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CELL–SUBSTRATE CONTACTS IN CULTURED CHICK EMBRYONIC CELLS: AN INTERFERENCE REFLECTION STUDY

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SUMMARY

Cell–substrate contacts in explants of different regions of early chick tissues were investigated using the technique of interference reflection microscopy. All the explants spread as epithelial sheets. During initial spreading a peripheral zone of 2–3 cells formed broad contacts with the substrate. In spread explants some cells in the centre made broad substrate contacts. A mat of extracellular material containing fibronectin was found under the explants. Focal contacts and focal adhesions increased in number during culture, and stress fibres were associated with them. These changes in cell contacts appeared more quickly in some tissues than in others. After 24 h, explants of hypoblast and definitive endoblast could easily be distinguished but by 7 days they were very similar. In the absence of serum, specialized cell contacts developed more quickly; in higher concentrations of serum, more slowly. Confrontations between explants were also examined. The most conspicuous feature was that cells in invading explants normally underlapped invaded cells. Invasion from above by an unspread explant could occur even if the invaded explant had formed many focal adhesions.

INTRODUCTION

During normal embryogenesis, there are many instances where cells of one tissue invade those of another. During endoderm formation in the chick, cells from the upper layer, the epiblast, penetrate the original lower layer, the hypoblast, and form the definitive endoblast, which later becomes the gut endoderm (Modak, 1965; Nicolet, 1971; Vakaet, 1970; Rosenquist, 1972; Fontaine & LeDouarin, 1977; Stern & Ireland, 1981). Previous reports (Sanders, Bellairs & Portch, 1978; Bellairs, Ireland, Sanders & Stern, 1981) examined the interaction between hypoblast and definitive endoblast grown in tissue culture. They showed that when a definitive endoblast explant was confronted with a hypoblast explant in culture, the definitive endoblast explant continued to spread, relatively unimpeded, into the area previously occupied by the hypoblast. This type of process has been termed 'invasion' in two-dimensional confrontations (Abercrombie, 1975). When the two tissues were challenged in a different situation, one being explanted on top of an already spread sheet of the other, the seeded tissue inserted itself and always continued to spread at the expense of the base tissue, whichever way round they were put (Bellairs *et al.* 1981). A great many factors are

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involved in the interaction of confronted tissues *in vitro* and those relating to fibroblastic cells have been reviewed recently (Stephenson, 1982).

When explants consist of cells with epithelial characteristics, one important factor is likely to be the relative strengths of the cell–cell and cell–substrate adhesions. The latter are considered here using interference reflection (IR) microscopy. This method, introduced by Curtis (1964), can provide information about cell–substrate contacts in living cells that is unobtainable with conventional light microscopy. The basis of the technique is that the medium between the cell and the glass substrate can act as a thin film and generate an interference pattern in reflected white light (Interference colour chart according to Michael-Levy, Zeiss). At high values of incident numerical aperture (INA > 1·0) the highest orders of interference are lost and the residual image is mostly zero-order (Izzard & Lochner, 1976). In monochromatic light, providing the image is zero-order, the blackest areas (darker than background intensity) represent regions where the ventral cell membrane is closest to the substrate, and the white areas (lighter than background intensity) regions further away. Shades of grey represent intermediate distances although some parts of the cell membrane further away than regions appearing white will not be seen at all. A full analysis of the technique has been presented by others (Izzard & Lochner, 1976, 1980; Gingell & Todd, 1979; Gingell, 1981; Bereiter-Hahn, Fox & Thorell, 1979).

Most of the work using this technique has been directed towards a study of cell–substrate contacts during fibroblast locomotion (Abercrombie & Dunn, 1975; Izzard & Lochner, 1976, 1980; Heath & Dunn, 1978; Couchman & Rees, 1979). Other cells have however been observed with the IR microscope, including mouse kidney epithelium (Cottler-Fox, Sparring, Zetterberg & Fox, 1979), chick corneal epithelium (Heath, 1982), metastatic and non-metastatic carcinomas (Cottler-Fox, Ryd, Hagmar & Fox, 1980; Haemmerli & Strauli, 1981), rat leukaemia cells (Haemmerli & Ploem, 1979), rat mammary adrenocarcinoma, PtK2 cells (Wehland, Osborn & Weber, 1979), chick sensory neurones (Letourneau, 1979), neutrophil leucocytes (Armstrong & Lackie, 1975; Keller, Barandun, Kistler & Ploem, 1979; King, Preston, Miller & Donovan, 1980), *Xenopus* tailfin epidermis (Radice, 1980) and the amoeboflagellate, *Naegleria* (Preston & King, 1978).

As well as investigating the cell–structure contacts of invasive tissues this study also provides new information on the spreading of epithelial sheets and a much needed comparison of normal early embryonic cells with the more commonly used fibroblasts.

MATERIALS AND METHODS

Cultures

Hens' eggs (Ross Rangers, Ross Poultry South) were incubated for 12–24 h to obtain stage XIII (Eyal-Giladi & Kochav, 1976) to stage 5 (Hamburger & Hamilton, 1951). Embryos were removed from their vitelline membrane in bicarbonate-buffered Tyrode's solution and small pieces dissected with steel knives or tungsten needles. Explants of chick heart fibroblasts were obtained from the distal tips of the ventricles of 8-day-old chick embryos. Explants were transferred, with fine siliconized glass pipettes, to glass chambers, each of which consisted of a glass microscope slide (79 mm × 39 mm × 1 mm) with a central circular aperture (diameter 12 mm) bound above and below with 35 mm square coverslips (Chance Propper no. 1) sealed with wax.

Each chamber contained 0·4 ml medium consisting of 9 ml medium 199 (Wellcome), 1 ml foetal calf serum (Gibco), 0·5 ml of a stock penicillin (500 i.u./ml) and streptomycin (50 μg/ml) solution (Gibco).

'Side-to-side confronted' cultures were produced by placing two explants close to one another so they met when spreading (Sanders *et al.* 1978). 'Vertical confronted' cultures were produced by allowing one explant to spread, and 24 h later explanting another on top of the first one (Bellairs *et al.* 1981). Tissue dissociations were carried out in Ca/Mg-free Tyrodes solution (CMF) in siliconized glass centrifuge tubes. The tubes were placed in a 37 °C water-bath for 45 min, during which time the tissues were pipetted up and down siliconized flamed round-tipped Pasteur pipettes twice, at 22·5 and 45 min. The dissociate was centrifuged at 1500 rev./min for 3 min, resuspended in medium and set up in the culture chambers.

In some control experiments, explants were grown in Tyrodes solution, in serum-free medium or in higher concentrations of serum (up to 70 %) in medium. In others hypoblast was grown on an area of glass previously occupied by another explant, which had then been removed by microdissection. Explants of different culture ages were also confronted. Some explants were set up in Rose (1954) chambers in order to be able to exchange the normal medium for one of lower ionic strength (King, Heaysman & Preston, 1979).

All cultures were maintained at 37 °C in a humidified incubator provided with CO_2.

Microscopy

Cultures were examined with a Zeiss IM35 microscope fitted with a super pressure mercury lamp (HBO 50), epi-illumination attachment (Zeiss part no. 47 17 60), narrow band pass interference filter (546 nm) and double reflector (HD). This provided the long illuminating path characteristic of the metallurgical version of the IM35. Two diaphragms were present: one situated nearer the HBO lamp, which enabled control over the illuminating numerical aperture (INA) of the objective, and the other nearer the objective, which functioned as a field stop. To minimize stray light the latter was kept nearly shut and the former was opened to just fill the objective. A modification, made by removing the mirror in the reflected dark-field position of the double-reflector, enabled rapid sequential observation with phase-contrast (PC) and IR optics. A polariser could be used, optionally, with very little effect on the image. Accurate positioning of the IR image was obtained by taking simultaneous PC and IR photographs. All IR observations were made with a Zeiss Planapo Ph3 100 × objective (NA 1·3) with a calculated operational INA of 1·25. Photographs were taken with an attached 35 mm camera using PanF film. In some control experiments the HBO 50 was replaced with a white light source (tungsten 60 W).

For fibronectin staining, half of the explant was dissected off the glass using tungsten needles to allow for access of antibody to the substrate, and then the coverslips were fixed in buffered formal saline for 30 min. They were then processed as described by Thom, Powell & Rees (1979) using rabbit anti-chick or rabbit anti-mouse serum fibronectin, and then incubated with goat anti-rabbit conjugated to fluorescein. After washing and mounting in saline the specimens were viewed using a fluorescein filter (Zeiss set 10) insert instead of the HD reflector.

RESULTS

Interpretation of the IR image

Because of the variety of optical systems used by others and differences in interpretation of IR images it was necessary to evaluate the particular IR system we used. For this purpose we examined chick heart fibroblasts, which have been well described by others. Three different types of contact region have been shown for these cells: *focal contacts* (black, spindle-shaped 10–15 nm substrate separation, formed sequentially during movement; Izzard & Lochner, 1976), *focal adhesions* (black, larger than focal contacts, more permanent and characteristic of stationary fibroblasts; Couchman & Rees, 1979) and *close contacts* (uniformly dark grey, 15–30 nm substrate separation, labile; Izzard & Lochner, 1976). These features are shown in Figs. 1 and 2. We are not

in a position to differentiate unequivocally between the specialized contacts (focal contacts and focal adhesions) without time-lapse cinemicroscopy. However, by analogy with published work, the smaller spindle-shaped black contact regions seen in 1-day-old fibroblasts (Fig. 1, arrowheads) are focal contacts whilst the larger darker contacts seen in older fibroblasts (Fig. 2) are focal adhesions.

In addition to these features we see (Fig. 1) a number of images corresponding to intracellular organelles: the nucleus, intracellular vesicles and microfilament bundles. These features would not be expected to appear in the IR image if the pattern was due to a simple single film and this highlighted the need for paired IR and PC observations of the same cells and for careful interpretation. These same features and others were observed in the chick cells described below.

We believe that the majority of patterns we observed in our system were zero-order, because the images were colourless when viewed with white light. Additionally, the image was near the level of focus of the substrate and could also be observed under spreading explants or in multilayered regions where the PC image was poor due to specimen thickness. However, if an INA < 1·0 was used, particularly with a 40× Planapo objective (NA 1·0), a series of concentric dark fringes was sometimes seen, which matched the cell outline, and we consider that these fringes were reflections from the upper cell surface/medium interface. They were rarely observed with the 100× objective if large aperture stops were used. That the darkest region in IR images did represent substrate attachments could be demonstrated by decreasing the ionic strength of the medium, thus partly lifting the cells off the substrate (King *et al.* 1979; Gingell & Vince, 1982). The darkest regions persisted after this treatment although other regions became white or disappeared. Gingell (1981) has argued, however, that if the thickness of the cytoplasm is less than 1 μm, then it will contribute to the image.

Fig. 1. Interference reflection image of 24-h-old chick heart fibroblast from 8-day-old embryo. Notice the focal contacts (arrowheads), retraction filaments (large arrows), stress fibres (small arrows), microspike (*m*) and image of nucleus (*n*) and vesicles (*v*). Bar, 10 μm (Figs. 1–6).

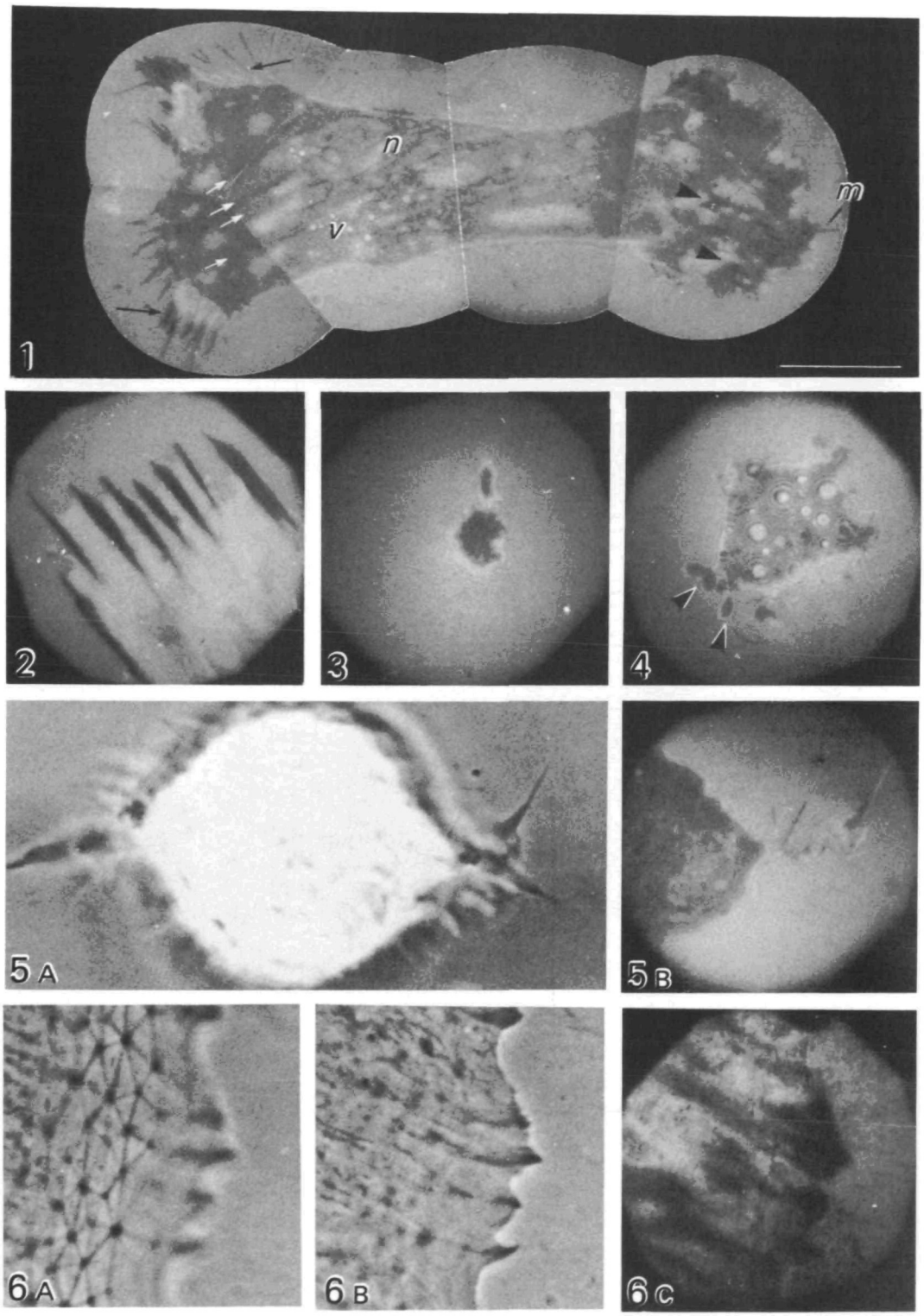
Fig. 2. Interference reflection image of part of chick heart fibroblast after 5 day culture from 8 day embryo. Focal adhesions are prominent.

Fig. 3. Interference reflection image of dissociated hypoblast cell settling in medium with serum. After 25 min, two small contacts are apparent in this cell.

Fig. 4. Interference reflection image of dissociated epiblast cell settling in medium with serum, after 35 min a broad edge of adhesion is apparent with small bleb-like contacts at the edge (arrowheads). Reflections from intracellular yolk granules are also apparent.

Fig. 5. Dissociated hypoblast cell after 3 h. A. Phase-contrast. Note rounded cell with filopodia. The cells in Figs. 3, 4 were also rounded but without filopodia. B. Interference reflection. A broad contact is present under the cell body and some filopodia are also seen.

Fig. 6. Part of a lamella from hypoblast cell in 5 day explant. A, B. Phase-contrast pictures at two different planes of focus. B. Focused near the substrate, and A further away. Radial struts found in the core of the cusps of the scalloped edge are associated with the polygonal network seen near the dorsal surface. C. Interference reflection. The cusps of the scalloped edge contain focal adhesions.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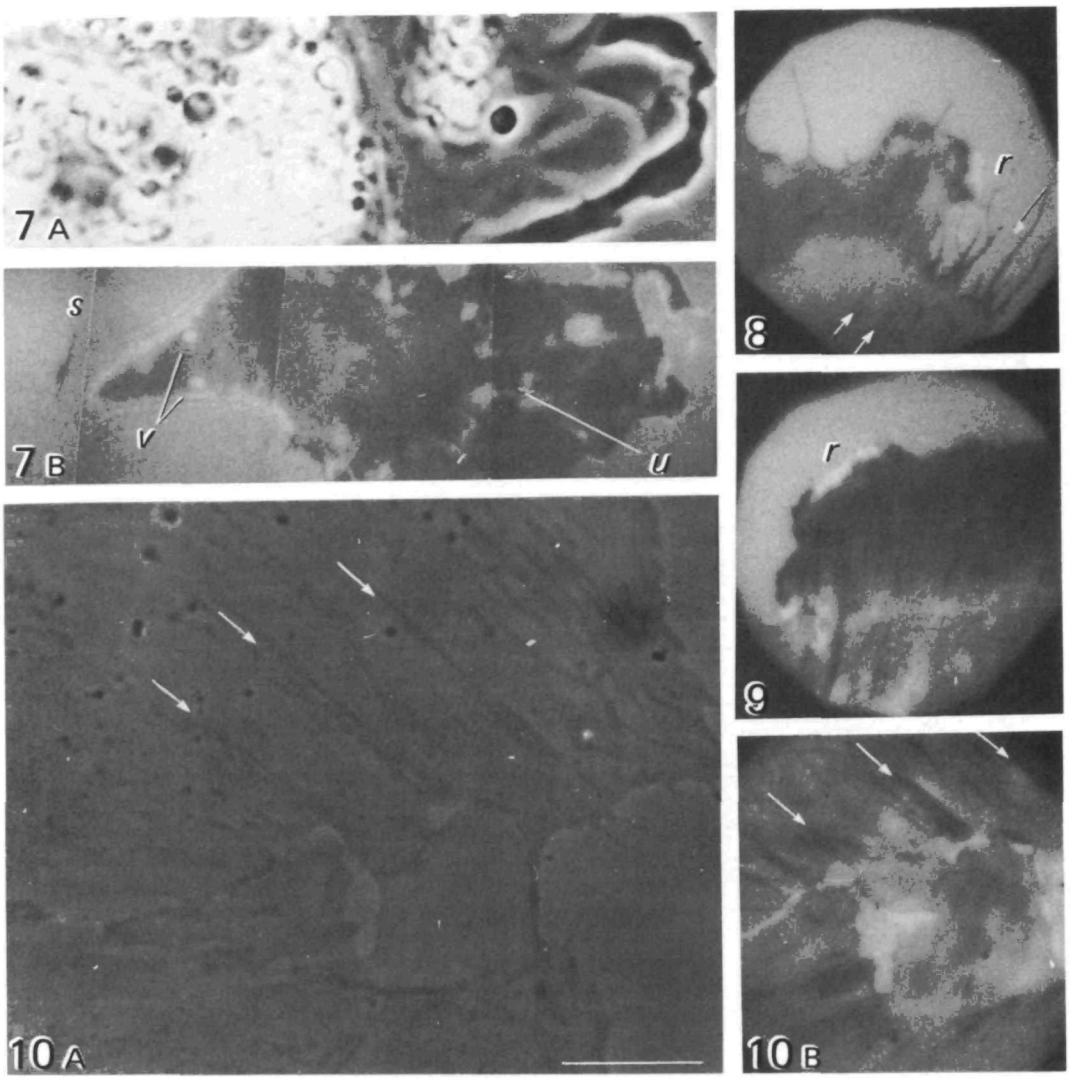

Fig. 7. Edge of a hypoblast explant (from stage XIV embryo) 3 h after explantation. A. Phase-contrast. The lamella region is on the right and the central yolky mass of the explant on the left. B. The interference reflection image is due to at least three cells. The outer marginal cell is underlapped by a spikey process of a submarginal cell (*u*). In the interior of the explant little contact is seen except for some spikes (*s*). Note the contribution to the image from the intracellular yolk vesicles (*v*). Bar, 10 μm (Figs. 7–10).

Fig. 8. Interference reflection image of part of the lamella of a hypoblast cell at the edge of a 24-h-old explant. The large arrow points to the dark retraction filaments. Note the ruffled membrane (*r*). Small arrows show submarginal focal contacts.

Fig. 9. Interference reflection of part of lamella of a definitive endoblast cell from a 24-h explant. This shows ruffling membrane (*r*) and submarginal spindle-shaped focal contacts.

Fig. 10. Part of the lamella of two adjoining hypoblast cells in a 3-day explant. A. Phase-contrast. Arrows indicate stress fibres. B. Interference reflection. Arrows show focal adhesions corresponding to the termini of the stress fibres seen in A.

Thus grey regions in the lamellae of cells may not necessarily represent regions of close contact with the substrate. This will be discussed below.

Substrate contacts during spreading of epithelial sheets

To provide a control for the vertical confronts we first needed to examine the normal spreading behaviour of explants. Explanted sheets first rolled up into a ball and during this stage poor IR images led us to examine dissociated cells in order to look at the first contacts formed. These were small dark grey circles surrounded by a white rim seen a few minutes after plating in all tissues studied (Fig. 3). These point contacts were mobile and were directly correlated with blebs visible by PC on the lower surface of the cells. After 30 min larger broad grey contacts were common beneath cells that were still rounded in PC (Fig. 4). In PC filopodia were commonly seen in the first hour of spreading and their distal tips were often associated with grey streaks in IR (Fig. 5).

Explants, particularly of hypoblast, spread on the substrate considerably faster than dissociated cells of the same tissues. By 30–45 min some cells of the hypoblast explants already had broad grey areas of contact visible by IR. One hour after explanting, most explants were rapidly spreading but the cells were closely packed and it was difficult to distinguish individual cells by PC optics. A yolk-free region of lamella at the edge of the explant was engaged in very active ruffling activity (Fig. 7A). Using IR optics a broad annular zone of cell–substrate contact was found. This consisted of an interlocking jigsaw of broad individual cell contacts involving both 'marginal' (those at the extreme outer edge) and submarginal cells (Fig. 7B). The innermost cells of this annular zone appeared mottled grey and white by IR. Marginal cells had a pattern consisting of uniform dark grey regions and patches of white, some of which corresponded to ruffles by PC. A consistent feature of many submarginal cells was a mottled dark grey region, towards the inside of the explant, and a uniform dark grey lamella region with spikes, towards the outside of the explant. These spikes lay under more peripheral cells where they were surrounded by a white area representing a region of underlapped cells away from the substrate (Fig. 7B). Towards the centre of the explant away from the zone of substantial contact, some grey spikes were seen in areas otherwise devoid of contact (*s*, Fig. 7B).

By 6 h after explanting the sheet was more fully spread and the individual cells were more flattened, particularly at the edge. The lamellae were broader and longer and the ruffling activity was less pronounced. In IR a corresponding increase in size of contact per cell, due to spreading, and a closer match between phase and IR images due to reduced ruffling activity was found. The only other difference was that more extensive contacts were apparent in the interior of the explant. These consisted of short processes, which could be identified in PC and were dark grey and white in IR, although part of the process seen in PC was not seen in IR.

Temporal and tissue differences of cell–substrate contacts

Table 1 summarizes our main findings obtained when different tissues were placed in culture and observed by IR over a period of up to 7 days. It shows that specialized

cell–substrate contacts formed more quickly in epiblast and endoblast than in hypoblast. Furthermore, the speed with which hypoblast explants made these contacts varied with the amount of serum present: the greater the amount, the slower the contacts were formed.

Table 1. *Timing of formation of specialized adhesions (days). For explanation see text*

	Hypoblast	Hypoblast −serum	Hypoblast + +serum	Epiblast	Definitive endoblast
Small focal contacts	2–3	1	—	1	1
Larger focal contacts (focal adhesions)	4	—	6	1	2–3
Patches (focal adhesions)	5–7	—	—	—	2–3

After 24 h hypoblast explants were well spread and consisted of a monolayered sheet. Under IR most marginal cells had large areas of close contact, but the margin of the lamellae of these cells had either a black edge with associated black spikes or a dark grey and white edge (Fig. 8). White areas were sometimes associated with regions that showed a concave margin in PC. Some focal contacts were seen behind the margin of edge cells (Fig. 8, arrows) but only in favourable cases was it possible to see associated stress fibres in the paired PC image. Submarginally and in the centre of the explant the few cells that were in contact with the substrate, as shown by IR, possessed broad lamellae similar to edge cells. Definitive endoblast explants attached less readily than hypoblast, but spread in a similar way, forming a monolayered sheet, although the centre of the explant could be multilayered. Rows of thin focal contacts were prominent at this time (Fig. 9). Epiblast and late hypoblast (entophyll crescent) cultures settled and attached even more poorly. Epiblast explants were still multilayered at the centre after 24 h although monolayered at the edge (Al-Nassar & Bellairs, 1982). The individual cells and their lamellae were smaller than those of the hypoblast. Focal contacts and focal adhesions with associated stress fibres were seen even earlier than in definitive endoblast explants. Late hypoblast explants had a more spikey appearance than the others, and the marginal cells had narrow lamellae in which focal contacts were prominent. The morphology of explants from anterior and posterior primitive streak was different after 24 h in culture. The marginal cells of anterior-streak explants resembled definitive endoblast in both PC and IR, whilst those of posterior streak had narrower and more spikey lamellae in PC, and larger focal contacts in IR.

In older cultures of hypoblast the main feature was the development of focal adhesions (Fig. 10B, arrows), associated either with the edge of cells or submarginally. They were usually orientated radially to the explant and did not cross one another. The general appearance of the IR image was now striped and in PC pairs the ends of stress fibres (Fig. 10A, arrows) were clearly seen to be associated with the focal adhesions. The definitive endoblast and epiblast explants had already achieved this appearance earlier (Table 1).

By 3–4 days, explants of hypoblast had reached their maximum degree of spread (Voon, 1980). Both focal adhesions seen in IR and stress fibres seen in PC were well developed but took two forms: the first consisted of two orthogonal arrays in which the fibres of the lower set terminated in focal adhesions. The second was a polygonal network (Ireland & Voon, 1981; and Fig. 6), the peripheral fibres of which terminated in the apices of clear scalloped margins, which formed corresponding black patches in IR (Fig. 6c). Definitive endoblast and epiblast achieved these forms earlier (2–3 days) and were usually visible by PC in all cell types. In IR images a dense meshwork of fine black fibrils was often seen both under and to the side of older hypoblast explants, and most remained when explants were removed mechanically from the substrate. These fibrils were only just visible with PC but clearly seen in IR (Fig. 11B), and were sometimes densely criss-crossing. They were more plentiful under definitive endoblast and epiblast explants. By 24 h immunofluorescent staining revealed the presence of fibronectin (Fig. 11A) in a similar pattern to the fibres seen by IR. Fibronectin was also demonstrated under hypoblast explants grown in the absence of serum. Definitive endoblast explants had considerably thicker fibres, which were closely associated with the cells and stained with anti-fibronectin antibody, whilst hypoblast explants had more diffuse and thinner fibres, which also stained with the antibody.

Explant confrontations

Side-to-side. Definitive endoblast and epiblast always displaced hypoblast in confrontations as previously reported (Sanders *et al.* 1978; Ireland, Bretland, Roe & Bellairs (1979). The boundary between the two explants (A and B in Fig. 12A) was not regular and there was some interdigitation, particularly in the central confrontation region (*c*, Fig. 12A). The main feature shown by IR was the underlapping of hypoblast cells by those of the invading explant. In the central zone two types of underlapping processes were seen: the first type was a broad lamella with large focal contacts (Fig. 15); the second type was a short spikey process, seen particularly with epiblast explants, which also contained focal contacts often associated with the spikes but sometimes submarginally (Fig. 16A, B). In the peripheral zone (see *p* in Fig. 12A) the arrangement often took the form of a whole edge of one cell underlapping another (Fig. 13), although mutual underlapping was also seen (Fig. 14). An important feature of epiblast invasion was the tongues of cells that radiated from the epiblast explants (Fig. 17), which in IR were clearly seen to underlie the hypoblast.

In confrontation between two definitive endoblast explants placed close together, a barrier was often formed in the zone of contact (Sanders *et al.* 1978; Bellairs *et al.* 1981). The cells became aligned normal to the line adjoining the centre of the two explants and tightly packed together (Fig. 18A). IR revealed that the cell bodies of many of these cells were not in contact with the substrate (Fig. 18B). Instead, they were underlapped by small processes from adjoining cells (arrows).

Vertical confrontations. In vertical confrontations (Fig. 12B) the 'base' and 'seed' cells were readily distinguishable. Bellairs *et al.* (1981) used the terminology of Explant I for the base sheet of cells and Explant II for the tissue placed on top of it, the seed. The following combinations were studied: definitive endoblast on hypoblast, epiblast

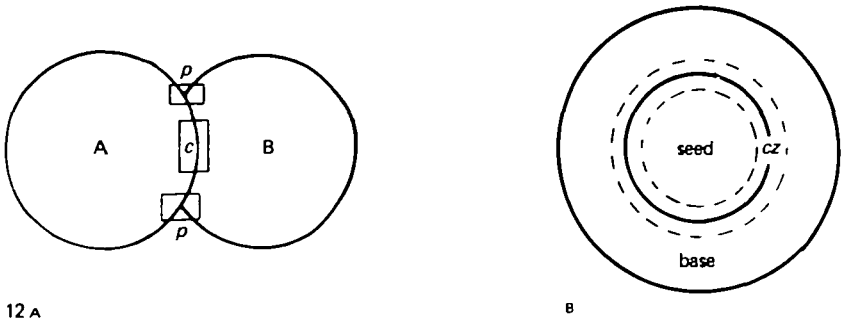

Fig. 12. A. Diagram to show situation found in typical side-to-side confronted culture. *p*, peripheral zones; *c*, central zone. B. Diagram showing situation found in typical vertically confronted cultures. *cz*, confront zone.

on hypoblast, hypoblast on hypoblast, hypoblast on endoblast and endoblast on endoblast. No tissues were seeded onto epiblast sheets, because these did not form large enough sheets to make seeding feasible. The seeded tissue had generally inserted into the base sheet, regardless of tissue type, one or two days after confrontation. The inserted pieces caused concentric unilateral alignment of the base cells in the confront zone (*cz*, Fig. 12B). When the confronted zone was examined with IR there were many instances of underlapping of base by seed cells. The types of contacts seen under the base and seed were similar to those present under single explants of corresponding ages. However, the analysis was complicated by the presence of fibrous material under the base.

It seemed important to determine how this insertion took place, so cultures were examined soon after the second explant was placed on the base. By PC little was seen due to the thickness of the seeded tissue (Fig. 20A), but IR showed that a substantial proportion of the edge of the inserting seed formed a complex IR pattern composed of many interlocking pseudopodia (Fig. 20B). In the confront zone, the area of contact just under the edge of the seed was far greater than that in a corresponding region just

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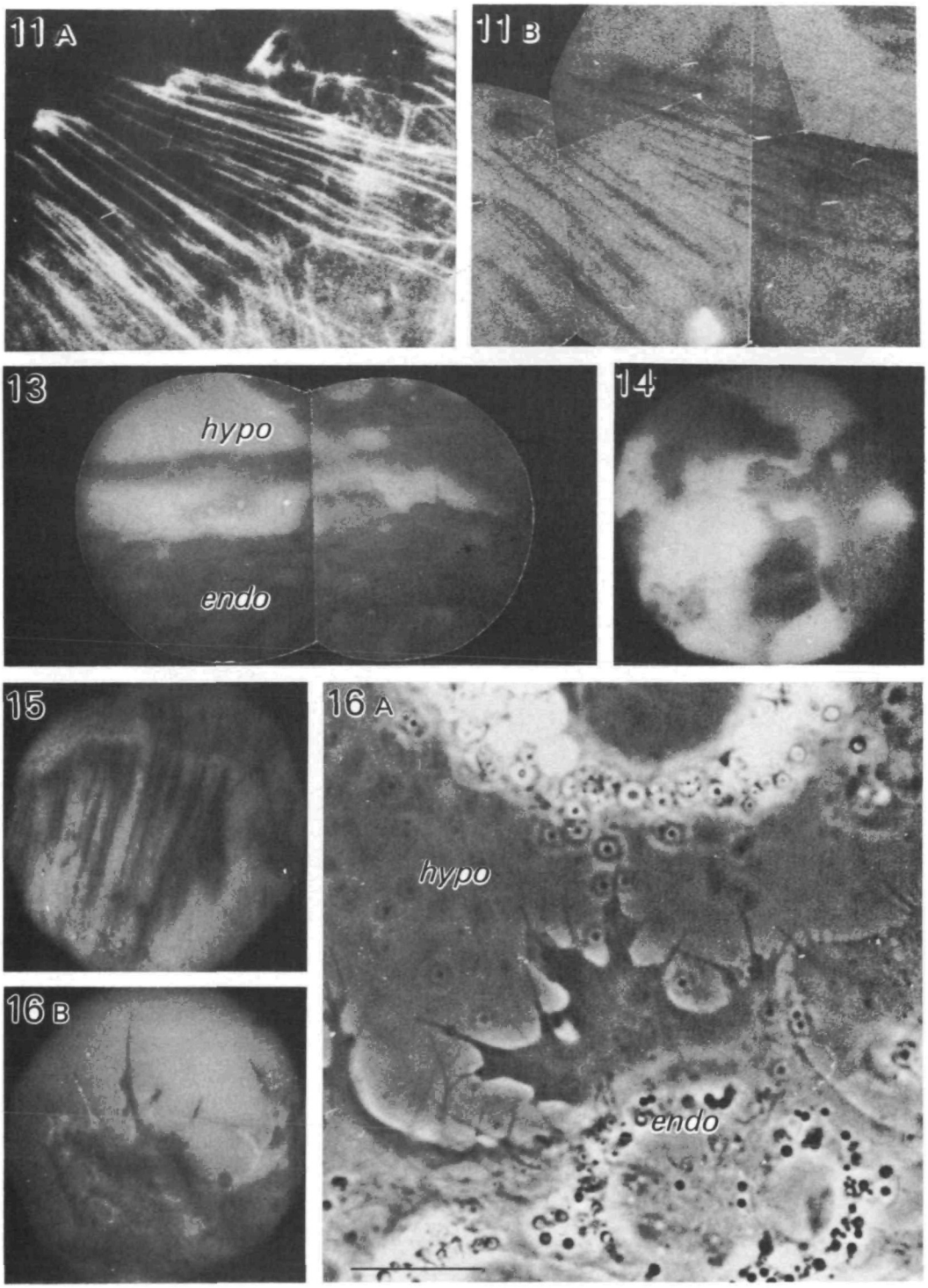
Fig. 11. Area of substrate from which a definitive endoblast explant (24 h) has been removed. A. Anti-fibronectin immunofluorescence. B. Interference reflection image of the same area. Note the close correspondence of the fibres in both images. Bar, 10 μm (Figs. 11, 13–16).

Fig. 13. Interference reflection montage of two cells in zone (*p*) (Fig. 12A). Endoblast cell (*endo*) underlaps hypoblast cell (*hypo*) along the entire length of the figure.

Fig. 14. Interference reflection image of two cells in zone *p* (Fig. 12A). Here a hypoblast cell and an endoblast cell show local mutual underlapping.

Fig. 15. Interference reflection image of two cells in zone *c* (Fig. 12A). A broad lamella of an endoblast cell underlaps the hypoblast explant. This lamella has many focal contacts.

Fig. 16A. Phase-contrast picture of confronted culture between endoblast (*endo*) and hypoblast (*hypo*). B. Interference reflection of part of the same area: a spikey process from an endoblast cell underlaps a hypoblast cell.


11 A
11 B
13
hypo
endo
14
15
16 A
hypo
endo
16 B

under the base. Another interesting feature was the strange IR pattern found under some cells of the base in the confront zone but not elsewhere (Fig. 19). This consisted of small dark grey point contacts, which may represent attachment points left on the substrate by retracting base cells or evidence for active detachment of base cells by invading seed cells, possibly due to lysis.

As a control, hypoblast explants were also grown on glass from which other explants had been removed. On these conditioned substrates spreading explants showed no difference in the types of contacts formed when compared to explants on unconditioned glass.

DISCUSSION

Evaluation of the interference reflection image

There are two aspects of the interpretation of the image that require further discussion. Firstly, the extent to which normal incidence theory can be applied has been questioned by Gingell & Todd (1979) and Gingell (1981). They suggested that cell thickness is important in contributing to the final image if the cytoplasm is < 1 μm, even at high INA. Thus flattened lamellae could appear dark, not because they were close to the substrate but merely due to thickness alone. Secondly, both Couchman & Rees (1979) and Bereiter-Hahn *et al.* (1979) have emphasized the possible contribution of local differences in refractive index within the cytoplasm to the IR image. Thus, they suggested that focal contacts may not necessarily be nearer the substrate than close contacts, and instead the darker image would be due to bundles of microfilaments. In a combined IR and electron microscopic (EM) study, Heath (1982) vertically sectioned fibroblasts examined live by IR before fixation and embedding. His findings agree with Izzard's cell–substrate separation values for black, grey and white parts of the IR image, except at the very distal tip of the leading lamella. Here a black rim seen in IR was due to thin cytoplasm as described by Gingell (1981).

We have confirmed that focal contacts and focal adhesions are in fact adhesions, for when we reduced the ionic strength of the medium, thus partially lifting the cells off the substrate, these same dark regions persisted. In addition, both black and grey areas

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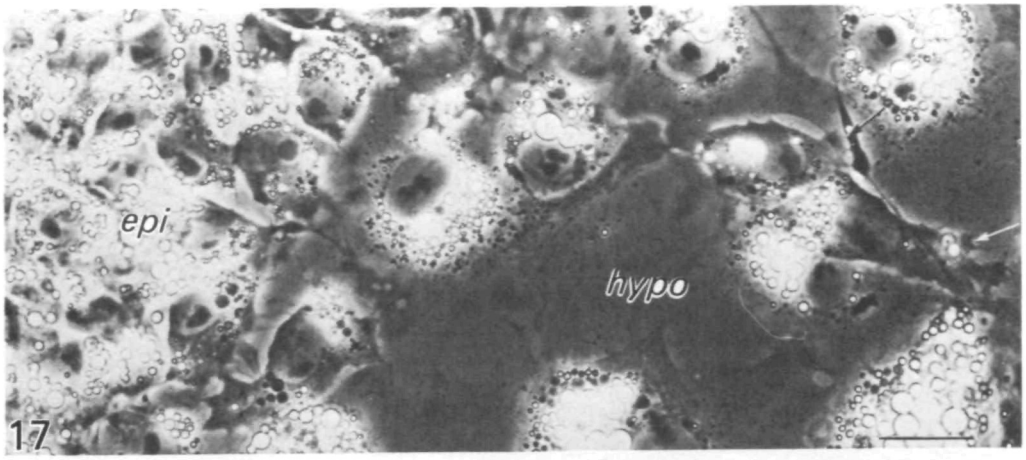
Fig. 17. Low-power phase-contrast picture showing tongues (arrows) of epiblast cells (*epi*) invading hypoblast explant (*hypo*) in a side-to-side confronted culture. Bar, 20 μm.

Fig. 18. A. Phase-contrast picture of barrier region where two definitive endoblast explants are confronted in a side-to-side culture. B. In interference reflection, the cell body of the cell in the centre is off the substrate and is underlapped by small processes from adjoining cells (arrows). Bar, 10 μm.

Fig. 19. Interference reflection image of part of a base cell in the confront zone (*cz*, Fig. 12B) of a vertically confronted culture. Small point contacts are seen as well as retraction filaments. Bar, 10 μm.

Fig. 20. A. Low-power phase-contrast picture of definitive endoblast explant inserting into hypoblast sheet in a vertically confronted culture. The rectangle shows part of the confront zone (*cz*, Fig. 12B) enlarged in the interference reflection montage. Bar, 50 μm. B. A complex zone of substrate contact can be seen at the edge of the inserting tissue. Much less substrate contact is seen under the base. Bar, 10 μm.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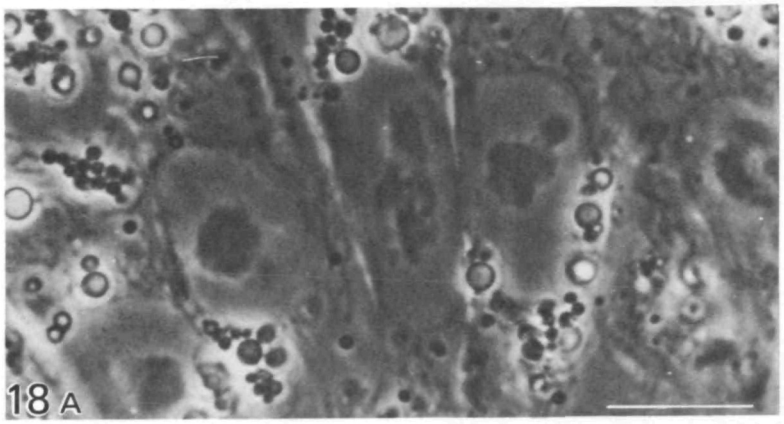
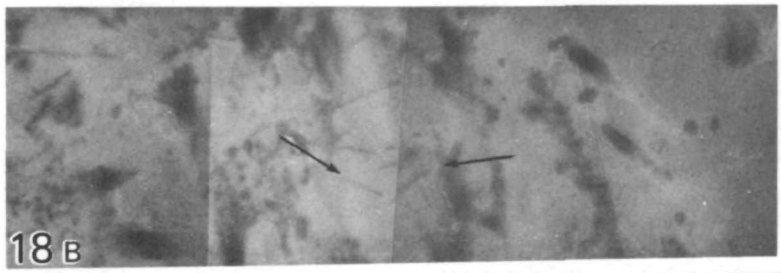
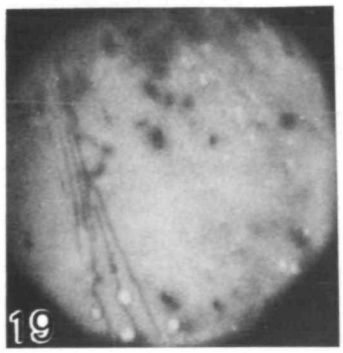
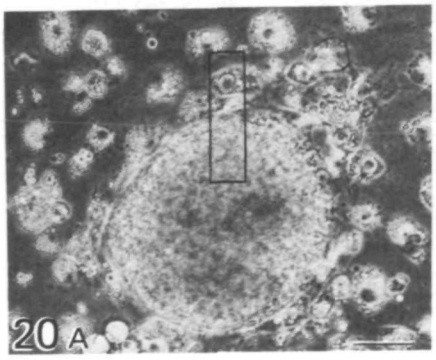
hypo
17


18 A


18 B


19


20 A


20 B

were seen under regions of considerably greater thickness than 1 μm as supported by EM (Al-Nassar & Bellairs, 1982). Intracellular organelles, however (particularly those near the ventral cell surface), do contribute to the image as can be seen by their appearance in our IR images. Some doubts must still remain as to the closeness to the substrate of thin processes of a high radius of curvature (e.g. filopodia and retraction filaments), since no theory exists to predict their behaviour, although microdissection often demonstrates that they are attached. For the reasons stated above we are confident that most of the dark regions seen in IR are adhesions.

Epithelial sheets

Epithelial sheets are often thought to be in contact with the substrate only through attachments made by marginal cells (Vaughan & Trinkaus, 1966), although attachment of cells just behind the margin has been demonstrated (DiPasquale, 1975). We had previously concurred with this view from observations made when dissecting explants from plastic dishes. However, using IR we have now demonstrated that not only do submarginal cells take part in the initial spreading but we have also established that after 24 h many cells under the central portion of a spread sheet had formed specialized adhesions to the substrate (although not as many as marginal cells). It is possible that some of these cells may be isolated highly motile mesoderm-like cells trapped under the epithelial sheet (Ireland, unpublished) but most seem to be part of the sheet. By contrast, Radice (1980), using *Xenopus* tadpole tail epithelium, and Heath (1982), using chick corneal epithelium, both found close contacts beneath submarginal cells, but few focal contacts.

Temporal changes of adhesions

The spreading behaviour of dissociated hypoblast cells is like that described for mouse fibroblasts (Vasiliev & Gelfand, 1977), but the initial substrate contact resembles fixed red blood cells settling on a hexadecane/saline interface (Gingell & Vince, 1980) and therefore may be a passive process.

During culture of explants, similar temporal changes in the types of cell–substrate contact were found with hypoblast, definitive endoblast and epiblast. The tendency was for large broad areas of attachment (close contacts), prominent during attachment and early spreading, to be replaced by numerous small areas (focal contacts) and later by larger but fewer contacts (focal adhesions). The timing of these changes, however, was different for each tissue. Thus, particularly after 24 h in culture, the tissue types could be identified by their IR patterns. Accompanying the development of specialized contact areas was a dramatic increase in stress fibres within cells, the termini of which were associated with the specialized contacts. Similar progressive changes have been observed in primary fibroblast explants (Couchman & Rees, 1979) and primary mouse kidney epithelial cells (Cottler-Fox *et al.* 1979). In the former this was interpreted as a change from an initial migratory phase to a later growth phase with reduced motility, but in the latter as irreversible differentiation. We have observed the reduction in motility but not the increased growth, but consider that such a morphological change

should not be called 'differentiation' without considerably more evidence as to what is taking place.

In low levels of serum the formation of specialized contacts in mouse kidney epithelium was delayed (Cottler-Fox *et al.* 1979), whereas we found the opposite results with chick cells. To us this suggests that if a component necessary for motility is present in serum at low levels, the decrease in availability during culture in 10% serum could explain the observed decreased motility and formation of specialized adhesions.

Couchman & Rees (1979) also observed an increase in fibronectin in parallel with immobilization. We also have evidence from IR of a build-up of substrate-attached material in the form of a fibrous mat beneath explants. We and others (Sanders, 1980) have detected fibronectin beneath explants of chick endoderm. Extracellular material has also been detected beneath and between these cells by Ruthenium Red staining and electron microscopy (Al-Nassar & Bellairs, 1982). Others (Hynes & Bye, 1974) have suggested that fibronectin production is related to cell density but with our explants cell number increases by only 25% in the first four days in culture (Voon, 1980).

Confronted explants

We had previously suggested that underlapping of the invaded tissue by the invading explant was a feature of invasion in confronted cultures grown on plastic substrates (Bellairs *et al.* 1981), based purely on our impression from phase-contrast microscopy. We can now confirm this from our IR studies of this tissue in both kinds of confrontation. Electron microscopic observations of confrontations between epiblast and hypoblast have also shown that the invading tissue (epiblast) underlies the invaded explants and is indeed closer to the plastic substrate (Al-Nassar & Bellairs, 1982). In side-to-side confrontations between young explants, where tissue-specific differences in behaviour have been demonstrated (Sanders *et al.* 1978; Ireland *et al.* 1979), our present data show a correlation between invasive ability and speed of formation of specialized substrate contacts during culture.

This is contrary to a reported correlation between failure to form focal contacts and invasiveness in chemically induced mouse carcinomas (Cottler-Fox *et al.* 1980). Underlapping has however been considered to be an important factor in explaining the differences in behaviour between 3T3 and polyoma-transformed 3T3 cells where invasion was thought to be due to exploitation of available non-cellular substrate (Bell, 1977).

As well as cell–substrate adhesion, another factor influencing invasion is probably cell–cell adhesion. Some idea of this can be deduced from the specialized cell–cell adhesions present in the different tissues. Recently, Stolinski, Sanders, Bellairs & Martin (1981) and Sanders & Prasad (1981) have shown differences in the types of junctions found between early chick tissues in culture. After 24 h desmosomes were found in definitive endoblast cells but not hypoblast cells. However, during prolonged culture of hypoblast cells a different junction consisting of colinear filament bundles developed between hypoblast cells similar to those described in pigmented retinal epithelial cells (Middleton & Pegrum, 1976).

Invasion assays routinely use side-to-side confrontations. In this study we have used both side-to-side and vertical confrontations with different results. In the latter the eventual insertion of the seeded explants is in general independent of tissue type (Bellairs *et al.* 1981) and we need to consider why it is more likely to be successful. There are a number of possibilities. The first is that from IR data we know that the marginal region of the sheet has a greater number of specialized adhesions, so if there is active breakdown of the adhesions by invading cells it will be more difficult there than at the centre of the explant. Focal contacts were not broken during underlapping of one fibroblast by another (Abercrombie & Dunn, 1975) or when neutrophil leucocytes moved under fibroblasts (Armstrong & Lackie, 1975). In our vertical confrontations the possession of focal adhesions does not prevent invasion of the base tissue, but it is probably the greater space available under this tissue, seen clearly with IR, which is exploited by the invading cells first.

The second possibility is the presence of a different substrate under the seeded tissue, i.e. that conditioned by the base sheet. Conditioned substrate has been shown to enhance the settling and spreading of mesoderm tissue (Sanders, 1980). Even though the presence of fibronectin has been demonstrated here, conditioned substrate does not seem to be a factor, since explants spread similarly on glass or on conditioned glass.

A third reason is that the behaviour of confronting cells is likely to be different at the edge of an explant, where active lamellae will meet, than in the centre of a sheet where the cells in the invaded tissue will be much less active. However, once penetration of the sheet occurs, cells with a free edge will arise in the centre of the sheet. Even then their contact behaviour may be important since contact inhibition might lead to an active retraction of the base sheet. Alternatively, they may find it more difficult to compete with the invading tissue, being on a concave edge and constrained by tension in the sheet.

Tension in the sheet may also help to explain the exact process of initial attachment of the top tissue to the substrate under the base. Holes develop in base sheets even without seeds, probably due to the tension generated by the expanding edge cells (Bellairs *et al.* 1981), and seeds may exploit these transient holes. Alternatively, they may make a hole actively by pushing in or by secretion of proteases, and this possibility is under investigation. The peculiar point contacts seen in the base cells in the confront zone of vertical confrontations may be a result of this.

The situation in vertical confrontations probably mimics that in the embryo more closely, as cells *in situ* are not normally as flattened as those at the edge of explants *in vitro*. Our studies using endoderm invasion models show that the precise nature of the tissues confronted both *in vitro* and in the embryo is not as important as the geometry of the arrangement in determining whether or not invasion will be successful.

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REFERENCES

ABERCROMBIE, M. (1975). The contact behaviour of invading cells. In *Cellular Membranes and Tumour Behaviour*, pp. 22–37. Baltimore: Williams & Wilkins.

ABERCROMBIE, M. & DUNN, G. A. (1975). Adhesions of fibroblasts to substratum during contact inhibition observed by interference reflexion microscopy. *Expl Cell Res.* **92**, 57–62.

AL-NASSAR, N. A. E. & BELLAIRS, R. (1982). An electron microscopical analysis of embryonic chick tissues explanted in culture. *Cell Tiss Res.* **225**, 415–426.

ARMSTRONG, P. B. & LACKIE, J. M. (1975). Studies on intercellular invasion using rabbit peritoneal neutrophil granulocytes (PMNS). I. Role of contact inhibition of locomotion. *J. Cell Biol.* **65**, 439–462.

BELL, P. B. (1977). Locomotory behaviour, contact inhibition and pattern formation of 3T3 and polyoma virus-transformed 3T3 cells in culture. *J. Cell Biol.* **74**, 963–982.

BELLAIRS, R., IRELAND, G. W., SANDERS, E. J. & STERN, C. D. (1981). The behaviour of embryonic chick and quail tissues in culture. *J. Embryol. exp. Morph.* **61**, 15–33.

BEREITER-HAHN, J., FOX, C. H. & THORELL, B. (1979). Quantitative reflection contrast microscopy of living cells. *J. Cell Biol.* **82**, 767–779.

COTTLER-FOX, M., RYD, W., HAGMAR, B. & FOX, C. H. (1980). Adhesion of metastatic and non-metastatic carcinoma cells to glass surfaces. *Int. J. Cancer* **26**, 689–694.

COTTLER-FOX, M., SPARRING, K. M., ZETTERBERG, A. & FOX, C. (1979). The process of epithelial cell attachment to glass surfaces studied by reflexion contrast microscopy. *Expl Cell Res.* **118**, 414–418.

COUCHMAN, J. R. & REES, D. A. (1979). The behaviour of fibroblasts migrating from chick heart explants: changes in adhesion, locomotion and growth, and distribution of actomyosin and fibronectin. *J. Cell Sci.* **39**, 149–165.

CURTIS, A. S. G. (1964). The mechanism of adhesion of cells to glass. A study by interference reflection microscopy. *J. Cell Biol.* **20**, 199–215.

DIPASQUALE, A. (1975). Locomotory activity of epithelial cells in culture. *Expl Cell Res.* **94**, 191–215.

EYAL-GILADI, H. & KOCHAV, S. (1976). From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology. *Devl Biol.* **49**, 321–337.

FONTAINE, J. & LEDOUARIN, N. M. (1977). Analysis of endoderm formation in the avian blastoderm by use of quail-chick chimaeras. *J. Embryol. exp. Morph.* **41**, 209–222.

GINGELL, D. (1981). The interpretation of interference reflection images of spread cells: significant contributions from the peripheral cytoplasm. *J. Cell Sci.* **49**, 237–247.

GINGELL, D. & TODD, I. (1979). Interference reflection microscopy. A quantitative theory for image interpretation and its application to cell–substratum measurement. *Biophys. J.* **26**, 507–526.

GINGELL, D. & VINCE, S. (1980). Long-range forces and adhesion: an analysis of cell–substratum studies. In *Cell Adhesion and Motility* (ed. A. S. G. Curtis & J. D. Pitts), pp. 1–37. Cambridge University Press.

GINGELL, D. & VINCE, S. (1982). Cell–substrate separation depends on salt concentration and valency: measurement on *Dictyostelium* amoebae by finite aperture interferometry. *J. Cell Sci.* **54**, 299–310.

HAEMMERLI, G. & PLOEM, J. S. (1979). Adhesion patterns of cell interactions revealed by reflection contrast microscopy. *Expl Cell Res.* **118**, 438–442.

HAEMMERLI, G. & STRAULI, P. (1981). *In vitro* motility of cells from human epidermoid carcinomas. A study by phase-contrast and reflection-contrast microscopy. *Int. J. Cancer* **27**, 603–610.

HAMBURGER, V. & HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

HEATH, J. P. (1982). Adhesion to substratum and locomotory behaviour of fibroblastic and

epithelial cells in culture. In *Cell Behaviour: a Tribute to Michael Abercrombie* (ed. R. Bellairs, A. Curtis & G. A. Dunn). Cambridge University Press.

HEATH, J. P. & DUNN, G. A. (1978). Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflection and high voltage electron microscope study. *J. Cell Sci.* **29**, 197–212.

HYNES, R. O. & BYE, J. M. (1974). Density and cell cycle dependence of cell surface proteins in hamster fibroblasts. *Cell* **3**, 113–120.

IRELAND, G. W., BRETLAND, N. P., ROE, M. & BELLAIRS, R. (1979). *In vitro* studies on the endoderm of the young chick embryo. *J. Anat.* **128**, 425.

IRELAND, G. W. & VOON, F. C. T. (1981). Polygonal networks in living chick embryonic cells. *J. Cell Sci.* **52**, 55–71.

IZZARD, C. S. & LOCHNER, L. R. (1976). Cell-to-substrate contacts in living fibroblasts: an interference reflection study with an evaluation of the technique. *J. Cell Sci.* **21**, 129–159.

IZZARD, C. S. & LOCHNER, L. R. (1980). Formation of cell-to-substrate contacts during fibroblast motility: an interference reflection study. *J. Cell Sci.* **42**, 81–116.

KELLER, H. U., BARANDUN, S., KISTLER, P. & PLOEM, J. S. (1979). Locomotion and adhesion of neutrophil granulocytes. Effects of albumin, fibrinogen and gamma globulins studied by reflection contrast microscopy. *Expl Cell Res.* **122**, 351–362.

KING, C. A., HEAYSMAN, J. E. M. & PRESTON, T. M. (1979). Experimental evidence for the role of long-range forces in fibroblast–surface interaction. *Expl Cell Res.* **119**, 406–410.

KING, C. A., PRESTON, T. M., MILLER, R. H. & DONOVAN, P. (1980). Cell–substrate interaction during amoeboid locomotion of neutrophil leukocytes. *Expl Cell Res.* **126**, 453–458.

LETOURNEAU, P. C. (1979). Cell–substratum adhesion of neurite growth cones and its role in neurite elongation. *Expl Cell Res.* **124**, 127–138.

MIDDLETON, C. A. & PEGRUM, S. M. (1976). Contacts between pigmented retina epithelial cells in culture. *J. Cell Sci.* **22**, 371–383.

MODAK, S. P. (1965). Sur l'origine de l'hypoblaste chez les oiseaux. *Revue suisse Zool.* **73**, 877–910.

NICOLET, G. (1971). Avian gastrulation. *Adv. Morphogen.* **9**, 231–262.

PRESTON, T. M. & KING, C. A. (1978). Cell–substrate associations during the amoeboid locomotion of *Naegleria*. *J. gen. Microbiol.* **104**, 347–351.

RADICE, G. P. (1980). Locomotion and cell–substrate contacts of *Xenopus* epidermal cells *in vitro* and *in situ*. *J. Cell Sci.* **44**, 201–223.

ROSE, G. (1954). A separable and multi-purpose tissue culture chamber. *Tex. Rep. Biol. Med.* **12**, 1074.

ROSENQUIST, G. C. (1972). Endoderm movements in the chick embryo between the early short streak and head process stage. *J. exp. Zool.* **180**, 95–104.

SANDERS, E. J. (1980). The effect of fibronectin and substratum attached material on the spreading of chick embryo mesoderm cells *in vitro*. *J. Cell Sci.* **44**, 225–242.

SANDERS, E. J., BELLAIRS, R. & PORTCH, P. A. (1978). *In vivo* and *in vitro* studies on the hypoblast and definitive endoblast of avian embryos. *J. Embryol. exp. Morph.* **46**, 187–205.

SANDERS, E. J. & PRASAD, S. (1981) Contact inhibition of locomotion and the structure of homotypic and heterotypic intercellular contacts in embryonic epithelial cells. *Expl Cell Res.* **135**, 93–102.

STEPHENSON, E. M. (1982). Locomotory invasion by tumour cells in explant confrontations. In *Cell Behaviour: a Tribute to Michael Abercrombie* (ed. R. Bellairs, A. Curtis & G. A. Dunn), pp. 499–527. Cambridge University Press.

STERN, C. D. & IRELAND, G. W. (1981). An integrated experimental study of endoderm formation in avian embryos. *Anat. Embryol.* **163**, 245–263.

STOLINSKI, C., SANDERS, E. J., BELLAIRS, R. & MARTIN, B. (1981). Cell junctions in explanted tissues from early chick embryos examined by freeze fracture. *Cell Tiss. Res.* **221**, 395–404.

THOM, D., POWELL, A. J. & REES, D. A. (1979). Mechanisms of cellular adhesion. IV. Role of serum glycoproteins in fibroblast spreading on glass. *J. Cell Sci.* **35**, 281–306.

VAKAET, L. (1970). Cinematographic investigations of gastrulation in the chick blastoderm. *Archs Biol., Liège* **81**, 387–426.

VASILIEV, J. M. & GELFAND, I. M. (1977). Mechanisms of morphogenesis in cell cultures. *Int. Rev. Cytol.* **50**, 159–274.

VAUGHAN, R. B. & TRINKAUS, J. P. (1966). Movements of epithelial cell sheets *in vitro*. *J. Cell Sci.* **1**, 407–413.

VOON, F. C. T. (1980). The morphology of early chick embryonic tissues *in vitro*. Ph.D. thesis, University of London.

WEHLAND, J., OSBORN, M. & WEBER, K. (1979). Cell-to-cell substratum contacts in living cells: a direct correlation between interference reflection and indirect-immunofluorescence microscopy using antibodies against actin and α-actinin. *J. Cell Sci.* **37**, 257–273.

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