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Distribution of antibody- and lectin-binding sites on dissociated blastomeres from mouse morulae: evidence for polarization at compaction

By ALAN H. HANDYSIDE[1]

From the Department of Anatomy, University of Cambridge

SUMMARY

The distribution of binding sites for rabbit anti-species antiserum, Concanavalin A (Con A) and peanut agglutinin (PNA) on dissociated blastomeres from 2- to 16-cell mouse embryos has been investigated using direct and indirect fluorescence techniques. With each ligand, paraformaldehyde-fixed blastomeres from 2- to 8-cell precompact embryos were uniformly surface labelled; the majority (77%) of late compact 8-cell blastomeres showed quantitative polarization of surface labelling; and 16-cell blastomeres were either polarized (53·3%) or uniformly surface labelled. Binding of fluorescein-conjugated PNA increased at the 16-cell stage. Labelling patterns on unfixed blastomeres were similar to those on fixed blastomeres except that surface label was patched and became internalized, most rapidly from the less heavily labelled areas of 8- and 16-cell blastomeres.

Quantitative polarization of binding sites at postcompaction stages was detected after (i) fixation, (ii) pretreatment and labelling in the presence of azide, cytochalasin D and/or colcemid, or (iii) labelling with monovalent Fab_1 antibody fragments. It is probably due, therefore, to the presence of microvilli at the heavily labelled pole, which increase surface area and are known to become localized to the outer surface of the compact morula (Ducibella, Ukena, Karnovsky & Anderson, 1977).

The possibility that the cleavage of polarized blastomeres into dissimilar daughter blastomeres could provide a mechanism for the spatial differentiation of the inner cell mass and trophectoderm of the blastocyst is briefly discussed.

INTRODUCTION

During early mouse development, the ovum undergoes a series of cleavage divisions until at the 8-cell stage the previously spherical blastomeres appear to maximize contact with each other and form a compact aggregate or morula (Lewis & Wright, 1935). As the next cleavage division proceeds, a small number of 16-cell stage blastomeres become completely surrounded by a monolayer of the other blastomeres (Barlow, Owen & Graham, 1972). Since rearrangement of blastomeres at this stage is minimal (Garner & McLaren, 1974), these enclosed 'inner' blastomeres and the surrounding 'outer' blastomeres are

[1] *Author's address:* Department of Anatomy, University of Cambridge, Downing Street, Cambridge, CB2 3DY, U.K.

considered to be the precursors of the inner cell mass (ICM) and outer epithelial trophectoderm layer respectively, the tissues which occupy equivalent relative positions at the blastocyst stage. Evidence consistent with this conclusion comes from an analysis both of the developmental fates of inner and outer cells (Hillman, Sherman & Graham, 1972) and of the molecular differentiation of separated inner and outer populations of blastomeres at the late morula and blastocyst stages (Van Blerkom, Barton & Johnson, 1976; Handyside & Johnson, 1978; Johnson, 1979*a*, *b*).

In association with the process of compaction at the 8-cell stage, ultrastructural observations have indicated that blastomeres become polarized along a radial axis: (i) microvilli are localized mainly to the outer surface of the morula, and (ii) microtubules and mitochondria are localized beneath apposed areas of adjacent lateral membrane (Ducibella *et al.* 1977). The possibility that the molecular composition of the membrane may also become polarized at this or later stages, as occurs in other transporting epithelia (Fujita *et al.* 1972; Isselbacher, 1975), has not as yet been extensively studied using specific antisera or lectins. However, binding of both a rabbit anti-ectoplacental cone antiserum (Searle & Jenkinson, 1978) and of ferritin-labelled Con A (Konwinski, Vorbrodt, Solter & Koprowski, 1977) was greater on the outer compared with the inner membrane areas of blastomeres in fixed, sectioned morulae. Other studies have been restricted to the accessible outer surface of intact morulae only, and several antigens have been described that either first appear or show maximal expression at this stage (Wiley, 1974; Wiley & Calarco, 1975; Artz *et al.* 1973; Konwinski *et al.* 1977; Willison & Stern, 1978; Solter & Knowles, 1978). This suggests that changes in the glycoprotein or glycolipid composition of the outer areas of membrane may be related to the early differentiation of the trophectoderm (see Jenkinson & Billington, 1977).

In the present report, the distribution of antibody- and lectin-binding sites has been examined on dissociated blastomeres from 2- to 8-cell precompact embryos and compact 8- to 16-cell morulae to study the effect of compaction on the distribution and redistribution of a broad spectrum of membrane components. The binding of a multispecific rabbit anti-mouse species antiserum (Gardner & Johnson, 1975; Handyside, 1978), and two lectins, Concanavalin A (high affinity for mannose and glucose residues) and Peanut Agglutinin (high affinity for galactose residues) has been investigated by direct and indirect fluorescence microscopy. Redistribution of membrane components induced by the binding of these multivalent ligands (Taylor, Duffus, Raff & de Petris, 1972; de Petris & Raff, 1973) has been assessed at each stage, by comparing unfixed blastomeres with others: (i) fixed before labelling, (ii) labelled in the presence of cytoskeletal or metabolic inhibitors, or, (iii) labelled with monovalent Fab_1 antibody fragments.

MATERIALS AND METHODS

(1) *Supply of Embryos*

Embryos were obtained from CFLP (Anglia Labs. Limited/Hacking and Churchill Limited) female mice after superovulation (Handyside, 1978) or mated naturally with CFLP, Balb/c or F1(A2G × C57B1) males. Natural matings were used predominantly for several reasons: (i) only small numbers of embryos were required and could be handled for disaggregation, (ii) the incidence of partially lysed embryos was considerably lower (defective embryos were always discarded), and (iii) synchronous ovulation was not necessary since embryos at slightly different stages could be accurately compared by reference to their cell number. Mating was detected on Day $\frac{1}{2}$ by the presence of a copulation plug. Two- to 4-cell embryos were flushed from the oviducts in the late afternoon or evening of Day $1\frac{1}{2}$; 8-cell precompact, 8-cell compact and mixtures of compact 8- and 16-cell morulae were flushed early morning, midday and late afternoon of Day $2\frac{1}{2}$, respectively.

Phosphate-buffered medium 1 supplemented with 10 % foetal calf serum (heat inactivated; Gibco Limited) (PB1 + 10 % FCS) was used for flushing and storage of embryos between manipulations (Whittingham & Wales, 1969). Medium 16 (Whittingham, 1971) supplemented with 0·4 % bovine serum albumin (Sigma Limited) (M16 + 0·4 % BSA) in the presence of 5 % CO_2 in air, was used for more prolonged culture at 37 °C.

Zonae were removed by a 15–30 sec incubation in acid Tyrodes solution at 37 °C, followed by extensive washing in PB1 + 10 % FCS. Comparison with cells obtained after mechanical removal of the zona indicated that binding of antisera and lectins was not affected by this treatment.

(2) *Disaggregation to single cells or small clusters of cells*

(i) *Calcium-free medium.* Embryos were transferred, with a minimal volume of normal medium, to 2–3 ml of calcium-free M16 + 0·4 % BSA in small Falcon petri dishes coated with 2 % agarose (Indubiose, L'Industrie Biologique Français) (dissolved in calcium-free M16) to prevent adhesion, and cultured for 20–30 min, at 37 °C in the presence of 5 % CO_2 in air. Disaggregation was accomplished by pipetting with a flame-polished micropipette and the blastomeres transferred to normal medium.

(ii) *Cytochalasin D (CCD).* Embryos were incubated in 1 μg/ml CCD (0·1 % dilution of 1 mg/ml stock solution in DMSO; Sigma Limited) in M16 + 0·4 % BSA and disaggregated as in (i).

(iii) *Trypsin/EDTA/calcium-free medium.* Embryos were washed free of serum in PB1 + 0·4 % BSA, and transferred to 200 μl drops of 5 % trypsin and 2 % EDTA in saline (Gibco Limited) diluted 1 to 10 or 1 in 50 with calcium-free M16 (i.e. 0·5 % trypsin + 0·2 % EDTA or 0·1 % trypsin + 0·04 % EDTA) on non-coated Falcon petri dishes under oil, pre-equilibrated for 30 min at 37 °C,

in the presence of 5 % CO_2 in air. They were incubated under the same conditions for 5–10 min. Immediately decompaction was complete as judged by the appearance of distinct cell outlines in the intact morula (see Fig. 3*b*), the trypsin-containing medium was drawn off and replaced by a large volume of PB1 + 10 % FCS.

(3) *Fixation*

Dissociated blastomeres were fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS) either for 15 min at 37 °C, or for 60 min at 4 °C, followed by extensive washing. Unlabelled fixed blastomeres did not autofluoresce.

(4) *Labelling*

(i) *Antisera.* Rabbit anti-mouse species antiserum (Gardner & Johnson, 1975; Handyside, 1978) was heat inactivated at 56 °C for 20 min, and uniformly labelled the outer surfaces of mouse embryos from the unfertilized egg to blastocyst stages as judged by indirect immunofluorescence. The antiserum was used in three forms: (a) whole serum (RAMS) diluted 1 in 10 to 15 in PB1; (*b*) fluorescein-conjugated IgG fraction (FITC-IgG RAMS) diluted 1 in 2 in PB1; and (*c*) fluorescein-conjugated Fab_1 fragments of the IgG fraction (FITC- Fab_1 RAMS) diluted 1 in 2, in PB1 (Johnson & Edidin, 1978).

Direct immunofluorescence. Blastomeres were incubated for 15 min at room temperature (21 °C) in 25 μl drops of FITC-conjugated antisera, washed extensively and mounted in separate wells of a tissue-typing slide (Baird and Tatlock Limited) in drops of medium without phenol red under oil.

Indirect immunofluorescence. Blastomeres were incubated in antisera as for direct fluorescence, washed, transferred to 25 μl drops of fluorescein-conjugated goat anti-rabbit IgG (FITC-GAR IgG; Miles Labs) diluted 1 in 15, in PB1 and incubated for a further 15 min at room temperature. After extensive washing these blastomeres were mounted in the same way. Both fixed and non-fixed blastomeres treated with conjugate alone were unlabelled.

(ii) *Lectins.* Blastomeres were incubated for 30 min at room temperature in: (*a*) fluorescein-conjugated concanavalin A (FITC-Con A; Miles Labs), stock solution 21 mg/ml in PBS, diluted 1 in 30, with PB1; or (*b*) fluorescein-conjugated peanut lectin (FITC-PNA; Sigma Limited) diluted to 1 mg/ml in PBS.

After extensive washing, blastomeres were mounted as for immunofluorescence.

(5) *Labelling of nuclei*

Fixed blastomeres were incubated for 15–30 min at 37 °C in the presence of the DNA-binding-fluorochrome Hoechst 33258 (50 μg/ml in PBS; Hilwig & Gropp, 1972), followed by extensive washing.

Table 1. *Numbers of blastomeres examined at each stage after labelling with various antisera or lectins*

Stage	Method of disaggregation	RAMS/ FITC-GAR IgG (indirect)	Labelled with FITC-RAMS	FITC-Fab RAMS	FITC-Con A	FITC-PNA
2-cell	Calcium-free medium	20 [2]	5	11	11	10
	+Trypsin/EDTA	5	—	—	—	—
	Total	25 [2]	5	11	11	10
4-cell	Calcium-free medium	18 [10]	5	5	15 [1]	10*
	+Trypsin/EDTA	— [37]	—	—	1 —	—
	Cytochalasin D	— [1]	—	—	— —	—
	Total	18 [48]	5	5	16 [1]	10*
8-cell	Calcium-free medium	128 [141]	50	60	30 [82]	11 [83]
	+Trypsin/EDTA	292 [501]	98	76	43 —	— —
	Cytochalasin D	40* [146]	—	—	— —	— —
	Total	450 [788]	148	136	73 [82]	11 [83]
16-cell	Calcium-free medium	305 [3]	50	62	77 [18]	39 [72]
	+Trypsin/EDTA	261 [278]	107	50	183 —	— —
	Cytochalasin D	80* [46]	—	—	— —	— —
	Total	646 [327]	157	112	260 [18]	39 [72]

[ ] Fixed with 4% paraformaldehyde before labelling.
* Number of blastomeres approximate.

(6) *Fluorescence microscopy*

A Zeiss epifluorescence microscope, incident source HBO 200 with excitation filter 427902 and barrier filter system 427903, was used to examine cells for FITC labelling. Double labelled cells were examined using filter set 487709 (plus additional excitation filter LP 425) for FITC and filter set 487705 for Hoechst 33258. Both phase and fluorescence micrographs were photographed on Kodak Tri-X 35 mm film. All fluorescence exposures were for 2 min.

RESULTS

The numbers of blastomeres examined at each stage, with the various antisera or lectins, method of disaggregation and any additional treatments are presented in Tables 1 and 2.

(1) *Rabbit antisera*

Since labelling patterns with RAMS did not vary with the method of disaggregation employed, the observations are presented together.

(i) *Fixed blastomeres.* 2-, 4- and precompact 8-cell blastomeres, fixed immediately after disaggregation and labelled by indirect immunofluorescence

Table 2. *Numbers of blastomeres examined at each stage after pretreatment with various inhibitors and labelling by indirect immunofluorescence**

Stage	Method of disaggregation	Labelled with		
		RAMS (indirect)	FITC-Fab RAMS	FITC-Con A
2-cell	Azide	2	11	—
4-cell	Azide	5	2	—
8-cell	Azide	23	1	14
	Cytochalasin D	94	—	—
	Colcemid	80	—	—
	Cytochalasin D + colcemid	80	—	—
	Total	277	1	14
16-cell	Azide	24	54	27
	Cytochalasin D	56	—	—
	Colcemid	40	—	—
	Cytochalasin D + colcemid	40	—	—
	Total	160	54	27

* Disaggregated in calcium-free medium alone or with added Trypsin/EDTA.

were labelled uniformly over their surfaces (Fig. 1*a*, *b*). Very occasionally in intermediate cleaving embryos, i.e. 3-cell or 5- to 7-cell stages, pairs of recently-divided blastomeres were found to be heavily labelled in the region of the presumed cleavage furrow (Fig. 1*a*, *b* and inset). This appeared, as judged by altering the plane of focus, to be due mainly to labelling by antibody of internal cytoplasmic structures in this region possibly as a result of the increased permeability to antibodies after fixation.

The majority of blastomeres disaggregated from early 8-cell morulae, classified macroscopically as semicompact, were also uniformly surface-labelled (Fig. 1*c*, *d*). However, an increasing proportion of these blastomeres disaggregated at later times, exhibited quantitative polarization of surface labelling (Table 3). One pole, typically occupying less than a complete hemisphere, was still heavily labelled but over the remainder of the surface the intensity was considerably reduced (Fig. 1*e*, *f*). The majority of blastomeres disaggregated from late 8-cell morulae, classified macroscopically as fully compact, were polarized in this way. After partial dissociation, the heavily labelled poles were always directed away from the point of contact between blastomeres (Fig. 1*e*, *f* inset). The detection of polarized labelling was not critically dependent on antibody concentrations.

The labelling patterns observed with blastomeres disaggregated from early compact 16-cell morulae fell into two subpopulations. Over half of the blastomeres at this stage showed polarized surface-labelling characteristics of the late 8-cell pattern, and this was often associated with localized regions of

internal cytoplasmic labelling at the opposite pole (Table 3). Other blastomeres were uniformly surface labelled, but again often had a localized region of cytoplasmic labelling (Fig. 1*g*, *h* and inset).

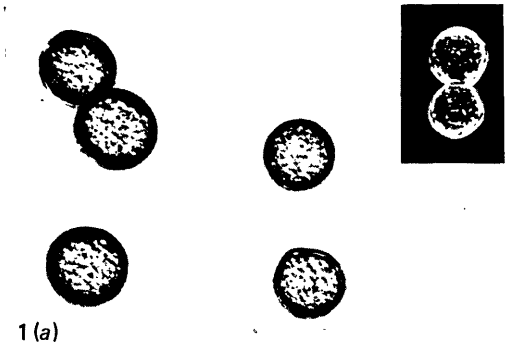
(ii) *Unfixed blastomeres.* At each stage examined, labelling of unfixed blastomeres by indirect immunofluorescence differed from that described above for fixed blastomeres in only two respects. First, the surface labelling was patched rather than continuous (Fig. 2*a*, *b*). And second, there appeared to be little or no cytoplasmic labelling, although it is possible that some of the observed patching could have been due to the early stages of absorptive pinocytosis. Otherwise, polarization of surface labelling at the 8-cell (Fig. 2*c*, *d*) and 16-cell stages (Fig. 2*g*, *h*) was equally evident.

(iii) *Unfixed blastomeres labelled directly with either FITC-IgG RAMS or FITC-Fab*$_1$ *RAMS.* The level of fluorescent labelling obtained with either of these preparations was very low even at high titres. Nevertheless, labelling appeared uniform on blastomeres dissociated from precompaction stages and polarized at compact 8- and 16-cell stages. In contrast to the appearance of unfixed blastomeres labelled by indirect immunofluorescence (Fig. 2*c*, *d*) labelling with both of the directly conjugated antibody preparations was continuous rather than patched (Fig. 2*e*, *f*).

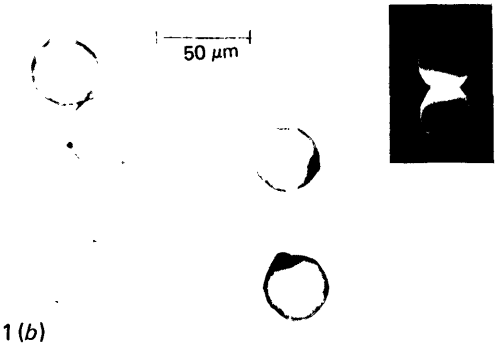
(iv) *Unfixed blastomeres labelled in the presence of inhibitors.* The proportion of polarized 8- and 16-cell blastomeres detected by indirect immunofluorescence was unaffected by the presence, and preincubation for 1 h prior to labelling, of colcemid (10 μg/ml), CCD (2·5 μg/ml), azide ($2{\cdot}5 \times 10^{-2}$ M), or a combination of CCD and colcemid. Both CCD (Fig. 3*a*, *b*) and azide treatment (Fig. 3*c*, *d*), however, appeared to cause an intensification and 'hairiness' of the heavily labelled pole compared to untreated blastomeres, and to reduce the patching in other areas.

(v) *Timing of polarization in relation to mitosis.* Polarized labelling of 8-cell blastomeres was first detected coincident with the first signs of macroscopic compaction at approximately 6–8 h before half of the population of embryos in culture had divided to the 16-cell stage. Thus, polarization would seem to be initiated within the first 4 or so hours after division to the 8-cell stage. Many blastomeres isolated from early compacting 8-cell embryos were only weakly polarized suggesting a progressive change in surface labelling pattern. Only in mixed populations of 8- and 16-cell compact morulae were the majority of 8-cell blastomeres strongly polarized. However, complete polarization was achieved before the blastomeres entered mitosis, as indicated by the demonstration of interphase nuclei using the DNA fluorochrome Hoechst 33258 (Fig. 3*e*, *f*, *g*).

(vi) *Orientation of polarization* in situ. In order to determine the positions of the heavily labelled poles of blastomeres following compaction *in situ*, decompact but undisaggregated late 8- and 16-cell morulae were preincubated in azide (to accentuate the polarized labelling – see iv), fixed and labelled by indirect immunofluorescence. After this treatment it was clear that the heavily



1 (*a*)



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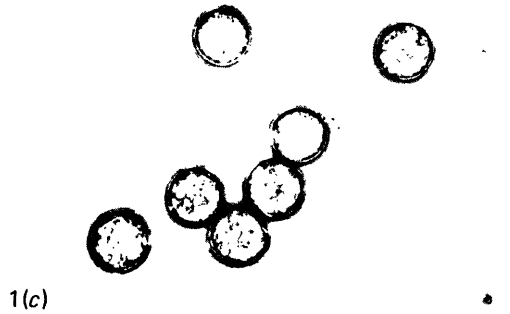
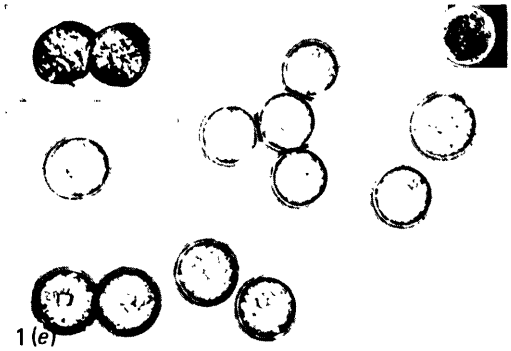


1 (*c*)

1 (*d*)



1 (*e*)

1 (*f*)

1 (*g*)

1 (*h*)

Table 3. *Proportion of polarized blastomeres detected after labelling by indirect immunofluorescence with RAMS (observations with both fixed and unfixed blastomeres included)*

Stage		Polarized (%)	Uniform	Total
8-cell	Precompact	9 (7·9)	105	114
	Semicompact	62 (34·1)	120	182
	Compact	188 (77·0)	56	244
16-cell	Compact	247 (53·8)	212	459

* Failure to detect polarized labelling in all blastomeres dissociated from 8-cell compact morulae is due to heterogeneity amongst blastomeres within an embryo and amongst embryos within a population. Disaggregation of individual late 8-cell embryos reveals all blastomeres to be polarized (unpublished).

labelled poles generally faced outwards and were not situated directly between adjacent blastomeres (Fig. 3*h*, *i*).

(2) *Lectins*

(i) *FITC-Con A.* At each stage examined, labelling of fixed or unfixed blastomeres with FITC-Con A corresponded to that observed with the anti-species antiserum by indirect immunofluorescence, i.e. polarization of surface

All embryos were completely disaggregated into individual blastomeres in calcium-free medium and pooled, unless otherwise indicated. In each case a phase contrast micrograph (large figure denotes embryonic stage) precedes the fluorescence (FITC) micrograph. (Scale bar = 50μm.)

FIGURE 1

Paraformaldehyde-fixed blastomeres labelled, by indirect immunofluorescence, with RAMS.

(*a*, *b*) 4-cell blastomeres dissociated with trypsin/EDTA in calcium-free medium. Note the typically uniform surface labelling in three of the blastomeres, and in the other two derived from a 3-cell embryo heavy (internal?) labelling in the presumed region of the cleavage furrow. Inset: two undissociated 8-cell blastomeres from a 5-cell embryo again showing heavy (internal?) labelling in the region of the cleavage furrow.

(*c*, *d*) 8-cell blastomeres from early compacting morulae. Note the uniform surface labelling.

(*e*, *f*) 8-cell blastomere from late fully compact morulae. Note intense labelling over one pole and relatively reduced labelling over the remainder of the surface (for comparison purposes *d* and *f* were photographed and printed identically from a single experiment). Inset: two undissociated late 8-cell blastomeres demonstrating that the region of heavy labelling is lateral to the presumed cleavage furrow.

(*g*, *h*) Early 16-cell blastomeres dissociated with trypsin/EDTA in calcium-free medium from an *individual* morula. Note the polarized surface labelling (arrowed) associated with internal cytoplasmic labelling at the opposite pole in half of the blastomeres. Inset: single polarized 16-cell blastomere at twice the magnification. The remainder of the blastomeres appear to be uniformly surface labelled but also show the internal cytoplasmic labelling at one pole.

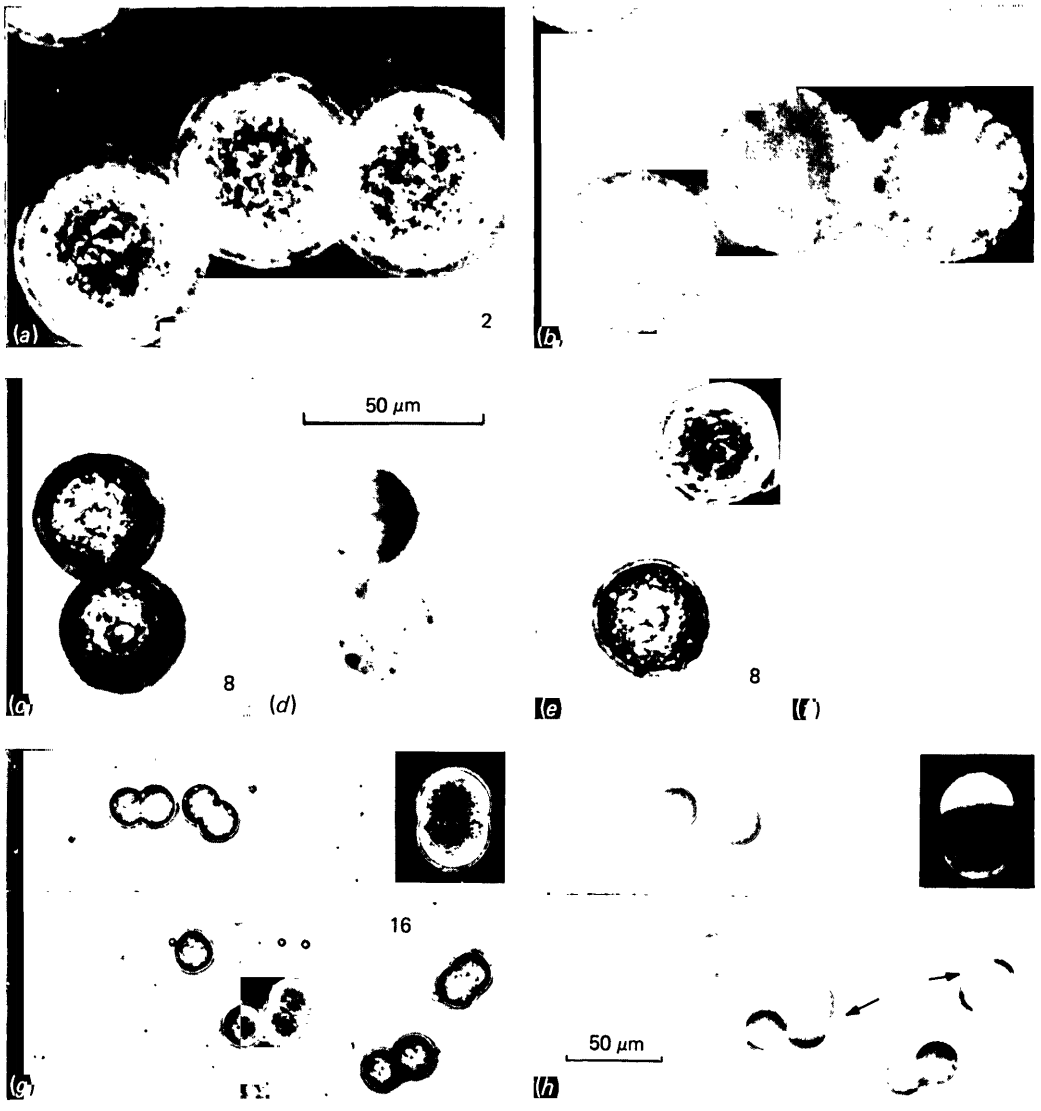


Fig. 2. Unfixed blastomeres labelled by indirect immunofluorescence with rabbit anti-mouse species antiserum (except *e*, *f*). Scale bar in *d* for figs *a–f* and for inserts *g* and *h*. (*a*, *b*) 2-cell blastomeres. Focused off the midline to demonstrate the patchy labelling distributed over the whole surface. (*c*, *d*) Compact 8-cell blastomeres. Focused off the midline to demonstrate the apparently reduced patching in the heavy label pole of the upper polarized blastomere. (*e*, *f*) Compact 8-cell blastomeres labelled with FITC-Fab_1 RAMS. Note the absence of patching on either the polarized (lower) or non-polarized (upper) blastomeres. (*g*, *h*) Pairs of blastomeres from 16-cell morulae, *partially* disaggregated in calcium-free medium. The polarized labelling observed with late 8-cell blastomeres appears to have been conserved on the pairs of daughter 16-cell blastomeres. In four of the pairs (including the two single cells) the polarized region has been inherited exclusively by one blastomere; in the other two cases (arrowed) the polar region appears to have been unequally bisected. Scale bar in *h* for figs *g–h* (not inserts).

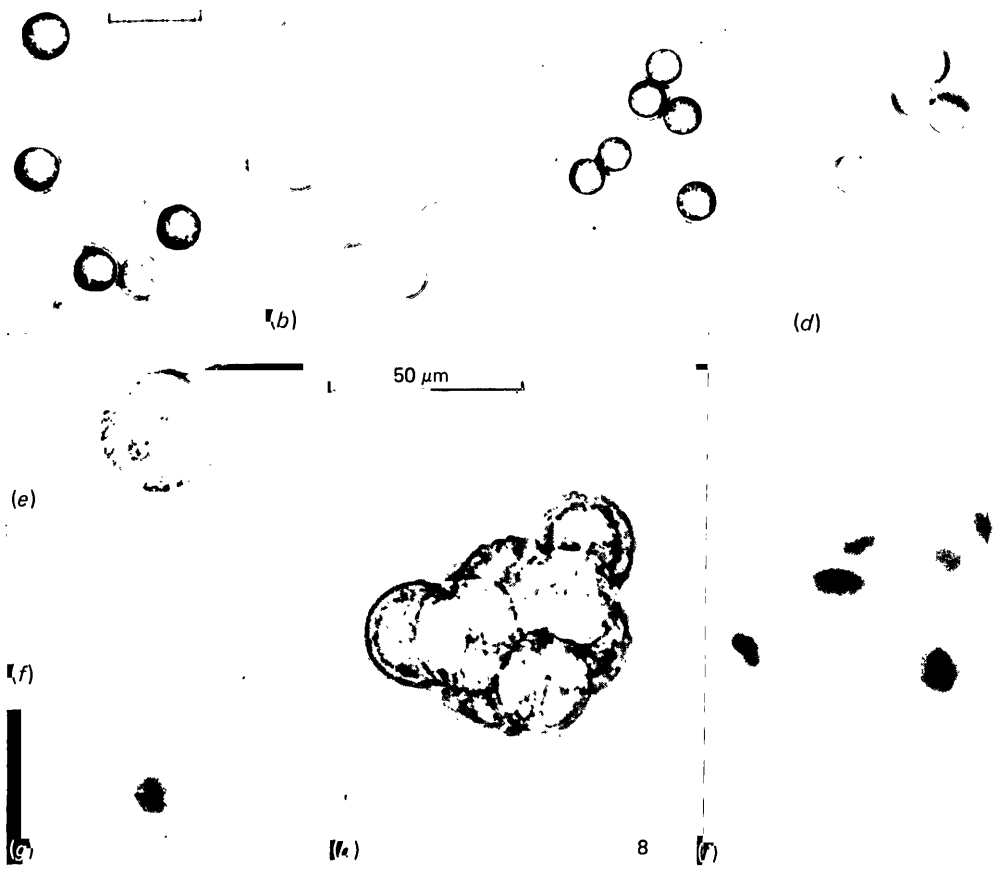


Fig. 3. Blastomeres and decompact morulae, treated in various ways, and labelled by indirect immunofluorescence with rabbit anti-mouse species antiserum. Scale bar in *a* for figs *a–d* and in *h* for figs *e–i*. (*a*, *b*) Late 8-cell blastomeres, pretreated and labelled in the presence of azide. Note the intense polarized labelling in some cases, compared with Fig. 2*c*, *d*. (*c*, *d*) Early 16-cell blastomeres, pretreated and labelled in the presence of cytochalasin D. Again note the intense polarized labelling in half of the blastomeres (compare with Fig. 2*g*, *h*). (*e*, *f*, *g*) Paraformaldehyde-fixed 8-cell blastomere, double labelled by indirect immunofluorescence (*f*) and with the DNA fluorochrome Hoechst 33258 (*g*). Note the interphase nucleus in the polarized blastomeres situated eccentrically away from the heavily labelled pole. (*h*, *i*) Late 8-cell compact morula, decompacted in calcium-free medium, treated with azide, fixed in paraformaldehyde and labelled by indirect immunofluorescence. Deliberately out of focus to demonstrate the predominantly outward facing heavily labelled poles of each of the blastomeres simultaneously.

labelling was restricted to blastomeres from 8- and 16-cell stages (Fig. 4*a*, *b*). However, amongst unfixed blastomeres disaggregated from 16-cell morulae there was a subpopulation which also showed considerable internalisation of label into discrete vesicles, in some cases leading to almost complete absence of surface labelling. Examination of pairs of blastomeres following partial

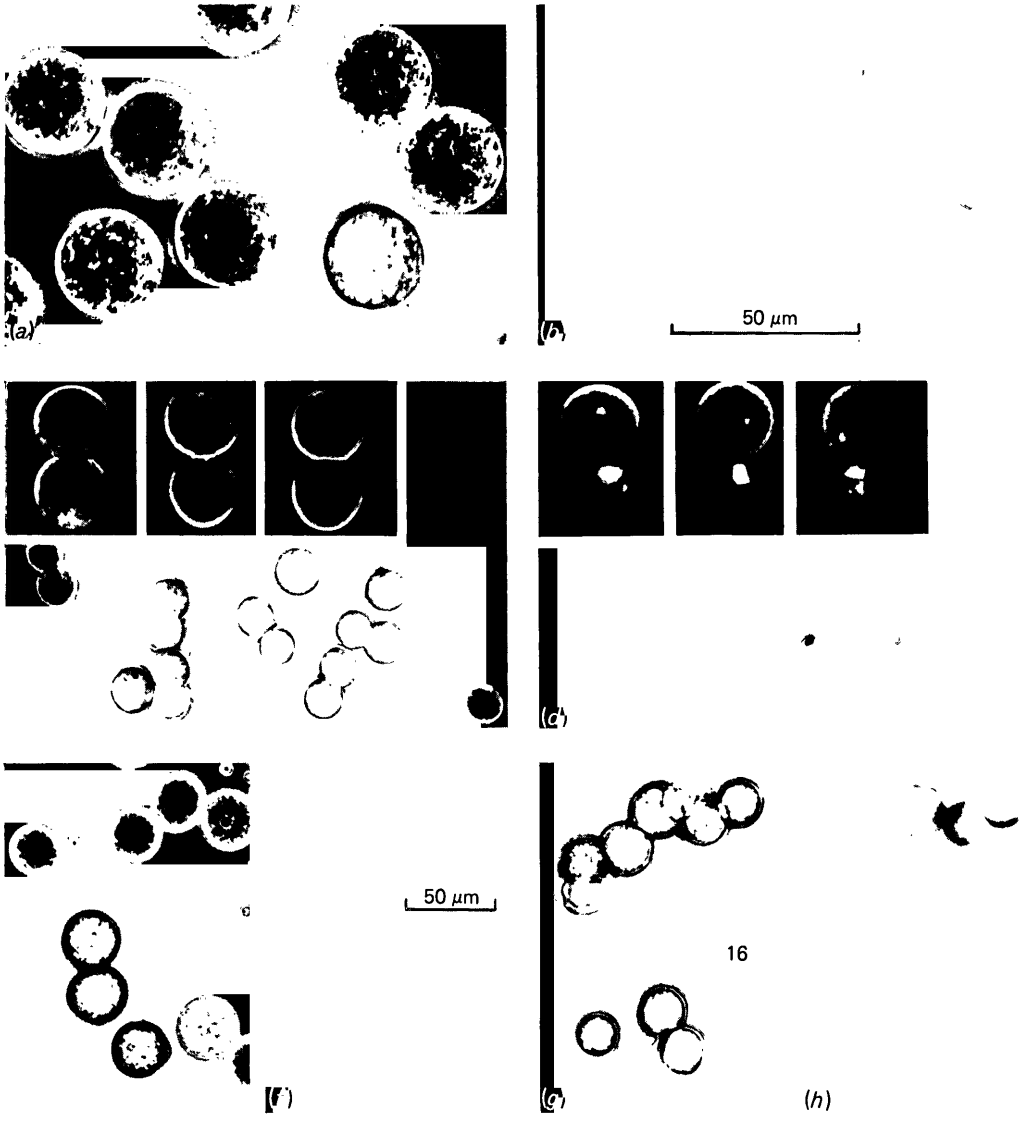


Fig. 4. Unfixed and paraformaldehyde-fixed blastomeres labelled with fluorescein-conjugated lectins. Scale bar in (*b*) for figs *a*, *b* and inserts. (*a*, *b*) Unfixed late 8-cell blastomeres labelled with FITC-Con A. Note the similarity with antibody labelled blastomeres (Fig. 2*c*, *d*) – again patching appears to be reduced in the heavily labelled region. (*c*, *d*) Unfixed early 16-cell blastomeres partially dissociated with trypsin/EDTA in calcium-free medium and labelled with FITC-Con A from an *individual* morula. Note the frequent occurrence of pairs of blastomeres one of which is heavily surface labelled, while the other is relatively unlabelled on the surface but contains internalized label localized in the region adjacent to the other blastomere. Inset: three more examples of these pairs of blastomeres from another individual morula, at twice magnification. (*e–h*) Late 8-cell (*e*, *f*) and early 16-cell (*g*, *h*) blastomeres fixed with paraformaldehyde, and labelled with FITC-PNA. Note the polarized labelling at both stages, but also the considerable quantitative increase in binding at the 16-cell stage. Scale bar in *f* for figs *c–h* except inserts.

disaggregation, indicated that this internalisation occurred predominantly in the non-polarized blastomeres leading to an accumulation of label in a region of cytoplasm adjacent to the other polarized blastomere (Fig. 4*c*, *d* and inset).

(ii) *FITC-PNA.* The intensity of fluorescent labelling resulting from the binding of FITC-PNA to fixed or unfixed blastomeres disaggregated from 2- to 8-cell-stage embryos was very low. Nevertheless, labelling appeared uniform with early cleavage-stage blastomeres and increasingly polarized after compaction at the 8-cell stage (Fig. 4*e*, *f*). However, unlike labelling with FITC-Con A which did not appear to show any stage-specific variation in overall intensity (over a wide range of concentrations), with FITC-PNA there was an increase in labelling intensity with fixed blastomeres at the 16-cell stage, irrespective of their state of polarization (Fig. 4*g*, *h*). As with Con A, surface bound FITC-PNA was rapidly internalised, apparently to a lesser extent from the heavily labelled pole.

DISCUSSION

The observations presented here have demonstrated that during compaction at the 8-cell stage, the density of binding sites for anti-species antibody and lectins on dissociated blastomeres becomes polarized. Four possible explanations for this feature of compaction have been considered.

(1) *Association with the cleavage furrow at mitosis.* Polarized surface labelling with FITC-Con A in the region of the cleavage furrow has been observed in J774 macrophages undergoing mitosis (Berlin, Oliver & Walter, 1978). However, binding at 4 °C was uniform, indicating that polarization was due to redistribution at physiological temperatures, possibly to the area associated with the actin filaments of the contractile ring. A similar phenomenon has been seen with cleavage-stage blastomeres dividing in culture *after* labelling with fluoresceinated Fab_1 antibody fragments or Con A (Handyside & Johnson, unpublished). Heavy labelling of the cleavage furrow of dissociated pairs of blastomeres, in which division had occurred *before* labelling, was very infrequent (Fig. 1*a*, *b*, and inset), was restricted to recently divided fixed blastomeres and appeared to be largely internal, as judged by altering the plane of focus. This may be due, therefore, to the treatment with paraformaldehyde causing relatively increased permeability to antibody in this area.

In contrast, the polarized labelling observed in the majority of 8-cell blastomeres dissociated from compact morulae, became progressively more marked towards the end of the cell-cycle, but was well established before actual mitosis (Fig. 3*e*, *f*, *g*). Also, the heavily labelled areas of decompacted but undisaggregated morulae did not appear to be situated directly between adiacent blastomeres (Fig. 3*h*, *i*).

(2) *Redistribution induced by the binding of multivalent ligands.* The binding of multivalent ligands, such as IgG molecules or lectins, to the surface of a variety of differentiated cell types is known to induce the redistribution of

receptors in a number of ways including: (*a*) the energy-independent process of 'patching' in which mobile multivalent membrane components are cross linked by multivalent ligands; and (*b*) the energy-dependent process of 'capping' in which cross-linked receptor/ligand patches are drawn to one pole of the cell (de Petris & Raff, 1973).

Labelling of unfixed blastomeres from all stages examined by indirect immunofluorescence (Fig. 2*a*, *b*) or FITC-Con A (Fig. 4*a*, *b*) was patched, indicating at least limited mobility and multivalency of some membrane components. On the other hand, labelling of unfixed blastomeres with FITC-RAMS or FITC-PNA appeared to be continuous. Similar variation in the ability of different lectins to induce patching on the outer surface of intact morulae and blastocysts has been reported (Brownell, 1977). Again FITC-Con A, and also FITC-RCA_{II} and FITC-RCA_{I} induced patching, whereas labelling with FITC-WGA was continuous.

The polarization observed with 8-cell blastomeres is unlikely to be due to capping, however, since it was evident on fixed blastomeres (Fig. 1*e*,*f*) and on blastomeres labelled with monovalent Fab_1 fragments (Fig. 2*e*,*f*) though patching was eliminated in both cases. Pretreatment with azide, cytochalasin and/or colcemid, which have been shown to inhibit capping of surface immunoglobulins in lymphocytes (Taylor *et al.* 1972; Schreiner & Unanue, 1976) or of H-2 in clone 1D mouse fibroblasts (Edidin & Weiss, 1972) was also without effect on the proportion of polarized blastomeres detected (Fig. 3*a–d*). Finally, the concentration of Con A used here exceeds the levels known to inhibit capping in lymphocytes (Yahara & Edelman, 1973).

(3) *Qualitative polarization of specific membrane components.* In other transporting epithelia, such as intestinal epithelium, certain membrane-associated enzymes and other antigens are known to be restricted to either the apical or baso-lateral membrane (Isselbacher, 1975). Since the 'outer' blastomeres of the morula are thought to be the precursors of the trophectoderm, the transporting epithelium of the blastocyst, comparable differences in the molecular composition of the internal and external surfaces of these blastomeres might also be expected to develop. Indeed, some authors have claimed that alkaline phosphatase activity is restricted to the internal surface membranes of blastomeres in fixed, sectioned morulae (Mulnard & Huygens, 1978).

Such an explanation is unlikely to apply to polarized labelling observed with blastomeres from postcompaction stages, however, since the polarization does not appear to be critically dependent on the specificity of the ligand. Every antiserum with activity against pericompaction embryos so far tested, including several rabbit antisera prepared against purified membrane-associated enzymes (Handyside & Ziomek, unpublished), a rabbit anti-embryonal carcinoma antiserum (Johnson *et al.* 1979), a monoclonal antibody to embryonal carcinoma (Bighouse, Johnson & McIlhinney, unpublished), as well as two lectins, Con A

and PNA, with contrasting affinities have shown quantitative rather than exclusive polarization at these stages.

In any case, since fixation of blastomeres *in situ* prior to decompaction and disaggregation was not technically possible, any molecules initially localized to restricted areas might be expected to redistribute by lateral diffusion after isolation, as has been demonstrated for some markers in intestinal epithelium (Ziomek & Edidin, 1978; Pisam & Ripoche, 1976). However, it should be noted that since 8-cell blastomeres are comparatively large cells, complete redistribution of membrane glycoproteins with average diffusion constants (Johnson & Edidin, 1978) would take of the order of 10 h, which approaches the cell-cycle time at this stage. Thus, localized insertion of membrane components as demonstrated with MDCK epithelial cells (Rodriquez-Boulan & Sabatini, 1978) could lead to the generation of cortical molecular gradients, which may play a role in the early differentiation of the embryo (Johnson, Pratt & Handyside, 1980).

(4) *Quantitative polarization due to membrane topography.* The fourth and most likely explanation for the polarization of binding-site density is that it corresponds to the polarization of microvilli, which thereby increase the unit density of surface area. Microvilli are known to become localised mainly on the outer faces of blastomeres *in situ* during compaction (Ducibella *et al.* 1977). This conclusion has been supported in several ways: (*a*) the heavily labelled poles appear to correspond to the outer faces of blastomeres in decompact but undisaggregated morulae (Fig. 3*h, i*); (*b*) selective labelling of the outer surfaces of blastomeres of intact compact morulae by brief incubations in fluorescein-conjugated antisera, followed by disaggregation and labelling of the dissociated blastomeres with rhodamine-conjugated antisera, has indicated that the heavily rhodamine-labelled poles coincide exactly with the exposed areas of the blastomeres that had been labelled *in situ* with fluorescein (Handyside, in preparation); (*c*) scanning electron microscopy of individual blastomeres has demonstrated a good correlation between degree of polarization of microvilli and that of ligand binding (Reeve & Ziomek, in preparation).

A similar explanation to that proposed for the observations reported here was also found to explain apparently reduced binding of FITC-Con A over the second metaphase spindle of unfertilized mouse eggs (Johnson, Eager, Muggleton-Harris & Grave, 1975), which is the only region lacking microvilli (Eager, Thurley & Johnson, 1977). However, it should be emphasized that since both the antisera and lectins used in the present study are multi-specific, polarization of a minority of membrane receptors by either of explanations two and three cannot be excluded.

The polarization of surface binding sites at compaction appears to be associated with an opposite polarization in the properties of adsorptive pinocytosis. Thus FITC-Con A in particular is rapidly taken into the polarized cell at the pole opposing the heavily stained, presumptive external face (see

Fig. 4*c*, *d* and inset). If substantiated by more quantitative techniques, this property will provide a functional marker typifying the internal membrane of the 8-cell blastomere.

A remarkable feature of these observations is the manner in which once polarity is established, it then appears to be conserved. Thus isolation of individual 8-cell blastomeres does not evidently disturb their overall polarity of structure, although some molecular distribution by diffusion may occur (vide supra). Furthermore, after division *in situ* to the 16-cell stage, each cell couplet formed from one 8-cell blastomere also appears to express an overall surface polarity (Fig. 2*g*, *h* and inset). This polarity could arise either by conservation of polarity at division or its regeneration anew after division. Thus, 16-cell couplets consist of either a polar plus an apolar cell, which could represent a cleavage plane approximately parallel to a line tangential to the embryo's surface, or, less frequently two polar cells, the poles not necessarily being of equal size, which might result from a cleavage plane approximately along the polarized axis of the cell (Fig. 2*g*, *h*). Experiments on the stability of polarization at division are presently underway. However, regardless of which mechanism is operative, since it is argued that the polar staining probably represents the externally exposed face of the cell, the ratio of polar to non-polar cells in a 16-cell embryo will provide a measure of the ratio of outer to inner cells (Handyside, in preparation). It has previously been proposed that the outer and inner cells of the morula have different cell fates, becoming trophectoderm and ICM respectively (Mintz, 1965; Tarkowski & Wroblewska, 1967). Thus, since outer and inner cells are from the moment of their formation identifiably different in their surface properties, it is possible that the polarity generated in 8-cell blastomeres at compaction is concerned with the initiation of the divergent differentiation of ICM and trophectodermal tissues. A 'polarization hypothesis' to explain the generation of cellular diversity within the morula and which incorporates these observations has been propounded recently (Johnson, *et al.* 1980).

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