J. Embryol. exp. Morph. 93, 239–255 (1986)

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The role of cell adhesion in the synchronization and orientation of polarization in 8-cell mouse blastomeres

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SUMMARY

A detailed investigation into the activity of the homotypic, Ca^{2+}-dependent cell–cell adhesion system (CDS) in the early mouse embryo has revealed its involvement in (i) the synchronizing of the time of polarization of 8-cell blastomeres, and (ii) the orienting of the axis of polarization. Since polarization marks an important and early event in the process of cell diversification in the mouse embryo, it is concluded that the CDS provides an important component of the system by which the temporal and spatial elements of normal development are integrated.

INTRODUCTION

The early development of the mouse embryo yields a blastocyst with an outer population of polar trophectoderm cells and an inner population of cells, the inner cell mass (ICM). These two tissues differ in both phenotype and developmental potential. It has been proposed that a key step in this process of cell diversification is the polarization of blastomeres that occurs at the 8-cell stage (see Johnson, 1985, 1986). The development of polarity, and the orientation of its axis, are influenced by cell interactions. Thus, when an early 8-cell blastomere is exposed to an asymmetry of contact with other blastomeres, an axis of polarity develops perpendicular to the contact regions (Ziomek & Johnson, 1980; Johnson & Ziomek, 1981*a*). Division of a polarized 8-cell blastomere can then generate polar and apolar progeny (Johnson & Ziomek, 1981*b*; Reeve, 1981*a,b*). The apolar 1/16 progeny also have the capacity to polarize if exposed to asymmetric cell contact (Ziomek & Johnson, 1981; Johnson & Ziomek, 1983). However, due to the tendency of cells to flatten on each other and maximize cell contact, apolar 1/16 cells *in situ* tend to be enclosed in the centre of the embryo and thus, being totally enveloped, do not polarize and do not therefore contribute to the cells of the trophectoderm. The outer polar 1/16 blastomeres, in contrast, give rise to the polar trophectoderm cells (Ziomek & Johnson, 1982; Randle, 1982; Suranyi & Handyside, 1983; Fleming, Warren, Chisholm & Johnson, 1984; Johnson, 1986).

Key words: cadherin, uvomorulin, blastocyst, polarization, mouse embryo, cell adhesion.

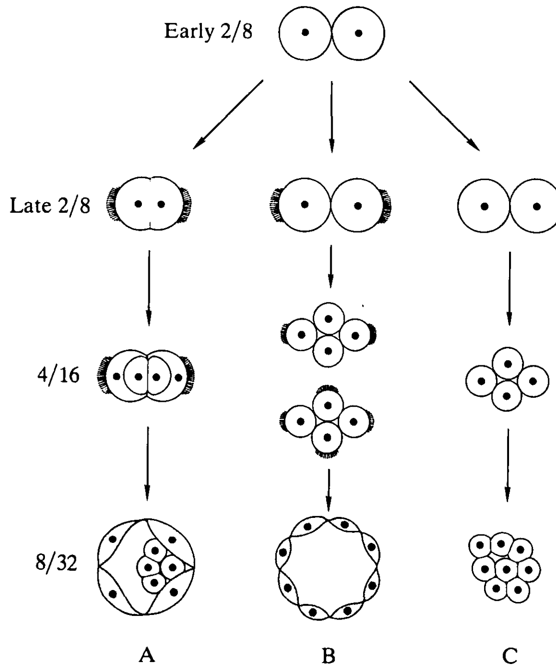

Fig. 1. Schematic outline of the predictions of the polarization hypothesis for (A) normal development, (B) inhibiting the CDS-mediated intercellular flattening characteristic of the early phases of compaction but not inhibiting polarization, and (C) inhibiting both polarization and CDS-mediated intercellular flattening – Johnson, 1986. Note: in case (B), where polarization occurs in the absence of CDS-mediated flattening, the orientation of the polarity will not affect the outcome as long as the polarized cells can rotate to align in an epithelial array.

If this proposal (summarized in Fig. 1A) for the generation of cell diversity in the blastocyst is correct, then certain testable predictions can be made (Johnson, 1986). One such prediction is that the inhibition of cell flattening should prevent apolar 1/16 cells from being totally enclosed, and so, being exposed to asymmetry of contact, they should polarize and generate an embryo in which all or most cells are trophectodermal (Fig. 1B). A second prediction is that suppression of polarization should lead to absence of trophectoderm (Fig. 1C).

In a recent paper, it was reported that a low-affinity monoclonal antibody (ECCD-1; Yoshida-Noro, Suzuki & Takeichi, 1984), directed against the Ca^{2+}-dependent cell–cell adhesion system (CDS) present on embryonic cells (E-cadherin; Hatta, Okada & Takeichi, 1985), prevented both cell flattening and polarization (Shirayoshi, Okada & Takeichi, 1983). The result of incubating cleavage-stage embryos in this antibody was blastocysts containing only trophectodermal cells. Thus, the first prediction was upheld but the second was not. This result therefore suggested that polarization might be irrelevant to the process of blastocyst formation. We report here a detailed re-examination of the effects of ECCD-1 on polarization at the 8-cell stage that leads us to a different result from that obtained previously, but which nonetheless suggests a central role for the CDS in the integration of time and space in early mouse development.

MATERIALS AND METHODS

(A) *Recovery and manipulation of embryos*

MF1 female mice (laboratory bred, OLAC derived, 3–5 weeks of age) were superovulated by injections of 5 i.u. of pregnant mares' serum gonadotrophin (PMSG; Intervet) and human chorionic gonadotrophin (hCG; Intervet) 48 h apart. The females were paired overnight with HC-CFLP males (Hacking and Churchill Ltd) and inspected for vaginal plugs the next day. Embryos were recovered as a mixture of 2-, 3- and 4-cell stages by oviducal flushing at 48 h post-hCG. Embryos were cultured in Medium 16 containing 4 mg ml^{-1} BSA (M16+BSA; Whittingham & Wales, 1969). Removal of the zona pellucida was achieved by brief exposure to acid Tyrode's solution (Nicolson, Yanagimachi & Yanagimachi, 1975) followed by a rinse in Medium 2 containing 4 mg ml^{-1} BSA (M2+BSA; Fulton & Whittingham, 1978). Embryo disaggregation was achieved by placing zona-free 2- or 4-cell embryos in Ca^{2+}-free M2+ 6 mg ml^{-1} BSA for 5–45 min, during which time they were disaggregated to single 2- or 4-cell blastomeres (1/2 or 1/4 cells) using a flame-polished pipette. Isolated cells were cultured in individual microdrops of M16+BSA and examined every hour for evidence of division to 2/4 or 2/8 pairs. In some experiments, newly formed 2/8 pairs were disaggregated to single cells as described above. Reaggregated pairs (1/8+1/8) were formed by briefly exposing the newly formed and isolated 1/8 blastomeres to a 1/20 dilution of Gibco stock phytohaemagglutinin (PHA) in M2+BSA, and sticking blastomeres together in pairs.

(B) *Immunocytochemistry*

Surface polarity was assessed by incubation of cells or embryos in 700 μg ml^{-1} FITC–ConA (Miles) or 1 mg ml^{-1} FITC–PHA (Miles) for 5 min at room temperature followed by thorough rinsing in M2+BSA. Labelled cells or embryos were then placed in specially designed chambers exactly as described in Maro, Johnson, Pickering & Flach (1984) for fixation with 3·7 % formaldehyde followed in some cases by extraction with 0·25 % Triton X-100. Extracted cells were incubated with antiserum to clathrin (Maro, Johnson, Pickering & Louvard, 1985) or actin (Johnson & Maro, 1984) followed by TMRITC-labelled anti-rabbit immunoglobulin antibody or with antiserum to cytokeratin ENDO-A (TROMA-1; Brulet, Babinet, Kemler & Jacob, 1980) followed by TMRITC-labelled anti-rat immunoglobulin antibody. Non-extracted cells were incubated with a rabbit antiserum to uvomorulin (Peyrieras, 1984) followed by TRMITC-labelled second layer.

Samples were mounted in Citifluor (City University, London) and viewed on a Leitz Ortholux microscope using selective filter set L2 for FITC and N2 for TMRITC. Photographs were taken on the Leitz Vario Orthomat System.

(C) *Special reagents*

(1) Monoclonal antibody ECCD-1 (ascites fluid; Yoshido-Noro *et al.* 1984) directed against the E-type of cadherin was diluted in M16+BSA and millipore-filtered prior to use. Preliminary experiments revealed that the antibdy was active to dilutions in excess of 1/200. All experiments in this report involved culture in 1/50 dilutions.

(2) An affinity-purified polyclonal antiserum (Peyrieras, 1984; gift of Nadine Peyrieras and Prof. F. Jacob) directed against uvomorulin, the same molecular species recognized by ECCD-1, was used at dilutions in M16+BSA as specified in Results. For immunochemical staining the antiserum was used at a dilution of 1/50.

(3) Embryos to be trypsinized were placed in M2 containing 4 mg ml^{-1} polyvinyl pyrrolidone (M2+PVP) at 37°C for 10 min and then divided into four groups. One group was retained in M2+PVP. A second group was placed in trypsin (0·1 mg ml^{-1} M2+PVP). A third group was placed in Ca^{2+}-free M2+PVP containing EGTA (0·1 mM). A fourth group was placed in Ca^{2+}-free M2+PVP containing both EGTA and trypsin. Embryos in all four groups were incubated for 30 min, rinsed in M2+BSA containing soyabean trypsin inhibitor (STI Sigma; 0·5 mg ml^{-1}) for 5 min and each group was divided into two populations. One population was placed in M16+BSA+STI and the other in M16+BSA+STI+cycloheximide (Sigma; 120 μg ml^{-1}).

Preliminary experiments were undertaken to determine the concentrations of the various reagents used in the above protocols.

RESULTS

(A) *The effect of ECCD-1 on the development of the blastocyst*

We first confirmed that prolonged exposure to ECCD-1 resulted in blastocysts that lacked, or were grossly deficient in, ICM cells. Intact early 8-cell embryos, isolated 8-cell blastomeres, and newly formed 2/16 couplets were placed either in normal medium or in medium containing ECCD-1 and cultured to the early blastocyst stage (approximately 32-cells, Chisholm *et al.* 1985). The intact embryos were then scored visually for the presence of an ICM, and the quartet of 32-cells (4/32) derived from 1/8 or 2/16 blastomeres were examined with TROMA-1, an antibody to a cytokeratin that reacts only with trophectoderm cells (Brulet *et al.* 1980). Of 82 intact embryos exposed to ECCD-1, 70 lacked evidence of an ICM (100% of 93 control embryos contained an ICM). Some intact embryos were allowed to continue in culture in the presence of ECCD-1 and all hatched from their zonae, attached to the culture dish, and formed a flat sheet of large cells devoid of a central ICM cluster. Of 21 4/32 clusters grown in ECCD-1 from the 1/8 or 2/16 cell stage, all consisted of four exposed cells that stained with TROMA-1 and none showed evidence of ICM cells (60% of 14 control clusters contained enclosed cells not reactive with TROMA-1). From these results, we conclude that ECCD-1 does indeed suppress development of ICM cells, and, moreover, the use of 1/8 and 2/16 cells shows clearly that cells are diverted from an ICM fate towards a trophectodermal course of development as a result of exposure to the antibody.

(B) *Exposure of ECCD-1 applied to newly formed 8-cell blastomeres*

There is considerable asynchrony within an embryo in the time at which blastomeres divide from the 4- to 8-cell stage. We therefore disaggregated late 4-cell embryos to single (1/4) blastomeres, observed the blastomeres every hour, and removed all that had divided to 2/8 blastomeres. Some were placed in ECCD-1 for 9 h, others were placed in control medium for 9 h, and some were placed in control medium for 6 or 8 h (by which time a stable axis of polarization is laid down and cell flattening has occurred; Johnson & Ziomek, 1981*a*) and then were transferred to ECCD-1 for the final 3 h or 1 h. Some late 1/4 blastomeres were placed in ECCD-1 and allowed to divide over the ensuing 1 or 2 h to 2/8 pairs, which were then cultured for a further 9 h in the presence of the antibody. At the end of the 9 h incubation period, the cells were scored for the extent of cell flattening and for surface and cytoplasmic polarity as assessed by surface binding of FITC-ConA and by distribution of cytoplasmic actin or clathrin. The results are recorded in Table 1, lines 1–7.

In control incubations a high incidence of polarity was recorded (Fig. 2A,B; Table 1, lines 1 & 6) regardless of whether the macromolecule used in the medium

was BSA or foetal calf serum (the latter having been used by Shirayoshi *et al.* 1983). As reported previously (Johnson & Maro, 1984; Maro *et al.* 1985), cytoplasmic polarity develops in advance of surface polarity, and thus the incidence of cytoplasmic polarity is scored as slightly higher in each condition. The incidence of cell flattening in controls was also high, being 95 % and 98 %, respectively. In contrast, cell flattening was prevented by exposure to antibody ECCD-1 when the antibody was present throughout, and was reversed when the antibody was added only during the final hour of incubation. However, although the incidence of polarity was reduced in the presence of antibody, a majority of blastomeres had polarized (Fig. 2C–F; Table 1, columns 3 & 4, rows 2–5, 7). In each case where a cell was judged to be polar both at its surface and within its cytoplasm, the poles were coincident and lay on the same axis through the cell. However, whereas in control pairs about 95 % of cells had an axis of polarity perpendicular to the point of contact with the sister cell (designated 'on-axis' and as described previously by Ziomek & Johnson, 1980), in 30–45 % of pairs treated with ECCD-1 for 9 h, the polarity developed off-axis with respect to the intercellular contact point (Table 1, column 5; compare Fig. 2A with 2C,E). This high incidence of off-axis poles in the absence of cell flattening was not an artefact arising from the movement of non-flattened blastomeres about their midbody connection during processing for microscopy as was shown by examination of pairs of cells that had flattened and polarized in control medium, but had then been decompacted in ECCD-1. These cells did not show a high incidence of off-axis polarity (Table 1, line 5, column 5). Moreover, amongst the patterns of off-axis polarity observed were many in which

Table 1. *Incidence and orientation of surface and cytoplasmic polarity in pairs of 2/8 blastomeres cultured for 9 h under various conditions*

Culture condition (1)	No. of cells scored (2)	% of cells with polar surface phenotype (3)	% of cells with polar cytoplasmic phenotype (4)	% of polar cells in which pole is off-axis with respect to contact point (5)
(1) M16+BSA	148	76	74	3
(2) ECCD-1 0–9 h	136	67	73	41
(3) 1/4→2/8 in ECCD-1	31	65	74	35
(4) ECCD-1 6–9 h	42	64	50	14
(5) ECCD-1 8–9 h	46	70	72	5
(6) M16+FCS	36	83	94	6
(7) ECCD-1+FCS 0–9 h	46	78	83	44
(8) Control for (9)–(12)	39	64	74	0
(9) 1/200 anti UV	50	46	58	23
(10) 1/100 anti UV	58	43	60	42
(11) 1/50 anti UV	50	52	70	42
(12) Ca^{2+}-free medium	68	47	56	52

BSA, bovine serum albumen at 0·4 mg ml^{-1}.
FCS, heat-inactivated foetal calf serum at 10 %.

the pole was adjacent to the midbody, a position that could not be achieved by the relative movement of cells in a couplet (e.g. see Fig. 2E,F). In addition, incubation of blastomeres in SSEA-1, a monoclonal antibody that reacts with the cell surface without inhibition of intercellular flattening (Solter & Knowles, 1978), did not cause poles to be off-axis.

(C) *Do other procedures that affect the CDS have similar effects to ECCD-1?*

We examined three other approaches to the neutralization of the CDS, namely the use of an affinity-purified polyclonal antiserum to uvomorulin (an identical molecule to E-cadherin: Kemler, Babinet, Eisen & Jacob, 1977; Hyafil, Morello, Babinet & Jacob, 1980), incubation in Ca^{2+}-free medium which inactivates the CDS (Hyafil, Babinet & Jacob, 1981; Ogou, Okada & Takeichi, 1982), and incubation in trypsin in the absence of Ca^{2+}, which cleaves an exposed 84×10^3 (M_r) fragment from the uvomorulin/cadherin molecule to destroy its activity as an intercellular adhesive (Hyafil *et al.* 1981; Ogou *et al.* 1982). All experimental treatments abolished intercellular flattening. The results from the first two

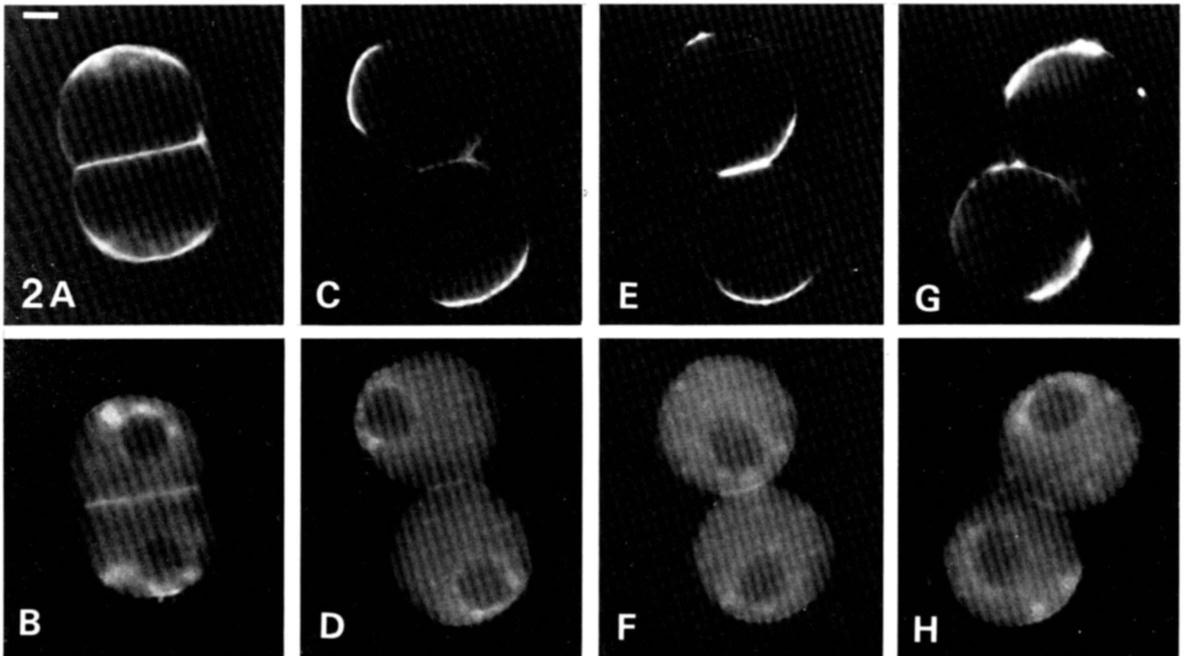

Fig. 2. Pairs of 8-cell blastomeres incubated for 9 h prior to assessment of (A,C,E,G) surface polarity as assessed from the binding pattern of FITC–ConA, and (B,D,F,H) cytoplasmic polarity as assessed from the distribution of intracellular clathrin. (A,B) Control. (C–F) Cells incubated in ECCD-1. Note off-axis poles in the upper cells in each case. In (F) the polar clathrin pattern is less focused, an indication that this cell is about to enter mitosis – Maro *et al.* 1985. (G,H) Cells incubated in anti-uvomorulin. Bar, 2·5 μm.

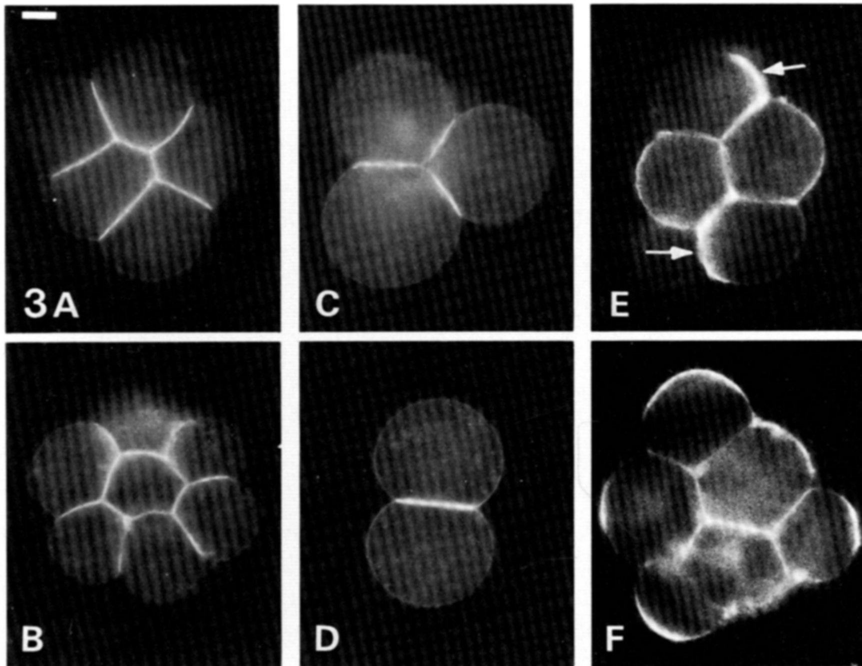

Fig. 3. (A,B) 8-cell embryos, (C) a 4-cell embryo, and (D) a 2/8 pair of cells that had been incubated in ECCD-1 for 8 h, all stained for the distribution of uvomorulin. Note the concentration of staining at intercellular interfaces and the persistence of staining elsewhere under any condition and for both stages of development. (E,F) Intact 8-cell embryos grown in the presence (E) or absence (F) of ECCD-1 from the mid-4-cell stage and examined for evidence of polarity at the late 8-cell stage. Note off-axis poles in (E) – arrows. Bar, (A,B,C,E,F) 1·3 μm; (D) 2·5 μm.

approaches using 2/8 pairs of blastomeres are summarized in Table 1, lines 8–12. It is clear that, as for ECCD-1, all treatments depressed the incidence of both surface and cytoplasmic polarity, but that nonetheless polarity did develop in the majority of cells. Most striking, however, is the effect of the treatments on the orientation of polarity which, as with ECCD-1, was disturbed severely (Fig. 2G,H).

The antiserum to uvomorulin, unlike ECCD-1 (Hatta *et al.* 1985), can be used for immunocytochemical staining, and its use both on pairs of 8-cell blastomeres and on whole embryos from 2- to 8-cell stages reveals that (i) staining with anti-uvomorulin is concentrated at, but not confined to, areas of intercellular contact regardless of which stage of embryo was examined (Fig. 3A–D), and (ii) after prolonged incubation in anti-uvomorulin, ECCD-1 or Ca^{2+}-free medium, surface staining with the antibody nonetheless remains evident (Fig. 3D).

Trypsin digestion of isolated early 8-cell blastomeres is difficult to perform, as the conditions require removal of macromolecules from the medium for an extended period and during this period the blastomeres tend to stick to the culture

dish and lyse. However, trypsin passes readily through the zona pellucida, the acellular porous investment surrounding the embryo, and so intact zona-enclosed 8-cell embryos were used in these experiments. First, the period of exposure to trypsin required to remove CDS activity was determined and the recovery period and conditions for regeneration of CDS activity established. Intact compact mid- to late-8-cell embryos were incubated under the conditions indicated in Table 2 (column 1) for 30 min, cultured under the conditions indicated in column 2 and analysed for the extent of cell flattening at various time points or for the incidence of cell polarity 2 h post-treatment. It is clear that EGTA, whether or not trypsin is present, decompacts the embryo, as would be expected. However, full compaction is re-established within 2 h on removal from EGTA alone, whereas after a combined pulse of trypsin and EGTA reinitiation of flattening occurred only after a delay of 6 to 7 h and completion only after 12 h (Table 2, line 5). This recovery of flattening only occurred in the presence of protein synthesis (Table 2, compare lines 5 & 6). No treatment destroyed the polar state existing at the time of incubation (Table 2, column 4). Control embryos, whether untreated or treated with EGTA or trypsin alone, stained strongly at their surface with anti-uvomorulin by immunofluorescence. Embryos treated with trypsin + EGTA stained less strongly, and the staining was restored after 5–7 h but only in the absence of cycloheximide.

We conclude from these observations that the CDS is regenerated in a form that is functional at the cell surface only after 7 h and in the presence of protein synthesis (see also Peyrieras *et al.* 1983). Next therefore, we took newly formed 8-cell embryos, exposed them to similar incubation and recovery conditions, and analysed them 7 h later. The results are indicated in Table 3. It is clear that a

Table 2. *Effect of various treatments on the flattening and polarization that have already developed in compacted 8-cell embryos*

Incubation condition (30 min) (1)	Culture condition postincubation (2)	No. of embryos analysed (3)	% with polarized blastomeres 2 h post-incubation (4)	% embryos compacted at various h post-incubation			
				0 (5)	2 (6)	7 (7)	12 (8)
(1) Control medium	Control	21	100	95	100	100	100
(2) Control medium	Cycloheximide	30	100	100	100	100	100
(3) Trypsin	Control	25	88	100	88*	100	100
(4) Trypsin	Cycloheximide	33	94	100	100	100	100
(5) Trypsin+EGTA	Control	22	70	0	0	34	90
(6) Trypsin+EGTA	Cycloheximide	34	89	0	0	0	0
(7) EGTA	Control	23	70	0	100	100	100
(8) EGTA	Cycloheximide	35	72	0	100	100	100

*These embryos were undergoing division to 16 cells, during which time they decompact transiently. Cycloheximide blocks division.

prolonged block to flattening occurs only after incubation in trypsin + EGTA and that some recovery from this block is occurring by 7 h when protein synthesis is permitted (Table 3, lines 5 & 6), a result that is not surprising in the light of the results in Table 2. However, after all incubation conditions polarity developed. The incidence of polarity was not so high when a trypsin + EGTA incubation was followed by recovery in control medium (Table 3, compare lines 5 & 6). This result is weighted heavily by the results from one experiment in which little polarization occurred at all in this subgroup compared with two others in which 100 % polarization was scored. However, it is clear that polarization can occur after such treatments, and can do so whether or not protein synthesis is present. When the nature and position of the surface poles were examined, however, they were rather paler and more 'ragged' when incubation had been in trypsin or trypsin + EGTA, possibly because of digestion of surface moieties reactive with the FITC–Con A probe. Moreover, after trypsin + EGTA many of the poles did not face outwards away from the contact points with other blastomeres, but were often directed laterally, apparently at random with respect to other blastomeres. Such a result appeared to be equivalent to the off-axis scoring described above for 2/8 pairs. We therefore examined the orientation of polarity in intact embryos placed in ECCD-1 at the mid-4-cell stage and cultured to the late 8-cell stage before analysis. Many blastomeres in these embryos also showed off-axis poles (Fig. 3E), a phenomenon we have never observed in control embryos (Fig. 3F).

Embryos treated with trypsin + EGTA did go on to form blastocysts, demonstrating that the procedures used did not damage the cells irreversibly. However, the blastocysts did contain ICM cells, an observation that is not surprising since the CDS is regenerated within 7 h to permit flattening unless protein synthesis is inhibited, in which case the embryos arrest as eight cells, thus preventing assessment of cell fate. Similarly, prolonged exposure to low calcium damages cell health and division (Ducibella & Anderson, 1976; Reeve, 1981*a,b*), and although ICM formation is impaired, the result is difficult to interpret with confidence.

Table 3. *Effect of treating newly formed 8-cell embryos in various ways on subsequent development of flattening and polarization.*

Incubation conditions (30 min) (1)	Culture condition postincubation (7 h) (2)	No. of embryos analysed (3)	% with polarized blastomeres (4)	% compacted (5)
(1) Control medium	Control	38	92	96
(2) Control medium	Cycloheximide	67	91	95
(3) Trypsin	Control	27	100	89
(4) Trypsin	Cycloheximide	43	92	90
(5) Trypsin+EGTA	Control	47	56	7
(6) Trypsin+EGTA	Cycloheximide	70	94	0
(7) EGTA	Control	38	95	85
(8) EGTA	Cycloheximide	41	89	92

Thus, use of four agents that affect the CDS, namely a polyclonal antiserum to uvomorulin, a monoclonal antibody to E-cadherin, prolonged exposure to medium low in Ca^{2+}, and pulsed exposure to trypsin + EGTA (with or without subsequent regeneration of the CDS) all yield the same result. First, intercellular flattening is abolished for as long as the adhesion system is inactivated. This result is in agreement with earlier results (Shirayoshi *et al.* 1983) and, taken with the observation that the ICM is reduced in the presence of the antibody, is consistent with the predictions of the polarization hypothesis. Second, development of polarization is not inhibited. This result contradicts that reported by Shirayoshi *et al.* (1983) but explains the ability of treated embryos to form trophectodermal vesicles, and is thus consistent with the predictions of the polarization hypothesis. Third, the orientation of the polarity that develops in pairs of 8-cell blastomeres and in whole embryos differs from that observed in controls, being unrelated to the points of contact made with other cells.

(D) *Exposure to ECCD-1 affects the time course of polarization*

It is important to determine why the present results differ from those reported by Shirayoshi *et al.* (1983). In the latter study, most experiments were performed by disaggregating 4-cell embryos to 1/4 blastomeres, placing these in ECCD-1, incubating cells through division to 2/8 pairs and then for a further 9 to 11 h into the fourth cell cycle. Late 1/4 cells treated by us in this way all divided to 2/8 within 1 to 2 h and showed a similar polarization pattern to newly formed 1/8 cells (compare lines 2 and 3 in Table 1). However, exposure of 1/4 cells to ECCD-1 earlier in the third cell cycle might yield a different result. We therefore took newly formed (0 h old) 1/4 blastomeres and placed them in ECCD-1 at 0 h, 5 h or 10 h postformation, and cultured them until the late 8-cell stage when control 2/8 were well compacted and polarized. The results are shown in Fig. 4B, and do reveal (i) a slight reduction in the incidence of polarization in the presence of ECCD-1 as was observed before, e.g. Table 1, and (ii) no very marked effect of the total period of exposure of cells to ECCD-1 on the incidence of polarization.

Next we examined the time course rather than the endpoint of polarization in the presence or absence of the antibody. Polarization is a continuous process, the time course of which can be monitored by serially sampling a population of blastomeres and scoring each for both cytoplasmic and surface reorganization. Were polarization to occur more slowly in the presence of ECCD-1, then a single-point sampling of the sort used both here and by Shirayoshi *et al.* (1983) would yield different conclusions depending on whether the samples were taken during or after the completion of the polarization process. We therefore took newly formed 1/4 blastomeres and placed them either in control medium or in ECCD-1 and cultured them until control blastomeres were 2, 6 or 10 h postcompaction. Samples were then scored for polarity (Fig. 5) and the results are presented in Fig. 4A). It is clear that at each time point there was less surface and cytoplasmic polarity in ECCD-1 exposed cells than in controls, but that the proportion of polarized cells increased with increasing time in both groups. It is important to

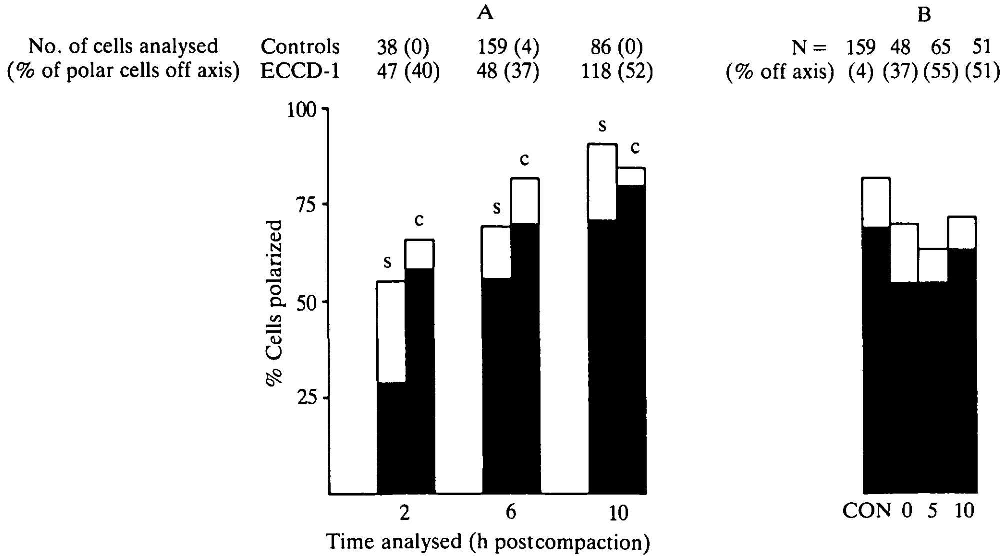

Fig. 4. (A) Newly formed 4-cell blastomeres were isolated and placed either in control culture medium (full height of bar in each case) or in medium containing ECCD-1 (solid bar height), cultured until controls were 2, 6 or 10 h postcompaction, and scored for either surface (s) or cytoplasmic (c) polarity. The numbers of cells analysed for each condition (and the percentage of poles scored as off axis) are recorded above each bar. (B) Newly formed 4-cell blastomeres were isolated and divided into four groups. One group was placed in control medium (CON) until the late compact 8-cell stage. The other groups were placed in ECCD-1 at 0, 5 or 10h postdivision to four cells, and cultured for the same period. All were then examined for cytoplasmic (full bar height) or surface (solid bar height) polarity. The numbers of cells analysed (and the percentage of poles off axis) are recorded above each bar.

stress that in these studies there was no evidence that ECCD-1 was affecting the length of the cell cycles since the times at which 1/4 cells divided to 2/8 pairs or 1/8 cells divided to 2/16 pairs were unaffected. Only the time course of polarization was affected.

(E) *A comparison of polarization in pairs of cells incubated in the presence of ECCD-1 with polarization in single cells*

Our results suggest that embryos or pairs of cells incubated in ECCD-1 differ from control cells in two ways. First, they polarize with an axis that disregards the position of their cell-contact pattern. Second, they polarize more slowly. The latter of these properties resembles that described previously for single, isolated blastomeres (Ziomek & Johnson, 1980). We therefore undertook a detailed comparison of the time course of polarization of single cells and pairs of cells incubated in the presence or absence of ECCD-1. Newly formed 2/8 pairs were disaggregated to 1/8 cells, all of which were exposed briefly to PHA (to facilitate subsequent aggregation) and then assigned to one of four groups. Some were placed in culture in either control medium or in ECCD-1, whilst others were aggregated in pairs and divided into identical groups for culture. Cells were

cultured for 7, 9 or 11 h before harvesting for analysis of surface polarty. The results are presented in Fig. 6. It is clear that the behaviour of pairs of cells incubated in ECCD-1 resembles that of single cells but differs from that of control pairs.

DISCUSSION

We have re-examined the effect of the monoclonal antibody to E-cadherin, ECCD-1, on the development of intercellular flattening and polarization of mouse 8-cell blastomeres. We were able to confirm that exposure to the antibody prevented the cell flattening that occurs normally at the 8- and early 16-cell stages, and did not prevent the postcompaction cell interactions that lead to junction formation and the production of a blastocoel. We have also confirmed the observation of Shirayoshi *et al.* (1983) that the development of embryos in the presence of the antibody leads to a reduced or absent ICM. Like them, we also failed to observe ICMs in both intact embryos and the outgrowths from them, and

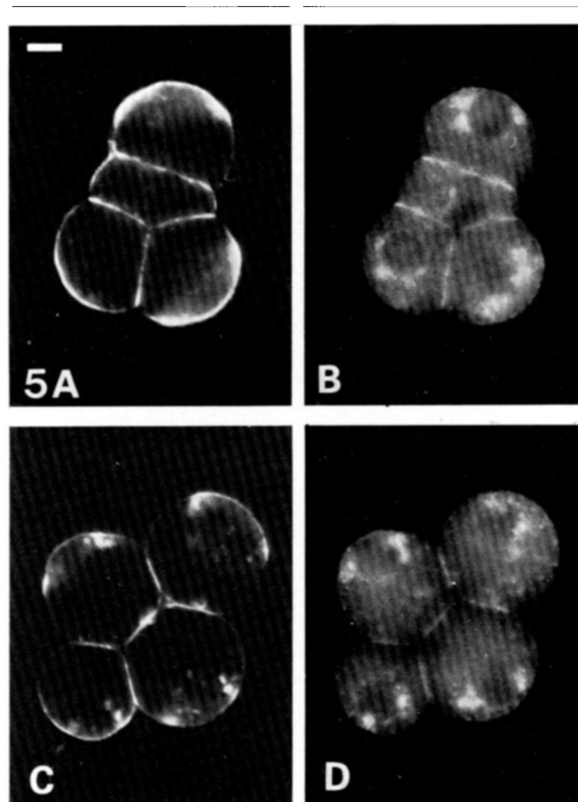

Fig. 5. Single, newly formed 4-cell blastomeres were cultured in the absence (A,B) or presence (C,D) of ECCD-1 to the mid-16-cell stage (10 h after compaction of the controls) and scored for the distribution of ConA receptors (A,C) or clathrin (B,D). Note three polar cells and one apolar cell in (A,B) and four polar cells in (C,D). Bar, 2·5 μm.

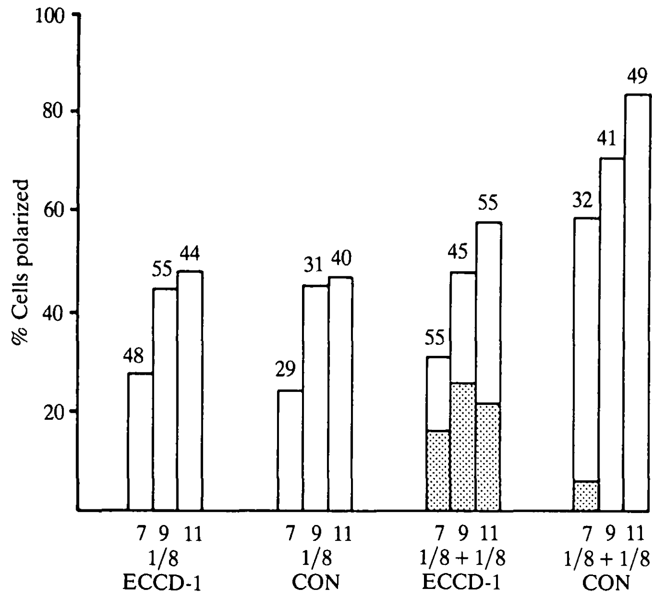

Fig. 6. Newly formed 8-cell blastomeres were disaggregated to 1/8 blastomeres. Half were reaggregated together in pairs (1/8+1/8). Aggregated and single cells were then cultured in control medium or in medium containing ECCD-1 for 7, 9 or 11 h before being scored for surface polarity. The proportion of poles scored as off axis are indicated by hatching. The numbers of blastomeres scored are recorded above each condition.

we extended their observation that ECCD-1-treated embryos were deficient in cells positive for alkaline phosphatase (diagnostic of ICM; Mulnard & Huygens, 1978), by showing that all cells present contained ENDO-A (the presence of which is diagnostic for trophectoderm; Brulet *et al.* 1980). Thus it has now been demonstrated by four different criteria that ECCD-1 treatment impairs ICM formation. It does so without impairing cell division and thus resembles treatment with a more complex polyclonal antiserum directed against multiple surface specificities which also disrupted intercellular flattening and ICM formation without affecting mitosis (Johnson *et al.* 1979). However, our results do differ from the previous study of Shirayoshi, Okada & Takeichi (1983) in two regards.

First, we did not find that exposure of embryos to ECCD-1 prevented polarization. We did find that the antibody delayed the attainment of polarization within a population of embryos but did so without any evidence that cell cycles were also lengthened. A detailed study of the time course of polarization in pairs of blastomeres incubated in the presence of the antibody showed that they behaved similarly to isolated blastomeres, which as a population also polarized over a more protracted time course (see also Ziomek & Johnson, 1980). Thus, it seems that in the presence of the antibody, blastomeres behaved as though they were unable, or less able, to recognize the presence of an adjacent cell. Evidence has recently been presented that suggests that an initiation signal for polarization is given on reaching the 8-cell stage and that this signal may involve the lifting of

an inhibitory effect exerted by a putative compaction-restraining factor (Levy, Johnson, Goodall & Maro, 1986). From the results presented here, we suggest that the rate of implementation of the initiated programme may be influenced by cell interactions mediated at least in part *via* the CDS, and which facilitate the temporal coordination of polarization among the blastomeres of an embryo. The CDS may achieve this synchronizing action by providing a focus for the initial intracellular events involved in polarization.

This focusing action also gives the CDS a crucial role in the spatial organization of compaction. Thus, exposure of 2/8 pairs to ECCD-1 had a radical effect on the axis of polarity, which appeared to be oriented randomly rather than perpendicular to the contact point(s) with other cells. This random orientation of the polar axis was also observed with other agents that suppressed intercellular flattening by interfering with the CDS. Such a randomization has been reported previously only during polarization in the presence of cytochalasin D (Johnson & Maro, 1984, 1985), an agent that also prevents intercellular flattening. Thus we conclude that cell adhesion normally not only increases the synchrony of polarization but also orients the developing polarity and does so through some property related to the cell flattening activity of the CDS. We assume that in the absence of a companion cell, or after the neutralization of its presence by ECCD-1, the blastomere initiates polarization either randomly or perhaps as a result of non-specific contact between the cell and physical features of its environment (see also Ziomek & Johnson, 1980). The CDS can be viewed as providing a localized lowering of a threshold for some intracellular change, which occurs later and randomly in the isolated cell. The CDS-mediated response may thus be envisaged as catalytic for an inherent, programmed developmental change. Such a conclusion has important implications for the design of experiments that attempt to assess the specificity of polarity induction.

Despite the delayed and disoriented polarity of blastomeres incubated in ECCD-1, the cells nonetheless do progress to form focal cell junctional associations at the late 16-cell stage and to form zonular junctions and a blastocoel at the 32-cell stage. This result implies that the disoriented blastomeres have rotated and sorted subsequently so that they become integrated within an epithelial sheet (see Fig. 1B). Such a sorting process is not surprising since a similar adjustment has also been observed when the spatial relationships of polarized cells are disturbed experimentally by various procedures (Johnson, 1986). The embryo displays a neat piece of developmental economy in using a cell surface adhesion system to orient and synchronize the initial polarization of cells, thereby reducing the requirement for reorientation and sorting of polarized cells subsequently.

The subcellular mechanisms by which the CDS achieves its orienting and synchronizing effects are not clear from these experiments. However, three pieces of evidence from previous observations do give us some clues. First, since gap junctions nonetheless develop in the presence of ECCD-1 (Goodall, 1986), they are not obviously involved in mediating the action of the CDS. Second, we have

reported that the stabilization of microtubules tends to retard the events of compaction, whilst their destabilization has a synchronizing effect (Maro & Pickering, 1984). It is therefore possible that one consequence of the intercellular flattening mediated *via* the CDS is a local depolymerization of microtubules, and indeed there is evidence to support such a possibility (see Johnson & Maro, 1986). Third, there is clear evidence both from the work presented here and from that already published (Hyafil *et al.* 1980; Shirayoshi *et al.* 1983) that the molecular species mediating the CDS activity is present at the blastomere surface from the 1-cell stage, and that it is not restricted to sites of intercellular adhesion. Thus, the extensive intercellular flattening that occurs first at the 8-cell stage is not likely to be controlled at the level of production of the CDS, but rather by its activation (Johnson, 1985). Such a conclusion is consistent with the observation that the initiation signal for compaction appears to function at a post-translational level (Levy *et al.* 1986). We have proposed that this initiation signal, acting *via* the influence of CDS-mediated flattening on subcortical organization, marks the first step in the process of cell diversification that leads to blastocyst formation (Johnson & Maro, 1986).

We wish to acknowledge the technical assistance of Gin Flach, Martin George, Sue Pickering, Ian Edgar and Sheena Glenister. The antiserum to clathrin was a gift from Dr D. Louvard, to actin from Drs E. Karsenti and P. Gounon, to Endo A (antiserum TROMA-1) from Dr Ph. Brulet and to uvomorulin from Dr N. Peyrieras and Pr F. Jacob. This work was supported by grants to M.H.J from the Medical Research Council and the Cancer Research Campaign, and to B.M. from the Fondation pour la Recherche Medicale. B.M. is an EMBO Fellow.

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(Accepted 10 November 1985)