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Cytocortical organization during natural and prolonged mitosis of mouse 8-cell blastomeres

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Summary

Late 8-cell blastomeres were harvested within the first 45 min after entering mitosis. Some mitotic cells were analysed within the ensuing 2 h for the organization of their surface in relation to their progress through mitosis. Whereas in most late interphase cells microvilli were restricted to a discrete polar region, in mitotic cells at all stages from early metaphase to immediately postcytokinesis microvilli were found to be present over more of the cell surface. Other mitotic cells were placed in nocodazole to arrest them in M-phase for up to 10 h. They were found to show an even more extensive distribution of microvilli over the whole surface, the longer periods of incubation yielding more extended coverage such that many cells no longer appeared to have any residual surface polarity. Removal from nocodazole at all time points from 1 to 10 h resulted in most cells completing mitosis to yield pairs of cells which, in most cases, resembled pairs derived from nonarrested blastomeres and in which a defined polar area of microvilli was restored. However, the percentage of differentiative divisions decreased after 6 h arrest. If, instead of removing cells from nocodazole, they were placed in both nocodazole and cytochalasin D (CCD) for periods of up to 3 h, most microvilli retracted to reveal a tight polar zone of CCD-resistant microvilli. This result suggests that a heterogeneity of cytocortical organization may still exist within the arrested mitotic cell. We propose a model to explain the origin of this heterogeneity of organization and its relationship to the generation of cell diversity.

Key words: cytocortex, mitosis, nocodazole, mouse embryo, cytochalasin D.

Introduction

Cell diversification in the early mouse embryo is first evident at the 16-cell stage. Two subpopulations of cells are present and differ in position, phenotype, properties and presumptive fate. It is believed that these two cell subpopulations originate at the preceeding division of the 8-cell blastomeres by a process of unequal cell division. In common with other developmental systems, in which unequal cell division is thought to play an important role in the generation of cell diversity, the mouse 8-cell blastomere displays (i) a clear asymmetry of organization prior to mitotic division, (ii) the retention of some elements of that asymmetry throughout the division process, (iii) the differential distribution of the conserved asymmetric elements to the two daughter cells, and (iv) the involvement of the differentially distributed elements in the developmental behaviour of the two daughter cells (reviewed in Davidson, 1986; Johnson *et al.* 1986).

The asymmetric or polar organization of the 8-cell blastomere is manifested at many levels within the cell, involving the cytoskeleton, cytoplasmic organelles and cell surface. However, at division of each 8-cell to yield a pair of 16-cells (2/16 pair), most of these asymmetrically organized features are redistributed throughout the cell, the only exception being elements of polarity within or associated with the surface membrane (Johnson & Ziomek, 1981; Maro

et al. 1985). It has therefore been suggested that there exists within the surface membrane or its associated cytocortex a distinct and relatively stable domain that directs the formation of the polar axis in interphase cells (Johnson & Maro, 1985), causes the cells possessing it to enter the trophectodermal lineage (Johnson, 1986), and serves as a 'memory' trace for polarity during mitosis such that one or both daughter cells, depending on cleavage plane orientation, inherit all or part of it (Johnson & Ziomek, 1981). In order for this putative cytocortical domain to function in the last of these capacities, it is important that its biological half-life should be significantly greater than the time taken to traverse mitosis.

In this paper, we describe experiments that examine the duration of the mitotic division from the 8- to the 16-cell stage, the nature of the changes in surface organization during this division, and the effect on these changes of arresting mitosis at metaphase for up to 10 h. We conclude that a discrete area of the cytocortex of the late 8-cell blastomere, coincident with, but not identical to, the overt pole of microvilli, could provide a stable focus for both the elaboration of cell polarity and the transmission of a polar axis to one or both daughter cells at division.

Materials and methods

Recovery of embryos

Follicular development was promoted in 3- to 4-week-old female mice (MF1 strain, Laboratory Animal Centre, Cambridge University) by injecting 5 i.u. of pregnant mare's serum gonadotrophin (Folligon, Intervet). Ovulation was induced by administering 5 i.u. human chorionic gonadotrophin (hCG; Chorulon, Intervet) 48 h later. Females were paired overnight with CFLP males (Interfauna) and those with a vaginal plug the following morning were retained. Embryos were flushed from the oviducts about 67 h post-hCG as compact 8-cells, or, when 4-cell embryos were required, were flushed at 48 h post-hCG and cultured overnight.

Preparation and handling of single cells

Well-compacted 8-cell embryos and late 4-cell embryos in some experiments were selected and exposed briefly to acid Tyrode's solution to remove the zona pellucida (Nicolson *et al.* 1975) then washed in Medium 2 containing 4 mg ml^{-1} BSA (M2 + BSA; Fulton & Whittingham, 1978) and cultured for 1 h to allow recovery. The embryos were then disaggregated to single cells by placing them for 15–30 min in Ca^{2+}-free M2 containing 6 mg ml^{-1} BSA, and gently moving the embryos up and down a flame-polished micropipette. Single 4-cell (1/4) or 8-cell (1/8) blastomeres in which a nuclear membrane was clearly visible were then transferred to drops of Medium 16 + 4 mg ml^{-1} BSA (Whittingham & Wales, 1969) under oil in Sterilin tissue-culture dishes pre-equilibrated at 37 °C in 5 % CO_2. Blastomeres were scored every 45 min on a Wild M5 dissecting microscope (×20 eyepieces, ×50 objective) fitted with a heated stage and any cells in which a nuclear membrane was not evident were harvested.

Arrest of cells in mitosis

Successful passage through mitosis requires a mitotic spindle, and disruption of the spindle halts the mitotic sequence of events at the metaphase–anaphase boundary. The drug nocodazole (NOC) disrupts microtubules (Hoeboke *et al.* 1976) and was used to dismantle the spindle. Nocodazole acts rapidly and is reversible. Putative mitotic cells were transferred to drops of M16 + BSA containing 10 μM-nocodazole (stock solution 10 mM in DMSO), a concentration effective in disrupting microtubule arrays in mouse embryos (Maro & Pickering, 1984; Johnson & Maro, 1985). Cells were incubated for 0–10 h in M16 + BSA + NOC under oil in 5 % CO_2 at 37 °C, after which time they were either stained and fixed, or were washed in M16 + BSA and transferred to nocodazole-free M16 + BSA. Cells in the latter group were fixed for analysis between 15 min and 24 h after release from mitotic arrest. In some experiments, cytochalasin D (CCD) at 0·5 μg ml^{-1} (stock solution 1 mg ml^{-1} in DMSO) was used to destabilize microfilaments.

Fluorescence microscopy

Cells were exposed to Concanavalin A conjugated to fluorescein isothiocyanate (FITC–ConA; 0·7 mg ml^{-1}, Sigma) for 5 min, followed by three washes in M2 + BSA. Nocodazole was present in all solutions used for staining and washing mitotic cells, since the effects of nocodazole are reversed rapidly in its absence. Similarly, CCD was also present in all solutions when cells were exposed to this drug at harvesting. The cells were then placed in specially designed chambers (Maro *et al.* 1984) which had been exposed briefly to phytohaemagglutinin to improve adhesion (PHA, Gibco, diluted 1:20 in PBS), washed three times with protein-free M2, and filled with M2 containing 6 mg ml^{-1} polyvinylpyrrolidone (M2 + PVP). The stained cells were then processed as described in Maro *et al.* (1984), fixed in 3·7 % formaldehyde in PBS for 30–40 min, washed in phosphate-buffered saline (PBS) and stained with the chromatin dye Hoechst 33258 (Sigma: 50 μg ml^{-1} in PBS) for 10 min. The cells were washed twice in PBS and left for 15 min in PBS before mounting in 'Citifluor' (City University, London). Samples were viewed on a Leitz Ortholux II microscope with filter set I2 for FITC–ConA and A for Hoechst, and Nomarski differential interference optics for bright field. Photographs were taken on Kodak Tri-X film using the Leitz Vario-orthomat photographic system.

Scanning electron microscopy

Cells were fixed for 30 min in a solution of 6 % (v/v) glutaraldehyde (Sigma) in millipored HPEM buffer (Pickering *et al.* 1988) and then transferred to HPEM buffer for at least 5 min for washing. These procedures, together with those for mounting cells and preparing them for viewing, are described in detail in Pickering *et al.* (1988).

Scoring of cells

Single FITC–ConA-stained interphase, mitotic and postmitotic 8-cell blastomeres were examined at all focal planes for the proportion of the surface with dense ConA staining. The mitotic state was recorded from the Hoechst staining pattern. With the SEM analysis, a similar classification was undertaken using the tilt and rotation facilities on each cell.

Results

(A) *Reliability of scoring for entry into mitosis*

In order to facilitate the study of mitotic cells, it was essential to obtain a subpopulation of cells enriched for recent entry into mitosis. Examination of a population of isolated late 8-cell blastomeres revealed some in which the nuclear envelope was not evident. It seemed possible that these cells had entered mitotic prophase recently. We therefore harvested all such cells and divided them into two groups. One group was fixed immediately and stained with the chromosomal stain Hoechst 33258. Of 59 cells examined, 48 (82 %) were in mitosis. The second group was cultured for 2 h and then examined for evidence of division; of 68 cells examined, 60 (88 %) had divided. Overall, these data indicate that there is a scoring error of between 10 and 20 %. This conclusion was born out in subsequent experiments. Interestingly, cells that were scored as lacking nuclear membranes and placed in nocodazole for up to 10 h, but which were then nonetheless found to be arrested in interphase, had an incidence of tight poles that was no different from that in cells late in interphase of the 8-cell stage (e.g. Fig. 5A,B). These cells, some of which must have had an interphase of up to 18 h (compared to 10–12 typically), show that the late interphase pole can be stable in the presence of the drug (contrast with the M-phase changes reported in Sections B and C below).

In all subsequent experiments, a population of late 8-cell blastomeres isolated from compact embryos, was examined at 45 min intervals. All cells scored as negative for nuclear membranes were assumed to be at some point in the mitotic cycle prior to cytokinesis. These cells were harvested for experimental use. Erroneously assigned cells were detected readily when analysis for ConA binding was used, as the nuclear staining revealed an interphase nucleus. However, scoring by scanning electron microscopy (SEM) could not detect such errors, and thus data from this approach must be assumed to include a group of up to approximately 20 % of erroneously scored cells. The consequences of this error are indicated in footnotes to the relevant data Tables.

(B) *Surface phenotype of cells passing through mitosis*

260 cells were harvested as lacking nuclear membranes and were fixed immediately or cultured for up to 2 h prior fixation and analysis either on the SEM or by staining of their surface with FITC–ConA plus the nuclear stain Hoechst 33258. Divided cells were found at all time points after harvest from 15 min onwards, and by 2 h *all* cells had divided. At time points up to 1 h most cells were found in metaphase, with some in anaphase or telophase. These data suggest (i) that the time taken for the entire population of sampled cells to complete mitosis is between 1 and 2 h from harvesting, which therefore represents the approximate duration of mitosis from late prophase to cytokinesis, and (ii) that metaphase occupies the major part of the mitotic period.

The surface of each cell (or pair of cells) examined in this experiment was then analysed for the extent and pattern of either its microvilli (SEM) or its ConA-binding sites. In addition, the ConA-binding patterns of populations of late interphase 1/8 cells and of 2/16 cells harvested 2–3 h after the *completion* of division are included for comparison. The results are recorded in Table 1, and representative cells are shown in Figs 1 and 2. It is evident that (i) during mitosis the area of the surface over which microvilli and ConA-binding sites are distributed tends to increase, (ii) this increase is not obviously restricted to a particular phase of mitosis, (iii) despite this increase, a clear asymmetry or polarity is usually evident throughout the process of mitosis, and (iv) after division, the proportionate area of the surface of the two 16-cell blastomeres over which microvilli and ConA-binding sites are distributed is restored towards that observed on 1/8 cells prior to mitosis. As we have reported previously (Johnson & Ziomek, 1981), most 2/16 pairs consisted of either one polar and one nonpolar cell (Fig. 2C–E) or of two polar cells, the poles being contiguous across the remnant of the cleavage furrow, reflecting the bisection of the pole at division (Fig. 2F–H).

(C) *The effect of arresting cells in mitosis on surface phenotype*

Nocodazole acts reversibly to sequester tubulin monomers and so causes the unravelling and disappearance of dynamic microtubules. Applied during mitosis, nocodazole will prevent assembly, and cause disassembly, of the spindle, thereby causing cells to arrest at the end of metaphase prior to chromosomal separation. Thus, if cells scored as lacking a nuclear membrane are placed immediately into nocodazole, those that are in prophase or metaphase cannot proceed into anaphase and therefore are arrested. Any cells that have passed the metaphase–anaphase

Table 1. *Proportion of cell surface occupied by microvilli or ConA-binding sites in 8-cell blastomeres undergoing mitosis and in pairs of 16-cell blastomeres soon after completion of mitosis*

Type of cell analysed	No. of cells analysed	% of cells in which the pole occupies: less than half surface area	half or more of surface area	whole surface
ConA binding				
Control late 8-cell (premitotic)	54	81	9	10
Metaphase	43	40	51	9
Anaphase/telophase	13	61	31	8
Postdivision cells*	59	64	32	4
2/16 pairs 2–3 h postcleavage	93	76	23	1
SEM				
Undivided cells	31	39	52	9
Dividing cells	13	31	62	7
Postdivision cells*	51	65	35	0

* The age of these cells postcytokinesis will range from minutes to a maximum of 2 h (see text). In the pairs, the proportion of the total surface of both cells occupied by microvilli is recorded regardless of whether the microvilli are distributed on one or both of the cells.

boundary at the time of addition of the drug progress through cytokinesis apparently unimpaired.

Cells scored as lacking a nuclear membrane were harvested and placed in nocodazole immediately. Any cells that divided within the next hour were assumed to have passed the metaphase–anaphase boundary and were discarded. The remaining cells were incubated for a total of 0 to 10 h in 10 μM-nocodazole, and were then fixed and examined either for the distribution of microvilli or for the distribution of ConA-binding sites. The scoring criteria used to classify the extent of ConA binding are illustrated in Figs 1 and 4, and examples of scoring criteria used in scanning electron microscopy are illustrated in Fig. 3. The results for each analysis are recorded in Tables 2 and 3.

The data in Table 2 reveal that with longer periods of exposure to nocodazole the distribution of ConA-binding sites becomes progressively more dispersed over the surface of the arrested cell, until by 4 h the majority of cells could not be scored with confidence as being polar. SEM analysis provides greater resolution than fluorescence microscopy and, as the data in Table 3 show, the proportion of cells assigned to the homogeneous group at each time point was lower, largely due to detection of a category of cells scored as having a graded distribution of microvilli across the cell surface, that gave these cells a residual axis of polarity (Fig. 3D). Close examination of microvilli under the SEM, particularly over the 2–5 h exposure period, revealed that, in general, the emergence of microvilli over more of the blastomere surface occurred by growth of new microvilli, whilst those at the polar region appeared to shorten slightly (Fig. 3, compare A,B with C,D).

Taken together, these results suggest that arrest in metaphase is associated with the loss of a clearly defined, tight, polar domain of microvilli and, indeed, at more prolonged time points with a progressive loss of all evidence of cell surface polarity.

Table 2. *Surface staining patterns assessed by fluorescence microscopy in 8-cell blastomeres arrested in mitosis for up to 10 h*

Duration of exposure to nocodazole	No. of cells analysed	Proportion of cell surface staining brightly with FITC–ConA (% of cells): quarter or less	quarter to half	more than half	homogeneous
Late 1/8 (controls)	59	39	42	9	10
0 h	31	3	53	38	6
1 h	56	0	52	30	18
2 h	46	4	42	39	15
3 h	77	1	20	33	46
4 h	80	0	4	29	67
6 h*	39	0	7	18	75
10 h*	20	0	5	30	65

* These groups are considered more fully by scanning electron micrography – see Table 3.

(D) *Phenotypes of 2/16 pairs formed on release from nocodazole arrest*

Cells exposed to nocodazole for up to 10 h were rinsed in medium lacking the drug and cultured for 2 h, during which time most of them divided (Table 4, column 2). The phenotypes of the 2/16 couplets produced were analysed, and the results are recorded in Table 4 with representative pairs of cells shown in Fig. 4K–P. It is clear that exposure to nocodazole for up to 6 h does not appear to influence the distribution of phenotypes produced on release from arrest. However, after 8 and 10 h of exposure to the drug, released cells generate a higher proportion of couplets in which both cells are either polar or nonpolar. In the latter case, both cells were found to be covered uniformly in microvilli and ConA-binding sites, all evidence of polarity being lost (Fig. 4I,J).

In order to determine whether those pairs of cells released from nocodazole after 6 or 10 h exposure were functionally similar to control 2/16 pairs, some were released from arrest and incubated in control medium for 10 or 24 h rather than just 2 h, and their

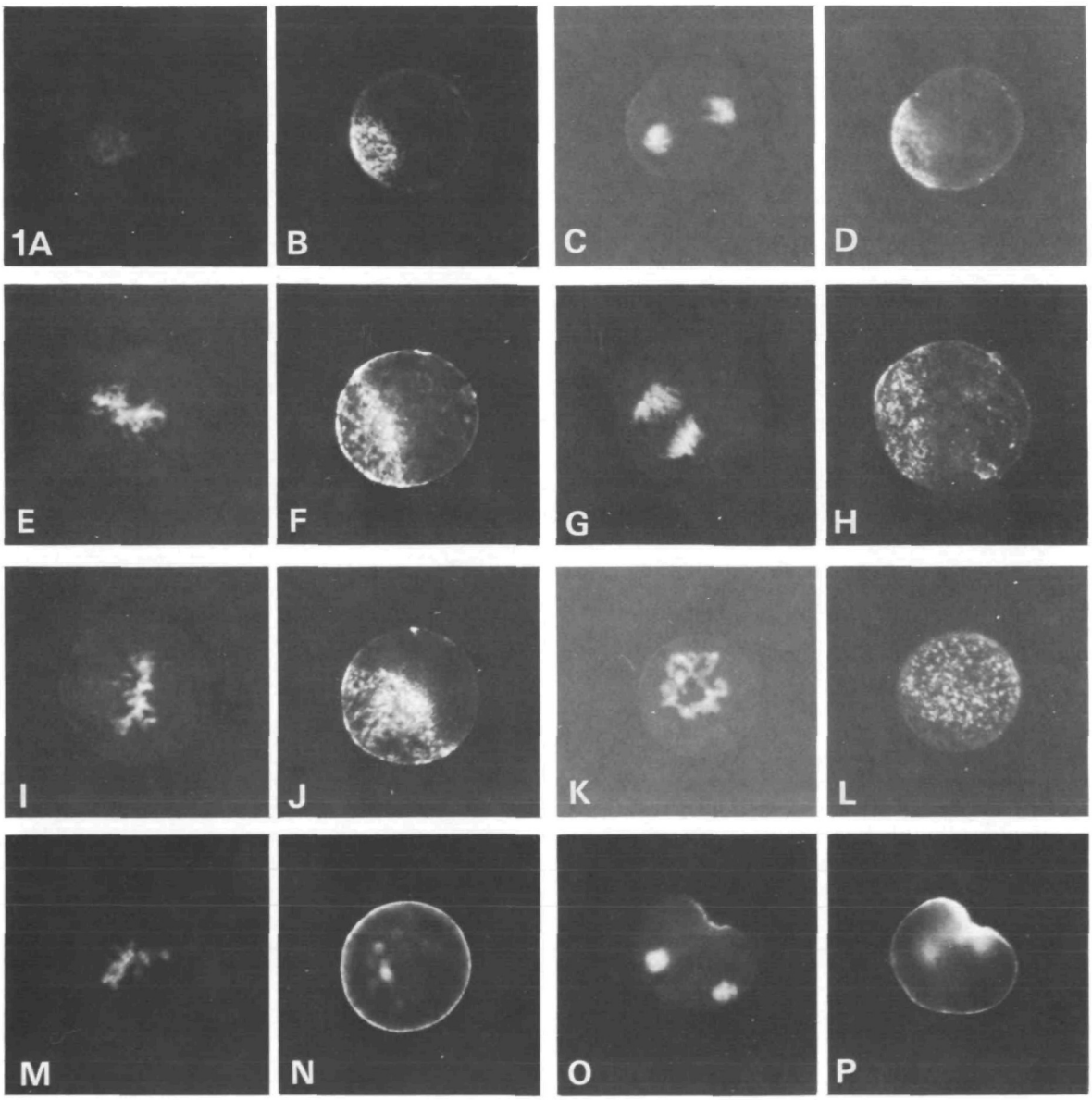


Fig. 1. 8-cell blastomeres harvested while undergoing mitosis, stained with FITC–ConA (panels B,D,F,H,J,L,N,P) fixed and stained with Hoechst 33258 (panels A,C,E,G,I,K,M,O). Note that in most cells there is evidence of surface polarity during mitosis. (A,B) Interphase cell with pole occupying less than 25 % of surface. (C,D) Anaphase cell with similar pole. (E,F) Metaphase cell with pole occupying approximately 25 % of surface. (G,H) Similar cell in anaphase. (I,J) Metaphase cell with pole occupying 50 % of surface. (K,L) Early anaphase cell with pole occupying 75 % of surface. (M,N) Apolar metaphase cell. (O,P) Telophase cell with pole occupying between 25 and 50 % of surface. ×32.

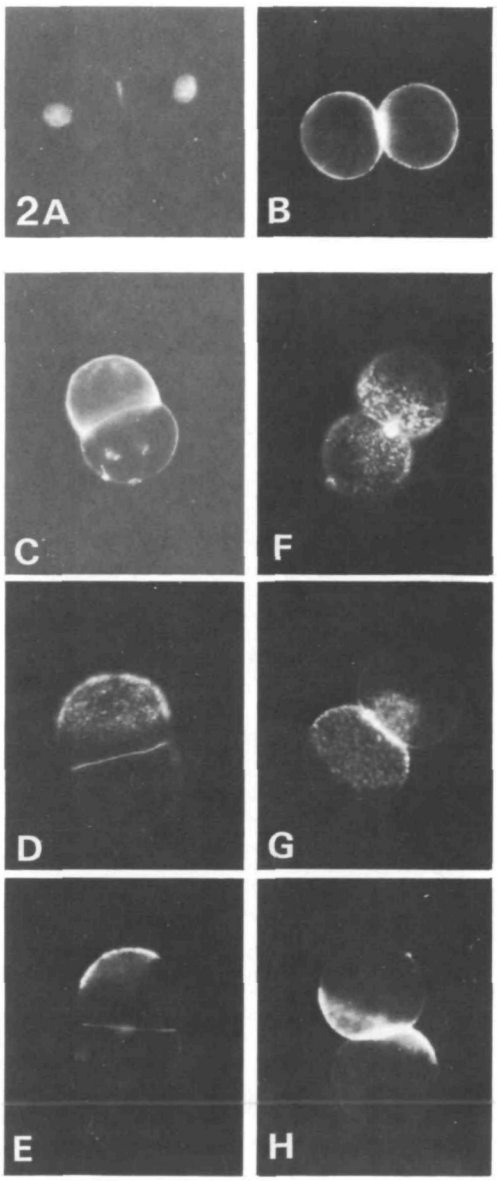


Fig. 2. Postmitotic 8-cells stained as in Fig. 1 (Hoechst panel A; ConA panels B–H) and in which the constituent 2/16 cells are (A,B) both nonpolar; (C–E) one polar and one nonpolar, the pole occupying 50 %, 25 % and less than 25 % of the total surface area; (F–H) both polar but with poles contiguous across midbody and poles occupying varying amount of total surface area. ×32.

phenotype and behaviour examined (Table 5). Pairs of 16-cell blastomeres derived from nonarrested mitotic 8-cells flattened rapidly on each other, and, in most cases, by 10 h one cell (the polar cell) had spread around the other (nonpolar) cell to envelope it. With further culture, a fluid-containing cavity formed in the centre of the cell cluster, yielding a miniblastocyst as described in detail previously (Ziomek & Johnson, 1981; Johnson & Ziomek, 1983). Mitotic 8-cells arrested for 6 or 10 h in nocodazole behaved similarly, but the proportion showing envelopment was reduced, and the incidence of blastocoel formation at 24 h was lower than in non arrested controls. In general, a subpopulation within the nocodazole-exposed cells looked less healthy than nonexposed cells, especially when exposure had been for 10 h. However, the general pattern of behaviour of the remaining released pairs was not grossly dissimilar from that of controls.

(E) *Surface phenotypes of 6 h nocodazole-arrested cells immediately after release*

Most 2/16 pairs derived after release of cells arrested in nocodazole resembled those derived from nonarrested blastomeres. Yet, prior to release, the arrested blastomeres were in many cases recorded as being nonpolar and in most remaining cases as having microvilli and ConA-binding sites that extended over more than 50 % of the cell surface. We enquired whether the restoration of a discrete polar organization to the surface of the released cells occurred before, during or after the completion of mitosis. Arrested cells were rinsed in nocodazole-free medium, and cultured for between 0 and 2 h prior to analysis. Table 6 records the surface phenotypes of released cells at different stages in their progress through mitosis, examples of which are shown in Fig. 4A–H. There appears to be little evidence of restoration of a restricted polarity during exit from mitosis, full restoration occurring after mitosis is completed. Thus, cells released from nocodazole arrest resemble cells exiting naturally from mitosis (Table 1).

(F) *Heterogeneity within the extensive microvilli of blastomeres arrested in nocodazole*

During mitotic arrest, microvilli and ConA-binding sites extend over most or all of the surface of the blastomere, yet after division a tight polar organization is restored to the 2/16 progeny. We examined whether the more extensive microvillous area of the surface present during arrest might differ in its nature or organization from the tight polar area present during interphase. Cytochalasin D (CCD) destabilizes microfilaments and affects dynamic, noncapped filaments more rapidly than capped stable filaments. We have shown previously that the tight polar microvilli, characteristic of the interphase cell, contain stable microfilaments (Handyside, 1980; Pratt *et al.* 1982; Johnson & Maro, 1984; Fleming *et al.* 1986). We therefore took blastomeres that had been arrested for 6 or 10 h in nocodazole, and placed them in nocodazole plus CCD for 15–180 min before fixing them for fluorescence or SEM analysis. The cells were scored for the nature and extent of their surface microvillous organization, and the results are summarized in Table 7, examples being shown in Figs 5, 6.

The outcome of this treatment depends upon the prior duration of exposure to nocodazole. Thus, in cells arrested for 6 h, it seems that exposure to CCD results in the regression of microvilli over most of the surface. Within the first 30 min of exposure to CCD, these regressing microvilli retracted to short stubby protrusions (Fig. 5G,H; 6A,B) and beyond an hour's exposure to CCD they were lost completely. Their regression revealed in most cells a discrete and limited area of polar microvilli (Fig. 5I,J; 6E,F) that resembled that present on premitotic polar 8-cell blastomeres. With further exposure to CCD, this polar area of microvilli became less well organized, in some cells assuming a ring-like structure as if those microvilli in the centre of the pole had regressed, leaving only a boundary ring of microvilli (Fig. 5O,P;

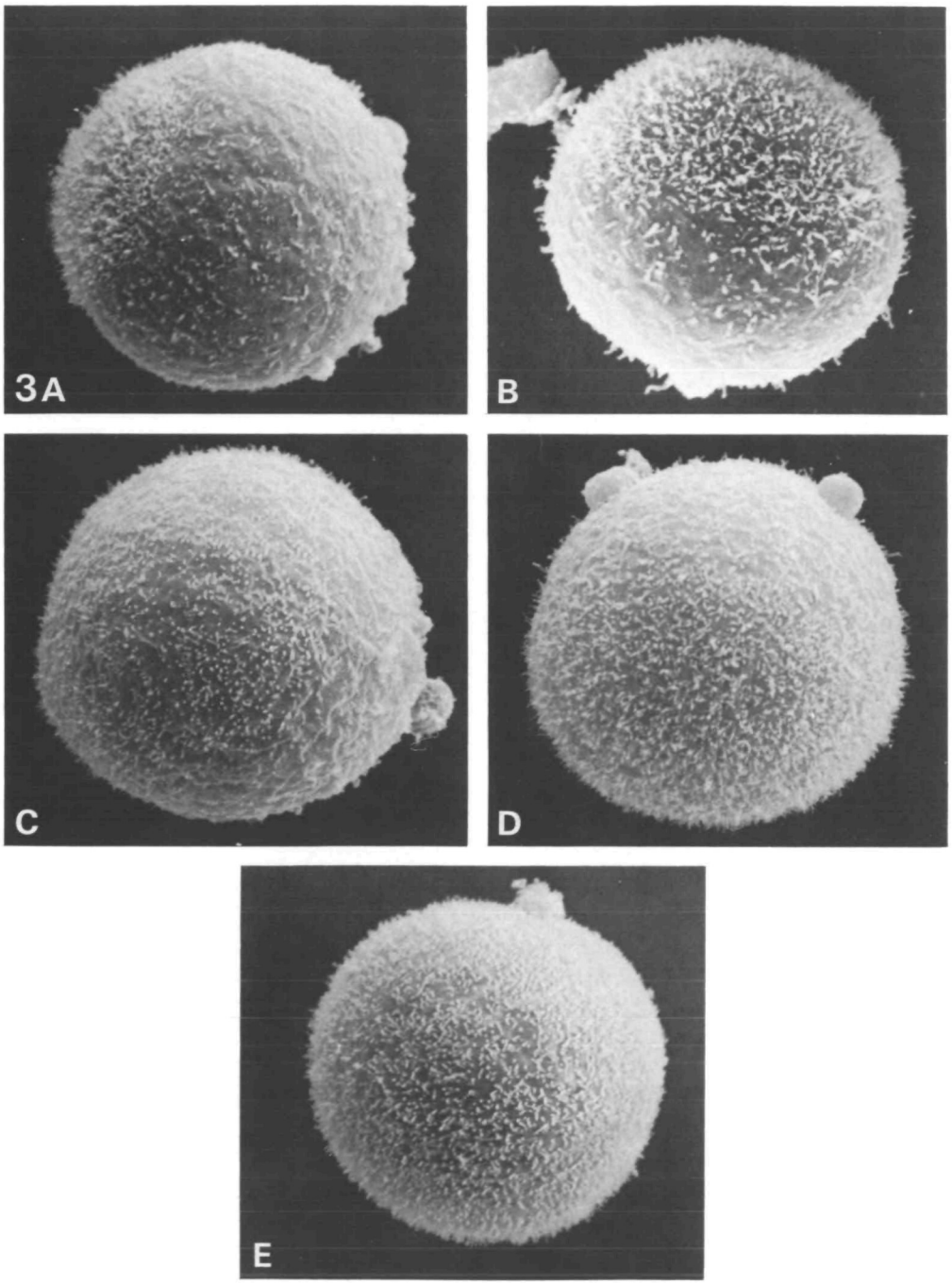


Fig. 3. Late 8-cell blastomeres treated with nocodazole as they entered mitosis and arrested for several hours before fixation and analysis on the SEM. (A) Tight polar distribution of long polar microvilli. (B) Microvilli long and organized in a pole occupying 50 % of cell surface. (C) Shorter microvilli covering 75 % of cell surface. (D) Microvilli over most of surface but graded in distribution being denser at one end than the other. (E) Uniform distribution of microvilli. ×2300.

6H), and in other cells leaving polar clumps of microvilli (Fig. 5K–N; 6G,I). Examination on the transmission electron microscope of cells treated with neither drug, with nocodazole for 6 h, or with nocodazole followed by CCD revealed microvillous distributions consistent with those seen on the scanning electron microscope, and confirmed that the microvilli in all cases had microfilamentous cores (data not shown).

In cells arrested in mitosis for 10 h before addition of CCD, the restoration of a tight polar organization over a limited area of the cell surface was observed less frequently. More cells tended to lose microvilli over the whole surface (Fig. 6J) although a graded loss was observed in many cells as though one end was losing them in advance of the other.

In order to demonstrate that these results were not some artefact of exposing any mitotic cell to the

Table 3. *Surface microvillous patterns assessed electron microscopically in 8-cell blastomeres arrested in mitosis for up to 10 h*

Duration of exposure to nocodazole	No. of cells analysed	Proportion of cell surface covered in microvilli (% of cells)				
		quarter or less	quarter to half	half to three quarters	graded*	homogeneous
Late 1/8 (controls)	66	38	41	6	6	9
0 h	23	0	65	26	0	9
2 h	23	4	44	52	0	0
4 h	34	0	24	50	15	11
6 h	64	7	17	23	35	18
8 h	48	0	17	27	31	25
10 h	39	0	3	31	43	23

* Gradation of microvilli across blastomere surface, being dense at one end and reducing to zero at the opposite end – see Fig. 3D.

Table 4. *Phenotypes of 2/16 pairs of blastomeres 2 h after release of parental cells from exposure to nocodazole for up to 10 h*

Time in drug prior to release	No. of cells that failed to divide	No. of cells that divided	Percentage of divided cells with phenotype that is		
			polar/nonpolar	polar/polar†	nonpolar/nonpolar
0 h	0	56	68	32	0
1 h	1	43	60	40	0
2 h	0	32	65	35	0
3 h	0	45	50	49	1
4 h	23*	50	59	41	0
6 h	1	29	59	34	7
8 h	2	55	22	67	11
10 h	8	48	33	29	38

* Most of these nondividing cells came from one experiment.
† Poles were contiguous across the midbody.

Table 5. *Patterns of behaviour of 1/8 blastomeres and their derivatives at various times after exit from mitosis*

Time in nocodazole	No. dividing on release	Period of culture (h)	No. surviving in culture	Percentage of surviving cell clusters that were				
				2 cells	3–4 cells	more than 4 cells	enveloping group	cavitating
0 h	51	10	49	97	3	0	77	0
6 h	54	10	52	94	6	0	42	0
10 h	24	10	23	77	23	0	57	13
0 h	42	24	39	3	90	3	90	100
6 h	24	24	22	18	77	5	68	67
10 h	28	24	24	64	36	0	50	50

combined drugs, we undertook a comparable set of experiments on arrested 4-cell mitotic blastomeres. The results in Table 8 reveal that the polarity effects described above are characteristic of the 8-cell stage and are not seen at the earlier 4-cell stage. In a few arrested 4-cells, a limited area of microvillous regression was observed, usually amounting to less than 25 % of the surface and frequently being adjacent to the residual midbody. It is possible, but not established, that such areas represented the limited areas

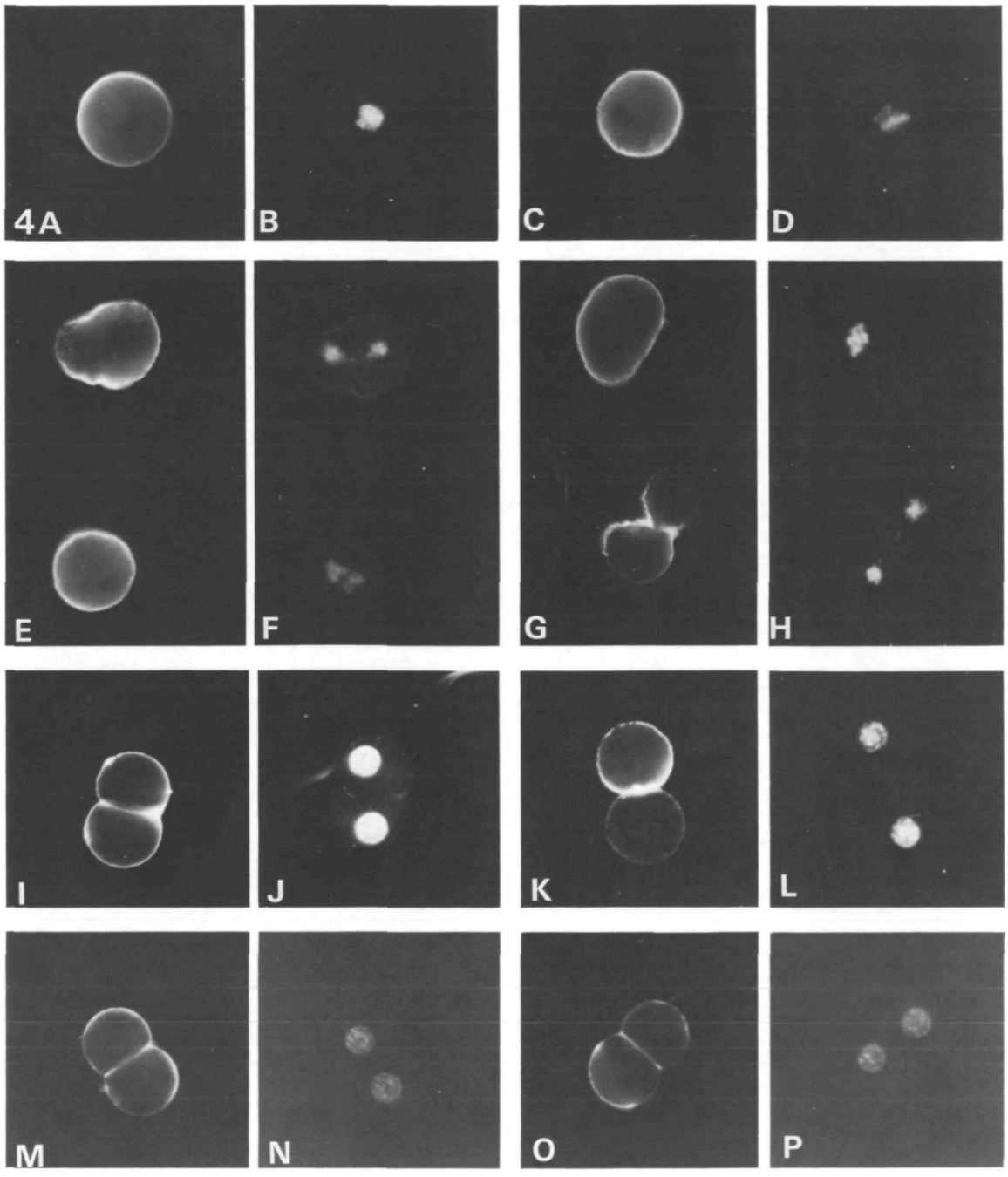


Fig. 4. Arrested mitotic 8-cell blastomeres at various times after the removal of nocodazole (expressed in minutes) and stained with ConA (panels A,C,E,G,I,K,M,O) and Hoechst 33258 (panels B,D,F,H,J,L,N,P). (A,B) 0 min, metaphase, graded distribution of ConA binding. (C,D) 30 min, very early anaphase, nonpolar. (E,F) 30 min, anaphase, nonpolar. (G,H) 60 min, lower cell telophase and 50 % pole across developing midbody, upper cell metaphase and nonpolar. (I,J) 120 min, postmitotic, both cells nonpolar (the parent cell had been exposed to nocodazole for 10 h prior to release). (K,L) 60 min, one cell stained more brightly than the other, bright staining occupying 50–75 % of total surface area. (M,N) 120 min, postmitotic, polar:polar pair. (O,P) 120 min, postmitotic polar:nonpolar couplet. ×32.

Table 6. *Proportion of cell surface covered by microvilli/ConA-binding sites in 8-cell blastomeres arrested in mitosis for 6 h by nocodazole and then released and analysed after between 15 min and 2 h*

Mitotic phase of analysed cell	No. of cells analysed	Proportion of cell surface covered with microvilli (% of cells)		
		less than half	half or more	homogeneous
ConA binding				
Metaphase	52	4	53	43
Anaphase/telophase	15	1	58	41
SEM				
Undivided	85	34*	58	8
Dividing	14	14	79	7
Postdivision				
ConA binding	93	62	32	6
SEM	26	54	23	23

* This figure will include any mis-scored interphase cells and thus may be artefactually high.

Table 7. *The effect of addition of cytochalasin D (CCD) on the surface phenotype of mitotic cells exposed to nocodazole for 6 or 10 h*

Time in nocodazole	Time in nocodazole + CCD	Scoring technique	No. of cells	Area of pole (%)		
				nonpolar	more than half	half or less (% with ring/clumped pole)
0 h (control interphase 1/8 cells)	0 h	ConA	59	10	9	81 (0)
	0 h	SEM	66	9	12	79 (0)
6 h	0 h	ConA	39	75	18	7 (0)
	0·25–0·5 h	ConA	30	10	13	67 (13)
	1 h	ConA	28	14	11	75 (33)
	2–3 h	ConA	5	20	0	80 (100)
6 h	0 h	SEM	64	18	58	24 (0)
	0·25–0·5 h	SEM	51	6	6	88 (11)
	1 h	SEM	21	14	0	86 (44)
	2–3 h	SEM	16	14	0	75 (33)
10 h	0 h	ConA	20	65	30	5 (0)
	0·5 h	ConA	15	33	53	13 (50)
	1 h	ConA	17	41	41	18 (33)
10 h	0 h	SEM	39	23	74	3 (0)
	0·5 h	SEM	13	46	0	54 (14)
	1 h	SEM	12	25	9	66 (13)

Table 8. *The phenotype of 4-cell blastomeres exposed to various conditions as a control for 8-cell blastomeres and examined on the scanning electron microscope*

Incubation condition	No. of cells	% of cells in which microvilli on the cell surface occupied		
		half or less	more than half*	nonpolar
Newly isolated interphase 4-cells	15	0	13	87
Interphase 4-cells cultured for 4–5 h after isolation	57	2	5	93
4-cells scored as lacking nuclear membrane e.g. mitotic	43	0	4	96
Mitotic 4-cells after 6 h in NOC	81	3	15	82
Mitotic 4-cells after 6 h in NOC and 0·5–1 h in CCD + NOC	116	6	25	69
Mitotic 4-cells after 6 h in NOC, release for 2 h during which they divided to 2/16 pairs	47	4	5	91

* In all cases, cells in this column had a small area devoid of microvilli amounting to no more than 25 % of the cell surface. This area was often adjacent to the remains of the midbody, and we suspect that it may represent a region that had been involved in intercellular contact.

of cell contact between blastomeres present at the 4-cell stage, at which microvillous loss has been observed. However, they clearly differed from the extensive areas of microvillous loss observed in equivalent 8-cell blastomeres.

Discussion

We have shown previously that when a polarized 8-cell blastomere divides, elements of its surface polarity persist throughout division (Johnson & Ziomek, 1981). In this paper, we have analysed a much larger number of 8-cell blastomeres undergoing mitosis. By use of double-staining procedures for chromosomes and surface microvilli, we have shown that the tight polar organization of the cell surface evident in the late 8-cell blastomere can persist into early metaphase, but that thereafter a greater, but variable, proportion of the cell surface becomes occupied by microvilli (Table 1). Only when mitosis is completed do microvilli again become restricted to a

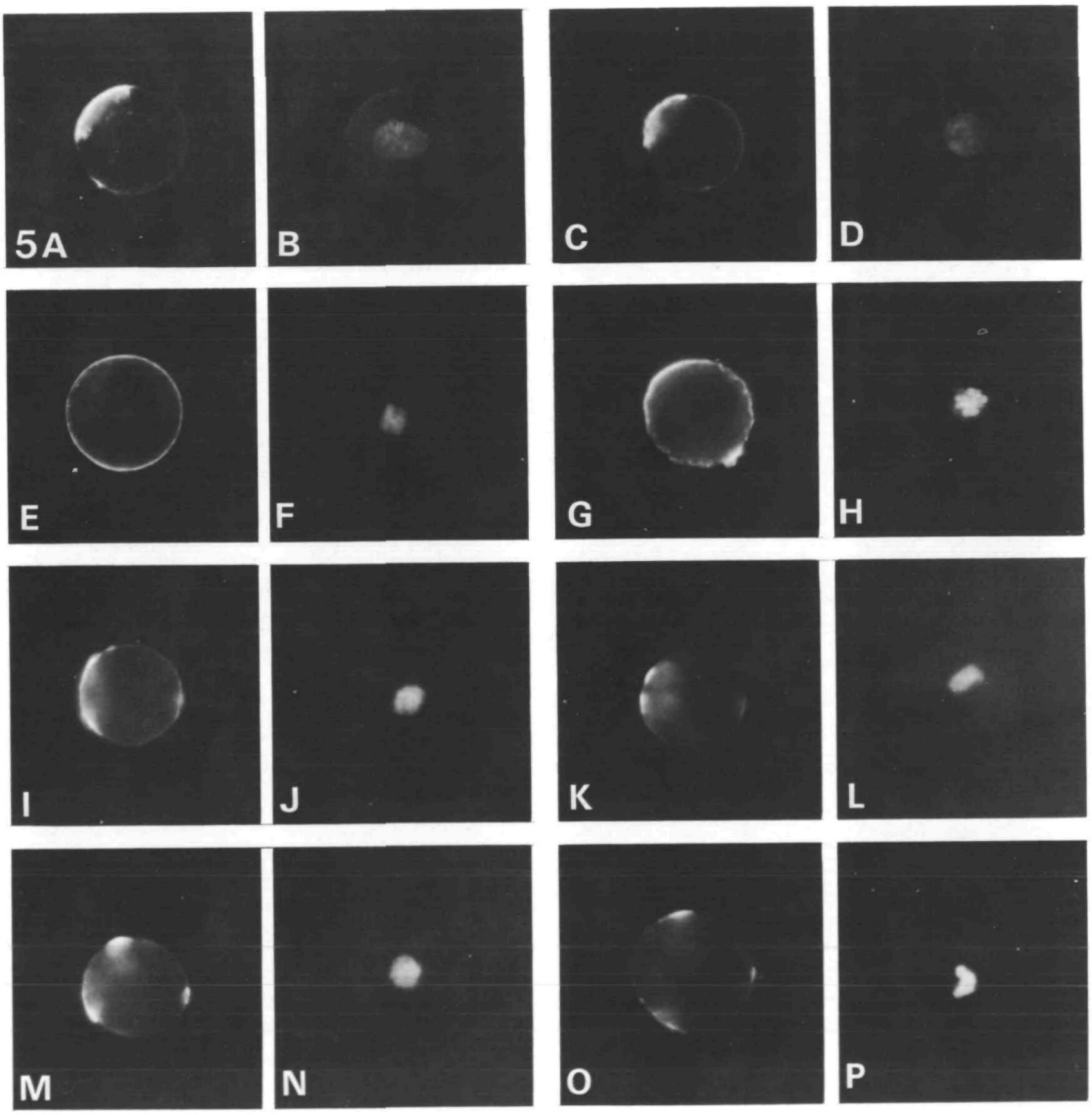
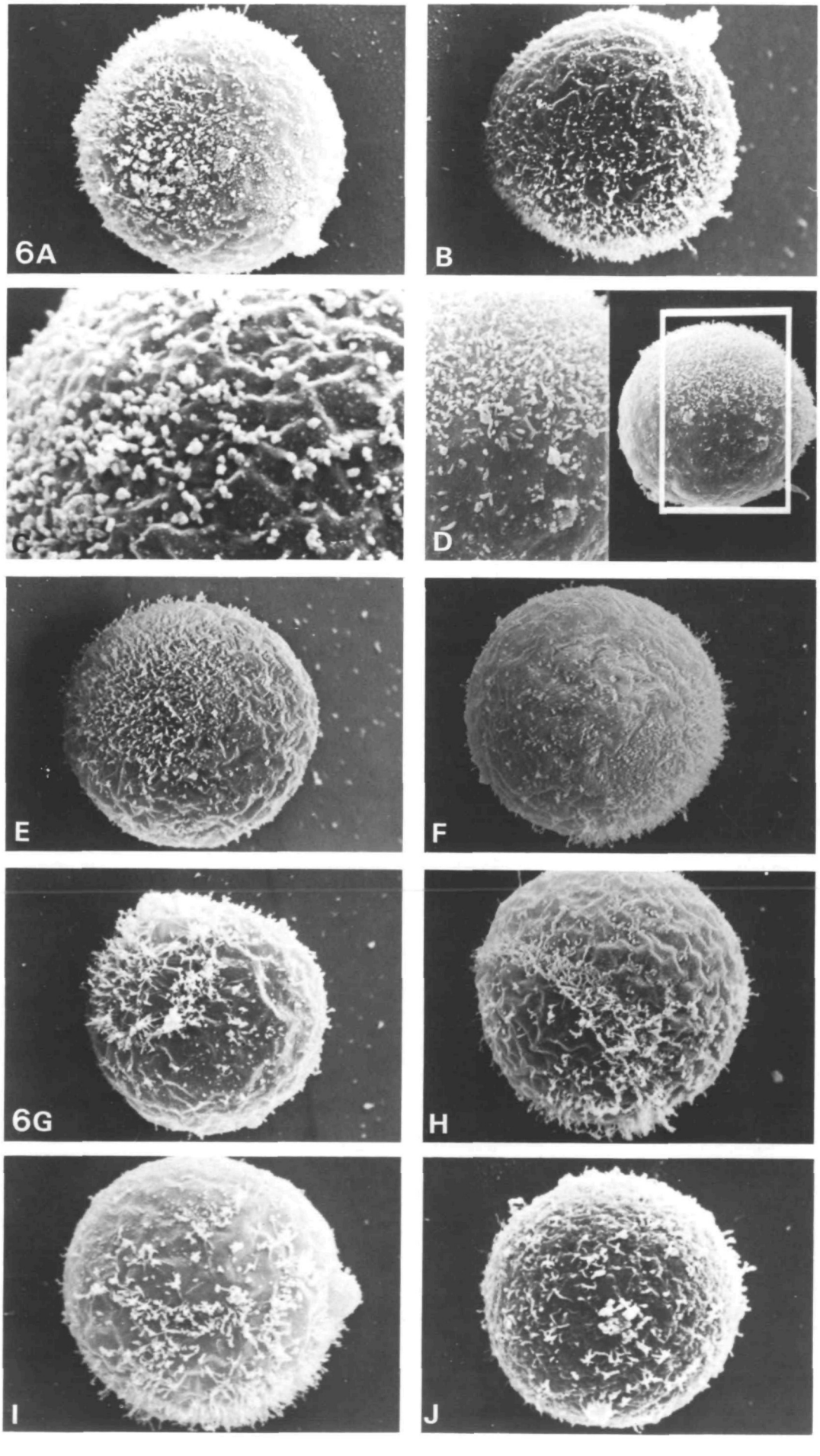


Fig. 5. Mitotically arrested 8-cell blastomeres exposed to nocodazole for 6 h and nocodazole + CCD for 15–120 min, before staining with ConA (panels A,C,E,G,I,K,M,O) and Hoechst 33258 (panels B,D,F,H,J,L,N,P). (A,B) Cell scored erroneously as mitotic and placed in nocodazole for 6 h where it remained in interphase, with its pole occupying less than 25 % of cell surface. (C,D) Similar cell exposed to both nocodazole and CCD after 6 h in nocodazole, similar pole. (E,F) Mitotic cell exposed to nocodazole for 6 h, metaphase and apolar. (G,H) Similar metaphase-arrested cell to that in E,F but exposed to CCD as well for a further 30 min; note the regressing level of stain over most of the surface revealing a polar ConA-binding region. (I,J) Similar cell in which clearly defined pole is evident. (K–N) Similar cells in which some heterogeneity or patchiness is evident within the polar area. (O,P) Similar cell in which the staining in the central region of the pole is reduced, giving rise to a ring structure. ×32.


6A
B
C
D
E
F
6G
H
I
J

tight polar domain. However, although microvilli occupy proportionately more of the dividing cell's surface, the dividing cell nonetheless has a clear axis of polarity throughout the whole process of mitosis. The retention of such an axis in the cell is clearly a critical requirement for the generation of cell diversity by unequal cell division.

There seem to be two interpretations of these events occurring at division. The appearance of microvilli over more of the cell's surface could represent a running down of polarity. If this were the case, then whether or not cell diversity was generated would appear to depend on a race between the process of run down and the time to complete mitosis. Alternatively, the overt surface pole of microvilli, which so clearly signals the cell's axis of polarity in late interphase of the 8-cell stage, may be simply one manifestation of an underlying polar axis. The changing pattern of microvillous distribution might then be explained if the transition from interphase to the M-phase state resulted in an uncoupling of the underlying covert polarity from its surface manifestation. After reentry into interphase, the association would be reestablished, and a tight polar organization of microvilli reimposed. We have undertaken experiments that attempt to distinguish between these alternatives by prolonging mitosis sufficiently to allow a process of run down to proceed to completion. Since we find here that nonarrested mitoses last between 45 and 90 min, in agreement with a previous report (Lehtonen, 1980), prolongation of mitosis for up to 10 h exceeds tenfold the period of the natural transition.

The passage of mammalian mitotic cells from metaphase to anaphase requires the presence of a spindle. If the spindle is destroyed by use of nocodazole, cells will nonetheless enter mitotic prophase but then will arrest at the metaphase–anaphase boundary. It has been shown previously that interphase polar 8-cell blastomeres exposed to nocodazole tend to have larger poles than control cells but nevertheless retain their polarity of surface microvilli for as long as they remain in interphase (Ducibella, 1982; Maro & Pickering, 1984; Johnson & Maro, 1985). We have shown here that nocodazole did not prevent or reverse the process by which microvilli occupy more of the cell surface during M-phase. Indeed, in the cells arrested in mitosis, this process became more marked so that most of the surface of the cell became microvillous, and in many cells no residual heterogeneity of microvillous presence was evident. The longer the duration of the arrest, the more extensive the distribution of the microvilli became, the precise extent depending upon the resolution of the scoring procedure used, scanning electron microscopy resolving heterogeneities better than fluorescence microscopy (Tables 2, 3). Careful examination of the increasing numbers of microvilli after various periods of exposure to nocodazole suggested that they grew *de novo*, initially adjacent to the polar microvilli and later towards the opposite end of the cell. We cannot exclude the possibility that some expansion of the area occupied by the interphase polar microvilli also occurred, perhaps achieved by a slight shortening of apical polar microvilli leading to microvillous membrane becoming intermicrovillous and so expanding the surface area occupied by the apical pole as a proportion of the total area of the blastomere. Such a mechanism might explain all or most of the limited polar expansion observed during most nonarrested mitoses, but cannot explain the more extensive changes observed during nocodazole arrest. It seems reasonable to conclude that the M-phase state is associated with a change in the cytoplasmic milieu that favours the generation of microvilli over the entire surface of the cell, thereby apparently rendering its surface increasingly nonpolar. Such a consequence of entry into the M-phase state is not surprising, since M-phase is known to be associated with uncoupling of gap junctional communication between blastomeres (Goodall & Maro, 1986) and loss of effective intercellular adhesion (Lehtonen, 1980; Goodall & Maro, 1986). In addition, entry into mitosis results in the complete and rapid loss of cytoplasmic and cytoskeletal polarity (Johnson & Maro, 1984, 1985; Maro *et al.* 1985; Fleming & Pickering, 1985; Houliston *et al.* 1987). The principal difference between the microvillous and cytoplasmic changes observed on entry into M-phase is the relative speed with which the loss of polarity of the latter occurs, being completed during prophase.

When nocodazole was removed from arrested blastomeres, most cells resumed progress through mitosis which they completed over the next 45–60 min. As in control nonarrested cells exiting from mitosis, this resumption of mitosis was not

Fig. 6. Mitotically arrested blastomeres exposed to nocodazole for 6 h and then to combined nocodazole + CCD for up to 2 h, before analysis by scanning electron microscopy. (A,B,C) Within the first 15 to 30 min microvilli regress over much of the surface, leaving long microvilli restricted to a polar area. This CCD-induced regression is similar to that observed in control interphase polarizing 8-cell blastomeres as shown in (D). (E–F) Within 30–60 min the regression is completed, leaving a clear pole of microvilli. (G–I) With further incubation, the polar region of microvilli begins to become disorganized, forming ring poles. (J) An example of a cell exposed to CCD after 10 h in nocodazole and in which no evidence of residual polarity is detected. (A,B,E–J) ×2300; (C) ×6000; (D) ×1500 – inset ×3000.

accompanied by any obvious immediate restriction of microvilli to a more limited area of the cell's surface (Table 6). However, after the completion of mitosis and the generation of two cells at cytokinesis, restriction of microvilli to a sharply defined pole did occur in most cases, and this polar region, like that in controls, was either restricted to one of the two 16-cell blastomeres or was divided to give two polar cells in which the poles on each were contiguous across the residuum of the cleavage furrow (Table 4). Thus, despite the run down of surface polarity in arrested cells, most of them nonetheless behaved like control cells on release. However, it is clear that those cells arrested for the longest periods in nocodazole behaved, as a population, less like controls than did those arrested for periods of up to 6h. First, on release after more than 6h exposure to nocodazole, fewer pairs were generated in which one cell was polar and the other nonpolar (Table 4), with correspondingly more polar:polar pairs (the poles being contiguous across the cleavage furrow remnant) and nonpolar:nonpolar pairs (in which both cells were microvillous over the whole surface). Second, the proportion of released couplets that went on to divide and form miniblastocysts with enclosed 'inner cell mass' cells was reduced (Table 5). There are two types of explanation for this result. The result could simply represent nonspecific deleterious consequences for some cells of prolonged culture in nocodazole. Alternatively, it could indicate that prolonged arrest leads to the loss of a polar axis. We cannot distinguish unequivocally between these alternatives on present evidence, but this question is addressed again below. However, the important observation to emerge from these experiments is that most blastomeres arrested for up to 6h, and some arrested for longer periods, went on to behave like nonarrested controls on release to interphase, and did so despite the major redistribution of microvilli over all or most of their surfaces that had occurred during the period of arrest. Thus, cells that had by all overt criteria lost their polarity had maintained an underlying memory of their polar axis. This observation leads to two conclusions. First, it implies the existence of an underlying polarity. Second, whatever the nature of this underlying polarity might be, it must have a half life that is well in excess of the time to traverse mitosis.

The existence of an underlying or covert polar axis that organizes the visible surface and cytoplasmic manifestations of polarity evident by the late 8-cell stage has been postulated previously to explain the result of a quite different type of experimental approach. A stable axis of polarity develops for the first time during the 8-cell stage. The earliest signs of polarization are observed in the cytoskeletal reorganization and in the redistribution of cytoplasmic organelles that accompanies it. Polarization of the surface was detectable later (Johnson & Maro, 1984; Maro *et al.* 1985; Fleming & Pickering, 1985; Houliston *et al.* 1987). However, it has proved possible to devise conditions that block all evidence of polarization within the cytoskeleton and cytoplasmic contents, but that permit development of surface polarity nonetheless. Moreover, if the conditions inhibitory for cytoskeletal and cytoplasmic polarizations are removed before, during or after the appearance of polarity within the surface, cytoplasmic polarity develops from a cytocortical focus (Johnson & Maro, 1984, 1985; Fleming *et al.* 1986). These observations suggested that early in the 8-cell stage changes of organization in, or closely associated with, the surface membrane were occurring that gave to the cytocortex a polar organization. This cytocortical polarity then served as the basis for the relatively rapid polar reorganization of the cytoskeleton and the slower reorganization of the surface microvilli to a tight polar array. It is possible that the cytocortical pole proposed to explain the pattern of polarity development also serves as the memory of the polar axis that persists during cleavage.

What might be the nature of this cytocortical polar memory? A fundamental requirement of such a memory is that it can function to organize both the cytoskeleton within the cell and the microvilli on its surface. It is thus likely that the pole will be a region of the cytocortex that is involved in stabilizing microfilaments and microtubules. We already know that microtubules preferentially regrow in the region of the polar cortex (Houliston *et al.* 1987) and that microfilaments in this area are preferentially accumulated and are relatively resistant to the action of CCD (Johnson & Maro, 1984; Fleming *et al.* 1986). We took advantage of this knowledge in experiments described here to investigate whether the postulated cytocortical memory of polarity might be revealed by a heterogeneity of stability within the microvilli on the surface of mitotically arrested cells. It was found that most microvilli on arrested cells are sensitive to the action of CCD and regress, leaving a tight polar cluster of microvilli corresponding in size and organization to that characterizing the late 8-cell interphase blastomere. The regressing microvilli resembled those observed during the development of polarity *de novo* in the interphase 8-cell blastomeres (Johnson & Maro, 1985; compare Fig. 6D with Fig. 6A–C), and it is reasonable to suspect that the CCD-sensitive microvilli might correspond to those generated recently during the period of arrest. More prolonged exposure to CCD induced loss of microvilli from the centre of the polar cluster, a phenomenon also described as

characteristic of late interphase poles under certain conditions (Johnson & Maro, 1985; Nuccitelli & Wiley, 1985). The revelation of such a cortical heterogeneity in arrested mitotic cells by the use of CCD was evident in fewer blastomeres arrested for 10 h than in those arrested for 6 h, which could be taken as support for the view, albeit not decisive support, that by this time the cortical memory was becoming erased (see above for discussion of this point).

We therefore wish to propose a model based on these and previous observations and developed from that outlined in Johnson & Maro (1986). During the early 8-cell stage blastomeres gain the capacity to respond to contact between blastomeres mediated at least in part by the calcium-dependent cell–cell adhesion molecule, uvomorulin. Their response is a change in cytocortical organization, within and spreading immediately beyond the confines of the contact point, that favours depolymerization of cytoskeletal elements. This results in local loss of the cytoskeleton and associated organelles within the cell and, less rapidly, in a loss of the overlying microvilli on the cell's surface. Depolymerization of cytoskeletal elements in the regions adjacent to the contact points might also, by elevating monomer concentration, drive the polar zone towards polymerization and ultimately to the accumulation there of further nucleating and stabilizing activities. By such a mechanism, each cell is driven from a nonpolar to a polar state in which cytocortical stabilizing activity becomes restricted to a polar zone most remote from the point(s) of intercellular contact. At entry into M-phase, the internal milieu of the cell changes in such a way that does not affect (at least for several hours) the polar cytocortical zone of stabilizing/nucleating activity, but which does permit growth of microvillous microfilaments elsewhere over the cell surface, as well as the extensive reorganization of the microtubules and other organelles observed during mitosis. On reversion to interphase conditions, the cytocortical pole reassumes its dominant role in the organization of the cell's contents and surface. The notion that a defined cytocortical domain can impose overall organization on a cell is supported by results from other developmental (*Fucus*: Brawley & Robinson, 1985; *Xenopus*: Gerhart *et al.* 1984; *Chaetopterus*: Jeffrey, 1985) and quasidevelopmental (MDCK cells: Nelson & Veshnock, 1986, 1987; Salas *et al.* 1986; Vega-Salas *et al.* 1987) systems. We are now exploring the molecular make-up of the cytocortex before and after polarization and in the polar cytocortical and the basolateral zones of polarized cells, to determine what the nature of the polar cytocortical organizing activity might be.

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