergo a form of polarization, since microvilli become concentrated within one hemisphere of the cell and form 'broad poles'. In contrast, CCD-treated blastomeres either develop a coherent or 'patchy' pole or distribute their microvilli in a disorganized manner all over the cell surface. These disorganized patterns could arise either from a generalized effect of the drug on the embryo membrane or from an interaction between CCD and the filament systems responsible for a progressive reorganization that precedes the development of an overt and stable pole (Johnson, 1981b). In support of this second possibility we have shown that early 8-cell blastomeres can polarize in the presence of CCD to the same extent as do controls (Table 3), though approximately half of these poles are 'patchy', implying that some terminal events of pole organization may be disrupted by the drug. Furthermore, the low incidence of polarity observed naturally in 4-cell blastomeres (Johnson & Ziomek, 1981b) increases during 16 h of CCD treatment and reaches levels similar to untreated 1/8 blastomeres, though again the majority of these poles are patchy and not apparently fully formed (Table 3). In contrast, CCD-treated 2-cell embryos exhibit only non-homogeneous patterns of labelling and microvilli irrespective of the duration of treatment. It is possible that the process of cytoplasmic reorganization that results in overt polarity begins considerably earlier than the stage at which a stable polarity is first detected since the capacity to induce polarity, as distinct from the capacity to respond to induction, has already been shown to develop progressively from the 2-cell stage onwards (Johnson & Ziomek, 1981b; Johnson, 1981b). The results described above are compatible with the notion that the early phases of the polarization process involve the gathering together of specific regions of the
cytocortex by CCD-sensitive microfilaments. The disruption of these microfilaments by CCD would then explain the non-homogeneous surface topography induced at early stages. The final phases of the process are either independent of microfilaments or involve microfilaments which are resistant to CCD, since apparently normal poles can develop in the presence of the drug and the stable polarized phenotype once achieved is not disrupted by CCD treatment (Handyside, 1980). The presence of CCD-resistant microfilaments is not unlikely since they have been described in a number of cell lines (Mak, Trier, Serfilippi & Donaldson, 1974; Morris & Tannenbaum, 1980) and intact microfilaments have been detected within the microvilli of CCD-treated embryos (Pratt et al. 1981). However, these experiments do not eliminate the possibility that the disorganized surface morphologies seen in all 2-cell and many 4-cell blastomeres treated with CCD are unrelated to polarization, and we shall only be able to discriminate between these two alternatives when the earliest stages of the polarization process have been analysed in more detail.

Intercellular apposition and the formation of zonular tight junctions both appear to be more sensitive to exogenous agents than does cell polarization. Two general points emerge from the data. First, although intercellular apposition, as judged by the ability to discern individual cell outlines, may appear at the light microscope level to be reduced by all of the agents tested, ultrastructural analysis at various times during drug treatment reveals that a considerable degree of intercellular apposition has occurred in several of them, namely 7-ketocholesterol (Pratt et al. 1980), anti-embryonal carcinoma antisemum (Johnson et al. 1979), concanavalin A (Reeve, 1981c) and tunicamycin. This increased apposition does not necessarily lead to the formation of zonular tight junctions, suggesting that specific configurations of glycoconjugates and lipids may be required for their assembly. Secondly, cell flattening, and the junction assembly that develops subsequently, both occur after the process of polarization (Ziomek & Johnson, 1980). Thus the apparent greater sensitivity of intercellular apposition and junction formation could be explained by an increased macromolecular complexity for those events that occur late during the process of compaction.

The value of this study lies not only in the light that it can shed on the macromolecular basis and interdependence of the different features of compaction, but also in the scope that it provides for exploration of the possible causal relationships between the events of compaction and those of blastocyst formation. It is already clear that agents such as anti-embryonal carcinoma antisemum and CCD, which have their main effects on the intercellular as opposed to the cellular (polarizing) aspects of compaction, also adversely affect the morphogenetic features of blastocyst formation (Ducibella & Anderson, 1979; Johnson et al. 1979; Surani, Barton & Burling, 1980; Pratt et al. 1981). However, these agents have no obvious effect on the molecular maturation of the blastocyst and the acquisition of ICM- and trophectoderm-specific profiles of polypeptide
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synthesis (Pratt et al. 1981; Johnson, unpublished). These experiments have suggested that the intercellular components of compaction are concerned primarily with the generation of a blastocoel and the organization of a fluid-transporting epithelium. On the other hand, polarization seems to be associated with the orientation of developmental information along a radial axis prior to its segregation into inner and outer cells at division (Ziomek & Johnson, 1980; Johnson & Ziomek, 1981a). These inner and outer subpopulations have distinct phenotypes that anticipate their presumptive fates as ICM and trophectoderm, and which are thought to direct their respective courses of cytodifferentiation (Johnson, Pratt & Handyside, 1981; Ziomek & Johnson, 1980; Ziomek, Pratt & Johnson, 1982). Our experiments provide a panel of reagents which can be used to continue the investigation of the relationship between polarization and differentiation of ICM and trophectoderm.

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