CELL MOVEMENT AND THE MECHANISM OF INVASIVENESS: A SURVEY OF THE BEHAVIOUR OF SOME NORMAL AND MALIGNANT CELLS IMPLANTED INTO THE DEVELOPING CHICK WING BUD

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SUMMARY
A survey of the behaviour of a variety of normal and malignant tumours and cells has been carried out to gain insights into the mechanisms of tumour invasiveness. The tumours and cells were implanted into the developing chick wing bud, which is a loose mesenchyme bounded by ectoderm. The distribution of the grafted cells was examined histologically after one or two days. The special feature of this assay is that the behaviour of cells is tested in a 3-dimensional tissue. Cells from 3 different carcinomas, mouse lung tumour, rat bladder tumour and human breast tumour did not invade the mesenchyme, whereas trophoblast, sarcoma 180, cultured hamster fibroblasts (BHK, PyBHK, Nil 8, HSV Nil 8) and neuroblastoma cells did. Cells from embryonic pigmented retina and heart ventricle were non-invasive. These results suggest that cell movement may not be a common feature of all invasive tumours.

The cells that did move into the mesenchyme appeared to do so by various mechanisms. Lack of contact inhibition of movement, although probably involved in the invasiveness of sarcoma 180 cells, does not appear to be necessary for invasion: cells that have been shown to exhibit contact inhibition of movement (BHK and PyBHK) also invade. Both normal and transformed cells (BHK and PyBHK; Nil 8 and HSV Nil 8) moved into the mesenchyme. Other invading cells, such as trophoblast, neuroblastoma and to a small extent, HSV Nil 8 cells, destroy the adjacent host tissue and this may be important in the invasiveness of these cells.

The patterns of invasion and interactions with the host tissue were varied. Trophoblast and the fibroblasts were often elongated along the basement membrane at the ectoderm/mesenchyme border and also closely apposed to the endothelial linings of blood vessels. Sarcoma 180 and neuroblastoma cells clustered around nerves. The embryonic tissues and neuroblastoma cells were often associated with blood vessels. These results are discussed in relation to tumour invasion.

A striking finding was that the carcinoma cells were frequently found positioned within the wing ectoderm on the basement membrane. This affinity of carcinoma cells for the epithelium rather than the mesenchyme leads to a reappraisal of the mechanisms involved in the invasiveness of carcinomas.

INTRODUCTION
The characteristics that distinguish malignant from benign tumours are that the malignant cells invade the surrounding tissue locally and metastasize: that is, spread to establish secondary tumours away from the site of the original tumour. These aspects of malignancy have surprisingly received scant attention compared to others.
such as the growth of tumours. The reasons why a cell becomes invasive and the mechanism of invasion, itself, are poorly understood. A pervading view is that invasiveness involves changes in the cell surface and cell motility: a loss of adhesion with neighbouring cells and active cell movement into the surrounding tissue.

There have been many attempts to try and pinpoint the special feature of tumour cell behaviour that leads to invasion. Some workers (Coman, 1944) have stressed the reduced adhesiveness of tumour cells to each other as the key change, while others (Loewenstein & Kanno, 1966) have drawn attention to the lack of communication between some tumour cells. It has been assumed that these could be primary events which are then followed by active migration of the tumour cells.

The view that tumour cells invade by active cell movement is widely held (Trinkaus, 1976; Strauli & Weiss, 1977). The appearance in tumour cells of microfilaments, believed to be necessary for cell locomotion, has supported this view (Franks, Riddle & Seal, 1969; Malech & Lentz, 1974). However, it is clear that even though many normal cells possess the intrinsic ability to undergo locomotion they remain in place and do not move around. Some malignant and transformed cells have been shown to secrete proteolytic enzymes (Reich, 1973) and the suggestion has been made that tumour cells could break down the surrounding tissue in this way. This would create free space into which the tumour cells could move. In another vein, it has been suggested that cancer cells invade because they are more adhesive to the surrounding cells than to each other. An elegant test of this hypothesis for the behaviour of neutrophil granulocytes has been made by Lackie & Armstrong (1975). Their results do not support such an explanation for the invasiveness of these cells. However, the major concept in the study of invasiveness has been that of contact inhibition of movement.

Contact inhibition of movement was originally described by Abercrombie & Heaysman (1954). They found that normal chick heart fibroblasts did not overlap in culture but tended to form a monolayer. They interpreted this as being due to the inability of normal cells to move over other normal cells. It was later shown that when the leading lamella of a normal cell came into contact with another cell its ruffling activity was paralysed locally where the 2 cells were in contact (Abercrombie & Ambrose, 1958; Trinkaus, Betchaku & Krulikowski, 1971). This has been termed contact paralysis (Gustafson & Wolpert, 1967). In contrast, S180 tumour cells appeared to be able to continue moving when in contact with normal cells (Abercrombie, Heaysman & Karthaus, 1957). This type of behaviour could lead to invasion of surrounding tissue by tumour cells. The finding that normal cells inhibit the locomotion of other normal cells has been put forward to explain why cells generally remain in place and do not invade.

The morphology of a dense cell culture has often come to be used as a criterion for the behaviour of the individual cells. If the culture forms a monolayer, the cells are assumed to show contact inhibition, whereas if the culture has a criss-crossed appearance, the individual cells are assumed not to be contact-inhibited in their movement (Sanford, 1967). Many, though not all, transformed cell cultures have a criss-crossed appearance.
Although the concept of contact inhibition has been seminal in directing work on invasiveness of tumours, what it describes is clearly not a simple phenomenon. The behaviour of a cell will be the upshot of a complex interaction between, for example, cell adhesiveness and local contractile forces, cell shape and contact paralysis (Gustafson & Wolpert, 1967; Steinberg, 1970). In addition, contact inhibition has been studied under conditions which differ in some important respects from those in vivo. The most obvious of these is that the cells move on a 2-dimensional artificial substratum provided by the culture vessel. Martz, Phillips & Steinberg (1974) have argued that many of the features of cell cultures may be understood in terms of differences in adhesiveness between cell-to-cell contact and cell-to-substratum contacts. It now seems that the overlapping of cells observed in culture is due to the ability of cells to move under each other (Bell, 1972, 1977; Erickson, 1976). In addition, it has been shown that the upper surfaces of both fibroblasts and epithelial cell sheets do not allow spreading of cells which are dropped on to them (Di Pasquale & Bell, 1974; Elsdale & Bard, 1974). These kinds of arguments tend to suggest that the gross morphology of the culture is the result of the morphology of the adhesions of individual cells to the 2-dimensional substratum of the culture dish, rather than the result of the contact behaviour of the cells. In fact, observations of some virally transformed cells show, contrary to expectation from the morphology of the cultures, that these cells are contact-inhibited in their movement (Bell, 1972; Guelstein et al. 1973; Erickson, 1976). It has been argued that the criss-crossed appearance of cultures of these cells reflects the amount of space available for underlapping (Bell, 1977).

The question of whether contact inhibition of movement occurs in 3-dimensional tissues has been little considered. It is clear that if it does not, then the phenomenon does not have any significance in vivo. Weston & Abercrombie (1967) showed that if 2 fragments of embryonic tissue were fused in culture there was no interpenetration of cells at the border, regardless of whether the cells were of the same type or of different types (embryonic chick heart and liver). It has been claimed, however (Armstrong & Armstrong, 1973), that cells from fused fragments of mesonephros do show intermingling. Armstrong & Lackie (1975) have recently investigated the hypothesis that contact inhibition is related to the invasiveness of granulocytes. They found that granulocytes do not exhibit contact inhibition with respect to embryonic fibroblasts and invade aggregates of such tissues in vitro. Wiseman & Steinberg (1973) and Gershman & Drumm (1975) have similarly looked at the ability of cells to invade aggregates and found that embryonic cells and cell lines move a small distance into aggregates. The interpretation of these results have been thrown into question by the finding that glass beads and other inert particles apparently invade aggregates (Wiseman, 1977) to the same extent as cells. Wolff & Schneider (1957) placed fragments of sarcoma 180 tumour in contact with various chick embryonic tissues in culture. It appeared that the sarcoma cells penetrated the embryonic tissues, particularly the mesonephros. In such cases it is not clear whether one cell type is invading or whether there is mutual intermingling and careful analysis is required on this point. A model for such analysis is that of Abercrombie & Heaysman (1976) on what happens in the zone where cells moving out from 2 explants interpenetrate.
Our primary interest in invasiveness is whether all malignant cells show invasiveness by cell movement and what characteristics are common to them. For this we required an assay which closely resembles the conditions in vivo. Our studies on the morphogenesis of the chick limb bud had suggested that this might be a useful site for implanting cells to test their invasiveness. The chick wing at early stages of development is a loose meshwork of mesenchyme cells bounded by a 2-layered ectoderm (Gould, Day & Wolpert, 1972; Searls, Hilfer & Mirow, 1972; Ede, Bellairs & Bancroft, 1974). There is ample space for cells to move into the mesenchyme and during normal development nerves and blood vessels do so. We have found this site to be an excellent in vivo culture chamber for a wide variety of tissues, since there is no immune response and it is well vascularized. Other recent studies (Easty & Easty, 1974; Scher, Haudenschild & Klagsbrun, 1976) have used the chorioallantoic membrane (CAM) of the chick embryo as a site to examine the invasiveness and growth of tumour cells.

Our aim has been to survey the behaviour of a range of different cells when implanted into the chick limb, rather than to concentrate on the behaviour of just one or two types. We were aware that fibroblast-like cells have been widely studied, whereas most common human tumours are of epithelial cells (carcinomas). We therefore were careful to include some carcinoma cells in our survey. Serving as a base-line for our experiments on invasiveness are our observations on what happens when a number of different normal embryonic tissues, such as mesonephros and liver, are implanted into the limb. Cells from these tissues do not invade the limb but stay in a compact group (Tickle & Goodman, in preparation).

We have chosen to examine the behaviour of the following cells in the chick limb, (a) Mouse trophoblast. This tissue is known to be highly invasive both in normal development during formation of the placenta and in ectopic sites in the mouse (Kirby & Cowell, 1968). It has recently been shown that trophoblast produces a proteolytic enzyme (Strickland, Reich & Sherman, 1977). (b) Sarcoma 180 mouse tumour and embryonic quail heart. The observations of these cell types in culture showed that embryonic heart fibroblasts were contact-inhibited in their movement, whereas the sarcoma 180 cells were not (Abercrombie et al. 1957). (c) Normal cell lines and transformed derivatives. BHK and PyBHK (Stoker & Macpherson, 1964), Nil 8 and HSV Nil 8 (Critchley, Chandrabose, Graham & Macpherson, 1974) cells were studied. These cell lines, particularly BHK and PyBHK, have been widely used as a model to compare normal and malignant cell behaviour and surfaces. There is a well documented difference in the appearance of mass cultures of BHK and PyBHK cells (Macpherson & Stoker, 1962). However, direct observations of cell collisions at high resolution have shown that both the normal and the transformed cells are contact-inhibited in their movement (Erickson, 1976). There is some evidence that transformed BHK cells may be less adhesive than PyBHK cells (Edwards, Campbell & Williams, 1971). All these cells have a fibroblast-like morphology. (d) Neuroblastoma. This is an established cell line of cells that do not have a fibroblast-like morphology. (e) Carcinomas. We have looked at the behaviour of cells from 3 different kinds of carcinoma. We have tested CMT 64 lung tumour. This mouse tumour is one of the few transplantable tumours that regularly metastasizes (Franks,
Cell movement and mechanism of invasiveness

Carbonell, Hemmings & Riddle, 1976). Two different rat bladder tumours induced by feeding rats with nitrosamines (Hicks & Wakefield, 1976) were investigated. This type of tumour has been studied as a model for chemical carcinogenesis. In addition, we looked at 5 different human breast carcinomas. These were both benign and malignant tumours and were included in our survey as examples of a common human tumour.

(f) Embryonic pigmented retina. This epithelium can be easily separated as a single cell layer from the adjacent neural retina.

METHODS

General

The standard operation was very simple. A hole was cut into the dorsal surface of the right wing bud of a chick embryo and into this the tissues or cells to be tested were grafted. After 1 or 2 days the distribution of the implanted cells was examined histologically. This allows time for invasion by cell movement but minimizes the contribution to invasion from cell division within the graft.

Table 1. Grafts of tissues assayed

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Form of graft</th>
<th>Solid</th>
<th>Pellet of cells</th>
<th>No. of grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse trophoblast</td>
<td>+</td>
<td></td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>Ectoplacental cone from 7-day embryos (day of vaginal plug = day 0)</td>
<td>+</td>
<td></td>
<td></td>
<td>17</td>
</tr>
<tr>
<td>Sarcoma 180</td>
<td>+</td>
<td></td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>Embryonic quail heart</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ventricle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHK</td>
<td>+</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>PyBHK</td>
<td>+</td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Nil 8</td>
<td>+</td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>HSV Nil 8</td>
<td>+</td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>+</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Mouse lung tumour</td>
<td>+</td>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Mouse lung tumour</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Rat bladder tumours</td>
<td>+</td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>Human breast tumours</td>
<td>+</td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Embryonic quail pigmented retina</td>
<td>+</td>
<td></td>
<td></td>
<td>6</td>
</tr>
</tbody>
</table>

Although our assay provides a test of cell behaviour within a 3-dimensional tissue there are certain problems associated with it. The technique, itself, is not easy and requires some practice. In about 10% of cases the grafts were not successful: either the host embryo died or we could not find the graft in the operated wing. In addition, our analysis of the results is complicated by the growth and expansion of the wing during the period when the behaviour of the grafted cells is being tested. We want to point out that some of the separation of the invading cells from the main graft will result from cell division in the intervening mesenchyme.

Preparation of tissues or cells used as implants (see Table 1)

The solid tissues were cut into small cubes with sharpened needles or scalpels. Pigmented retina was peeled off as a sheet of cells with needles. In some cases, the separation of the pigmented retina from adjacent tissues was performed after 30 min incubation in 2% trypsin
at 4 °C. The pieces of tissue used to make grafts were approximately 200 × 200 μm, so as to fit neatly into the holes in the chick wing bud. Some of the solid tissues, however, contain large amounts of matrix material such as collagen. Cutting pieces or breaking off pieces often resulted in grafting just matrix with only the occasional cell. This was a particular problem with the human breast tumours obtained from patients at the Middlesex Hospital. On one occasion, 6 grafts from a malignant tumour were made. On examining the results histologically only one graft was found to consist mostly of cells. We therefore decided to separate the cells from the matrix of these tumours and use these as our implants. We found that if the tumour was teased apart with forceps and needles, many cells were released. These cells were either in small clumps or single. Cells could thus be collected and centrifuged to form a firm pellet. Pieces of this pellet were then grafted into the chick wing bud, and thus every implant consisted of cells. In some cases we were able to ‘pop’ clumps of cells out of connective tissue capsules and graft these. We cannot be sure, however, that all the cells we grafted were tumour cells and that there were not some normal cells included too.

The cell lines, usually just subconfluent, were obtained from many different sources (see acknowledgements). The cells were removed from the glass or plastic vessel in which they were growing by a standard treatment with 0·1 % trypsin (Difco 1:250) and 0·001 M EDTA in calcium- and magnesium-free Hanks’ saline (pH 7·4) at room temperature. The cells were watched under an inverted microscope and when they started to detach from the substratum upon gentle rocking (usually at around 5 min) chicken serum (Gibco-Biocult) was added to give a final concentration of serum at around 10 %. The cells were then washed by centrifugation with tissue culture medium (Basal medium Eagle’s with HEPES buffer (Gibco-Biocult) + 10 % chicken serum) at least twice. The cells were then resuspended in medium and packed into a small firm pellet by centrifuging in small tubes (50 × 6 mm) at approximately 2000 g for 10 min. The pellets were then left at 38 °C for at least 1 h to consolidate. In addition pellets were made which were a mixture of the test cells and embryonic quail wing mesenchyme cells. This was done for 3 reasons. Firstly, the test cells often stuck together very poorly and were therefore difficult to graft. The addition of quail wing cells acted to bind the pellet together. Secondly we have found (Tickle & Goodman, in preparation) that quail wing mesenchyme cells do not invade the chick wing, since we can recognize quail cells by the staining properties of their nuclei (Le Douarin, 1973). Thus we can estimate the invasion of the test cells by comparing their position with that of the implanted quail cells. The quail cells were prepared from the mesenchyme of wings of stage 20–22 (Hamburger & Hamilton stages) quail embryos obtained from our own breeding stock of Japanese quails (Coturnix coturnix Japonica). The wings were cut off the embryos and soaked in 2 % trypsin (Difco 1:250) in calcium- and magnesium-free Hanks’s saline for 1–2 h at 0–4 °C (Szabo, 1955). After this treatment the ectoderm can be readily removed from the mesoderm in medium by gentle manipulation with sharpened tungsten needles. The mesoderms were then washed several times in medium and disaggregated mechanically into a cell suspension by flushing through a narrow-bore Pasteur pipette. The quail cell suspension was then added to the suspension of test cells and thoroughly mixed. The cells were then centrifuged to form a composite pellet. After at least 1 h incubation at 38 °C, the pellets were flushed free from the bottom of the centrifuge tubes by a jet of medium blown out of a Pasteur pipette and removed into a dish containing medium. There the pellets were cut into cubes (200-μm side) to be grafted into the chick wings.

**Preparation of host embryos**

White Leghorn fertilized chicken eggs were incubated at 38 °C for about 3 days. Then they were windowed and staged. The eggs were then reincubated until the embryos were at stages 20–21. These embryos were used as hosts for implants of test tissues and cells. The wing bud at this stage of development consists of a hemispherical bulge about 1·5 mm across and protruding 1 mm. Most embryos lie on their left sides, thus presenting their right wing buds uppermost. A square hole of side approximately 200 μm can be readily cut into the dorsal surface of the right wing bud with sharpened tungsten needles, after tearing away the membranes from over the wing with forceps. Between a third and a half of the depth of the wing mesenchyme was removed, together with the overlying ectoderm.
Grafting procedure

The solid pieces of tissue were carried into the egg on small spatulas made out of beaten nickel chrome wire. Pieces of pellet were often too fragile to pass through a meniscus without breaking up. These were usually transferred into the egg in a Pasteur pipette. The pieces of tissue or pellet were placed into the hole cut into the wing bud. In some cases the graft was kept in place by a platinum wire pin (25 \( \mu \text{m} \) thick); in other cases the graft was not pinned at all. If the graft was extremely fragile it was sometimes bridged over in the limb by a staple made out of platinum wire. After the operation was performed the window in the egg shell was re-covered with sellotape and the egg was reincubated for a further period, usually 1 day or 2 days at 38 °C. The wire pins and staples can usually be removed safely about 4 h after the operation, but this was not always done.

Histology

At known times following grafting, usually at 1 day and at 2 days, the host embryos were removed from the eggs and placed in cold half-strength Karnovsky's fixative (Karnovsky, 1965). The right wings were cut off and fixed in half-strength Karnovsky's fixative overnight at 4 °C. The wings were dehydrated through a graded series of ethanols, and stained during the process with 0.5 % Alcian green in 70 % ethanol for 1 h so that they could easily be seen. The wings were cleared in propylene oxide and then embedded in Araldite. (At the same time a series of grafted wings was prepared for electron microscopy. The results will be presented elsewhere.) One- to two-micrometre sections were cut either serially and arranged in groups of 4 sections or there was an interval of 30 \( \mu \text{m} \) between groups of 16 sections. These were stained with 1 % aqueous toluidine blue. When sections of grafts containing quail cells were cut, the sections were placed in parallel on 2 different sets of slides. One set of slides were subbed with gelatine (Rogers, 1967). The sections on these slides were treated with a modified Feulgen technique so that the quail cells could be distinguished from the chick cells. The other sets of sections were stained with toluidine blue in the usual way. In each experiment pieces of tissue and pellet similar to those used as grafts were fixed, embedded and sectioned in the same way as the wings bearing grafts to check the histology of the grafted cells. The outcome of over a hundred grafts was examined (see Table 1 for list of grafts).

RESULTS

General results

The grafts tended to end up in the proximal part of the wing, which will give rise to the humerus. After 1 day the wing tissue was only just beginning to differentiate. After 2 days, however, there was cartilage with matrix, rimmed with perichondrium and well defined muscle masses as well as loose connective tissue. Nerves had also invaded the developing limb.

We found that we could readily recognize the graft cells histologically in all cases. Often the grafted cells were much larger and had different staining properties from the host wing cells. In all cases, except where stated to the contrary, many mitoses were seen among the graft cells. However, we did notice differences in the ability of cells to survive in the limb.

Our criterion for invasiveness was the presence of single cells or small groups of cells away from the graft. Refer to Table 2 for a summary of the behaviour of the cells that were tested.
Trophoblast

The cells in the pieces of ectoplacental cone used for grafting appeared rather heterogeneous. Some of our grafts almost certainly contained proximal endoderm cells.

Table 2. Summary of behaviour of grafted cells

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Invasion into mesenchyme</th>
<th>Invasion into ectoderm</th>
<th>Damage to host</th>
<th>Aligned along basement membrane at ectoderm mesenchyme interface</th>
<th>Other special features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trophoblast</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Around blood vessels</td>
</tr>
<tr>
<td>Sarcoma 180</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Around nerves</td>
</tr>
<tr>
<td>Quail heart</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Associated with blood vessels</td>
</tr>
<tr>
<td>BHK</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Around blood vessels</td>
</tr>
<tr>
<td>PyBHK</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Around blood vessels</td>
</tr>
<tr>
<td>Nil 8</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Around blood vessels</td>
</tr>
<tr>
<td>HSV Nil 8</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>Around blood vessels</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>Around nerves associated with blood vessels</td>
</tr>
<tr>
<td>Mouse lung tumour</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Rat bladder tumour</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Human breast tumour</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pigmented retina</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Associated with blood vessels</td>
</tr>
</tbody>
</table>

* 2 out of 10 grafts.

In 15 out of 18 grafts several individual cells were found away from the graft in the chick mesenchyme (Fig. 1). In the other 3 grafts one cell, only, in each case was found away from the graft. It seems that these particular grafts contained a high proportion of cells which would not normally be invasive, such as proximal endoderm cells and vacuolated 'frothy' giant cells. However, in these grafts and in others, tongues of trophoblast cells were seen fanning out into the limb while still retaining contact with the main graft.

The pattern of invasion of the implanted trophoblast cells had several interesting features. Trophoblast cells were often (10 cases out of 18) found elongated along the interface between the ectoderm and the mesenchyme. Sometimes a string of trophoblast cells were seen streaking away from the graft under the ectoderm. It was interesting that in 2 cases trophoblast cells were found lying under the apical ectodermal ridge, which controls limb outgrowth (Saunders, 1972) and the limb had apparently continued to develop normally. The invading cells most frequently were aligned,
when this could be determined, parallel to the long axis of the limb. In many cases the trophoblast cells under the ectoderm were extremely attenuated and did not appear to affect the usual relationship of the ectoderm and the mesenchyme. In other instances, however, the ectoderm was buckled away from the mesenchyme as though 'uncoupled' and there was a space in which the trophoblast cell was lying (Fig. 2). The mesenchyme cells around the lower edge of the space seemed to be drawn together as if with a purse-string. In one case we noticed that the basal surfaces of the ectoderm cells appeared to have blebs on them (Fig. 2). We have investigated the

intimate relationship between the trophoblast cells and the basal lamina of the ectoderm in thin sections in the electron microscope and will present the results in another paper.

Trophoblast cells were often found near blood vessels. In some cases the trophoblast cells appeared to border directly on to a blood vessel and make up part of the vessel wall together with the endothelial cells. There was no indication that trophoblast cells tended to 'home in' on blood vessels. There were several instances where
invading trophoblast cells were away from the graft with blood vessels in the intervening mesenchyme.

There was frequently erosion of the chick tissue associated with the trophoblast cells. We have already mentioned the space around trophoblast cells under the ectoderm and places where endothelial cells have been destroyed or usurped. Also trophoblast cells often lined a crater within the limb (Fig. 3). It is possible that similar widespread erosion by trophoblast cells could be responsible for the huge blood blisters in some of the host wings. In 2 experiments where a piece of trophoblast had been pinned to the outside of the limb rather than implanted into a hole cut into the dorsal surface, the limb was denuded of ectoderm over a large area in the region of the graft. This suggests that the ectoderm was either eroded away or perhaps could not heal. In several cases a group of trophoblast cells came to lie along the edge of a cartilage element (Fig. 3). The cartilage itself was not invaded or broken down and there was a firm edge to the cartilage matrix where it abutted trophoblast cells. There was no perichondrium present in this region (Fig. 3).

Sarcoma 180 tumour

The sarcoma cells were much larger than the chick mesenchyme cells and were readily recognized. The cells both at the graft and away in the mesenchyme appeared rounded in section. Sometimes the invading cells were seen to have a blunt protrusion at one end. It is interesting that the morphology of the sarcoma cells in the limb closely resembles the rounded appearance of these cells in culture (Abercrombie et al. 1957).

The cytoplasm of many sarcoma cells was vacuolated but the significance of this is not clear. These vacuoles could either result from pinocytosis or be a sign that the cells were secreting. Yet another possibility is that these vacuoles represent the first stages of degeneration, as they do in fibroblasts in an 'old' tissue culture.

In 12 out of 17 grafts cells were found to have invaded the chick mesenchyme from a main tumour graft (Figs. 4, 5). In the 5 other cases a few sarcoma cells only were found dotted around in the limb as if the graft had fallen out.

A series of grafts was also done to look at the time course of invasion. The host embryos were reincubated for varying lengths of time from 3 h up to 9 h after the

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Fig. 4. Sarcoma 180 graft left for 1 day. Several cells (arrowed) invading the mesenchyme. Note dividing cell within main graft. Scale bar 50 μm.

Fig. 5. Sarcoma 180 graft left for 2 days. Invading cells (arrowed) in cartilage and soft tissue. Note smooth border of main graft (gr). Scale bar 50 μm.

Fig. 6. Sarcoma 180 cells (arrowed) associated with nerve (n). Scale bar 50 μm.

Fig. 7. Sarcoma 180 cells in differentiated cartilage. Scale bar 50 μm.

Fig. 8. 7-day embryonic quail heart in the chick limb. Note smooth border between graft and surrounding mesenchyme. Graft left for 2 days. Scale bar 50 μm.

Fig. 9. Adjacent section to that in Fig. 8, Feulgen-stained to show quail cell nuclei (with darkly staining nucleoli). Confirms that there are no quail cells away from graft. Scale bar 50 μm.
Cell movement and mechanism of invasiveness
operation. In 2 grafts that were examined 3 h after implantation, there was no evidence for invasion. In 1 case out of 2 that had been left for 7 h, however, 2 cells were found away from the graft. In cases, examined after 8 and 9 h, several cells had invaded the mesenchyme.

Usually there was a compact main graft with individual sarcoma cells scattered in the surrounding tissue. The distribution of sarcoma cells that invaded has been examined in detail in 4 cases. One case was of a graft implanted for 9 h, 2 were of grafts left 24 h and the other was a 48-h graft. The total number of cells away from the graft that had been implanted for 9 h was 13 and the mean distance away from the graft was 71 μm; the farthest cell was over 180 μm from the graft. In the grafts left for 1 day, 28 cells in one case and 30 cells in the other were found away from the graft. The mean distances moved were 68 and 100 μm and the farthest were over 240 and 340 μm. In the other case examined where the graft had been left for 2 days the number of cells away was 88; the mean distance moved was 120 μm, while the cell farthest away was at a distance of 540 μm. This cell was actually 200 μm further away from the graft than any other cell.

There are several interesting features of these measurements. The number of cells away from the graft increases the longer the graft has been left in the chick wing bud. This increase could be due both to more cells emigrating out of the main graft and also to the division of cells that had already moved away. It is striking that not all the sarcoma cells invade the tissue even over a period of 48 h and that often many remain as a healthy compact group at the original site of the graft. The mean distance moved by the invading cells tends to increase the longer the graft has been implanted into the limb. We should emphasize again that some of the separation of sarcoma cells observed will result from the growth of the limb tissue between them. The longer the graft has been implanted into the limb, the greater the distance the farthest cell has reached. Perhaps this is a more telling measure of invasion than the mean distance moved.

We have already mentioned that in many cases there is a compact main graft. The graft may make a smooth border with the chick limb tissue in some places but may show interdigitation along other edges. It is clear, in this case, that within the same graft, individual cells show different interactions with the surrounding mesenchyme.

An interesting feature of the pattern of invasion of the sarcoma 180 cells was that they tended to end up adjacent to nerves in the older limbs. In fact, in grafts that were left for 2 days, some sarcoma cells were found associated with nerves in 6 cases out of 7 (Fig. 6) and in 1 very proximal graft that was left for 1 day sarcoma cells were found near nerves.

In 1 graft sarcoma cells were found in the cartilage (Figs. 5, 7). It is not clear whether these cells had invaded the cartilage. It seems more likely that they were 'trapped' there during differentiation of the surrounding tissue.

Embryonic quail heart ventricle

Four grafts were made of fragments of quail heart ventricle from embryos of stages 22-24 (Hamilton & Hamburger). The grafts were compact and no cells were
found away from the graft. This was confirmed by studying parallel sets of sections Feulgen-stained to show the quail nuclei. In 2 cases it was clear that the grafted tissue was of 2 main types, developing heart myoblasts and connective tissue cells. The muscle tissue tended to be separate from the loose connective tissue cells which were arranged in a swirling pattern. Neither of these cell types had invaded. Grafts contained large spaces filled with blood in 3 of the 4. For instance, one of these grafts looked like a sponge with blood-filled sinuses. In 2 grafts some of the endothelial cells lining the sinuses appeared to be of graft origin, i.e., quail.

Additional grafts were made so that we would be looking at the behaviour of heart cells of the same age as those studied by Abercrombie & Heaysman (1954). In 7 grafts the heart tissue was compact (Fig. 8) although the edges of the graft were not always smooth. The grafts again contained 2 cell types, swirling connective tissue and developing heart muscle. No cells were seen away from the graft, even in the Feulgen-stained parallel set of sections (Fig. 9). The muscle tissue did not look as healthy as that in grafts of younger tissue because the cells were vacuolated. Even so, cells in mitosis were observed within the graft. In 6 out of the 7 grafts there were blood vessels associated with the graft, either at one side of the graft or actually running through the graft itself. As in grafts of pieces of young ventricle, some of the endothelial cells lining these vessels may come from the graft.

**BHK and PyBHK**

Six grafts of BHK cells were examined in detail. In 1 case only a few cells were found at the graft site. In all other 5 cases, BHK cells have been found away from the graft in the chick mesenchyme. The cells often appear elongated parallel to the long axis of the limb and seem to be streaking off (Fig. 10).

In 2 cases BHK cells were found flattened along the mesenchyme-ectoderm border (see trophoblast). It is clear for instance that one such cell was extremely attenuated underneath the basement membrane of the ectoderm (Fig. 11). In other instances it looked as though BHK cells might be flattened along the ectoderm side of the basement membrane (Fig. 12). In no case was there any sign of damage or bulging of the ectoderm as had been observed in some regions where trophoblast cells were lying under the basement membrane.

In all the grafts made of PyBHK cells there were many cells away from the graft. Some cells were more than 500μm away. The cells invaded the mesenchyme both singly and in streams where cells were stretched out along their neighbours (Fig. 14).

In 5 cases (out of 7) PyBHK cells were found near blood vessels and apparently almost lining the vessels (Fig. 13) and in 4 cases they were found at the ectoderm/mesenchyme interface (see trophoblast, BHK).

In 3 cases cells from the graft were found in cartilage. In one, a large graft of PyBHK cells came to lie close to the differentiating cartilage and a tongue of cells extended from the graft into the cartilage matrix. This was the only case in any of the grafts in which it did look as though cells might be invading cartilage. In the
2 cases, the cells found in cartilage might well have been stranded there (see sarcoma 180). However, PyBHK cells have been found in cartilage in more experiments than have any other type of cell, and also in higher numbers. The small clumps of cells in cartilage could have arisen by division of a stranded cell.

The grafts of PyBHK cells were often very extensive and many cells in mitosis were observed. The bulk of the graft was usually avascular. It looked as though the centre of the graft might be necrotic in one case. This might be expected if the graft became larger than a few millimetres in diameter (Moskowitz, 1964; Folkman, 1974). The cells within the graft appeared to be tightly apposed to each other and in a swirling pattern reminiscent of the morphology of some tumours such as fibrosarcomas. It is interesting that such whorls and spirals are seen in dense fibroblast populations in 2-dimensional culture (Elsdale & Wasoff, 1976).

In some places the border between the graft and the chick limb mesenchyme is quite smooth. The border, in other places, is uneven, with streams of cells fanning out from the graft into the mesenchyme (see sarcoma 180).

Nil 8 and HSV Nil 8

There was evidence of invasion of the mesenchyme by the Nil 8 cells in every case. However, the number of cells found away from the graft and the distance they had moved was small in some cases. This must be partly due to the relatively few cells in the graft. Cells were found flattened along the interface between mesenchyme and ectoderm in 5 out of 7 cases (Fig. 15).

Ten grafts were made of HSV Nil 8 cells or mixtures of HSV Nil 8 cells and quail mesenchyme cells and there was ample evidence for invasion, with cells more than 500 μm away from the graft. In every case some cells were found flattened along the mesenchyme/ectoderm border (see trophoblast, BHK and PyBHK) (Fig. 16). Some cells were found a long way from the graft under the ectoderm and in some grafts the cells that were farthest away from the site of implantation were those under the ectoderm. This could be due to there being fewer obstacles to cell movement along the basement membrane than in a pathway through the meshwork of mesenchyme.
cells. On this latter route invading cells would have to weave between the cells. An alternative possibility is that there is a passive component to the translocation of cells under the ectoderm. This could be due to the forward sliding of the ectoderm that occurs during limb outgrowth (Amprino, 1965), HSV Nil 8 cells were sometimes found under the apical ridge (Fig. 17 and see trophoblast). Another feature of the pattern of invasion was that the HSV Nil 8 cells often came to lie very close to blood vessels, just separated from the blood vessel lumen by a thin rim of endothelial cells (see also PyBHK).

HSV Nil 8 cells were found in nearly every part of the limb (Fig. 18) and in all orientations. The regions that were more or less free of graft cells were the ectoderm and the cartilage. No cells have been found in the ectoderm and only 3 cells in 1 graft were found in well differentiated cartilage with metachromatically staining matrix. In 2 cases there were signs of erosion of the chick limb mesenchyme by the implanted transformed cells and the centre of the graft region appeared to be eroded away.

The transformed Nil 8 cells were very elongated and fibroblast-like in morphology as they appear in culture, in contrast to the rounded shape of the sarcoma 180 cells.

**Neuroblastoma**

Neuroblastoma cells were found away from the graft. In 2 cases the invasion was limited both in the numbers of cells involved and the distances moved from the main graft. These were the exceptions and most grafts had many cells that had invaded.

Neuroblastoma cells were found associated with nerves (Fig. 19) in 5 out of 7 cases where the graft had been left for 1 day and ended up rather more proximally then usual (see also sarcoma 180). Another characteristic feature was that neuroblastoma cells often appeared to be associated with blood vessels. Little clumps of neuroblastoma cells within the loose connective tissue of the limb seemed to be accompanied by an attendant blood vessel.

Of particular interest was the observation that the neuroblastoma cells, particularly
when in small clumps, tended to lie in clear spaces (Fig. 20). These spaces could result from the digestion of the surrounding tissue by the neuroblastoma cells. For instance, in 1 case a small group of neuroblastoma cells were found lying within a space at the edge of developing cartilage. It was difficult to tell in other cases whether blood vessels adjacent to the grafted cells had been breached too, thus contributing to the space around the neuroblastoma cells.

The neuroblastoma cells were very distinctive and readily recognized in the limb because they were large and also rather darkly staining. None of the cells seemed to have long processes but they were rounded.

**Mouse lung tumour**

The mouse lung tumour was implanted into the chick wing in 2 forms. One set of grafts was of pieces cut from a tumour which had been grown subcutaneously in a mouse. The other set of grafts were pieces of pellets of cells that had been growing in tissue culture.

**Cell line.** Five grafts were made of a mixed pellet of lung tumour cells and quail wing mesenchyme cells. In only one of these grafts was there any invasion of lung tumour cells into the mesenchyme and 2 cells were found away from the graft in developing cartilage.

In all 5 grafts lung tumour cells were positioned in the chick ectoderm. In none of these grafts were there any quail mesenchyme cells in the ectoderm. In fact, there was a striking partition of lung tumour cells into the ectoderm and quail cells into the mesenchyme (Figs. 21, 22). In groups of lung tumour cells within the mesenchyme the cells tended to be loosely organized into epithelia. We considered that the cells did not form perfect epithelia because, for instance, the position of the nucleus within the cell was variable. In addition, few of the loosely formed tubules had lumina. This contrasts with the behaviour of reaggregated embryonic mesonephros. These cells reconstituted beautiful tubules with a well defined lumen (Tickle & Goodman, in preparation). It may be that these cultured lung cells lack intrinsic polarity and external cues are needed to organize them into epithelia. Mesonephros cells, on the other hand, appear to maintain polarity when disaggregated.

**Solid tumour.** Out of 6 grafts, 1 cell was found away from the graft in the chick mesenchyme in 2 cases. In the other grafts no cells had moved into the mesenchyme.

A striking feature of the behaviour of the Lewis lung tumour cells was their interaction with the ectoderm when the graft came to lie adjacent to it. This occurred in 5 out of the 6 grafts. Mouse lung tumour cells were found positioned within the wing ectoderm in register with the wing cells. It appeared that the tumour cells were sitting on a common basement membrane with the ectoderm cells (Fig. 23). The grafts were partially excluded from the chick wing mesenchyme during healing. This meant that the ectoderm, over some extent of the graft, had come to lie between the graft and the limb mesenchyme. This exclusion of the graft from the limb was found in some of the other cases where carcinoma tissue was implanted, and is reminiscent of the self-isolation behaviour of disparate tissue masses from amphibian embryos (Townes & Holtfreter, 1955).
Rat bladder tumours

Two types of bladder tumour were used as grafts. One was a domed outgrowth of the type usually produced by feeding nitrosamines to rats and the other was a more extensive and branched structure, not typically produced (Hicks & Wakefield, 1976). We also took pieces of each of the bladders in which the tumours occurred. These regions of the bladder had been exposed to carcinogen but had not developed tumours.

Bladder tumours. One bladder tumour consisted of large cells and there was very little stroma present, while the other contained much more debris as well as cellular regions. The results of 7 grafts were examined: in 2 cases cells were found away from the graft; in 1 graft 1 cell had invaded, and in the other graft, 4 cells. In the 5 remaining grafts no cells had invaded. The distances that the invading cells had moved were small, ranging from 10 to 60 μm. Although these few cells showed invasive behaviour, the majority of cells remained at the graft.

In all 5 grafts which ended up next to the ectoderm, tumour cells were found within the wing ectoderm (see mouse lung tumour). The positioning within the ectoderm could be due to an interaction with the basement membrane. In one case tumour cells which were adjacent to the underside of the ectoderm appeared to be positioned on the wrong side of the basement membrane (Fig. 24). It was not clear whether the tumour cells had actively taken up this position or whether the chick ectoderm had healed over them.

The border between the grafted tumour and the chick mesenchyme was smooth in some places. In other regions in the same graft, bladder cells appeared to be putting out long processes into the mesenchyme (see also sarcoma 180). The outer edge of the tumour which protruded from the wing rounded off so that it was a smooth edge.

Fig. 21. Graft of mouse lung tumour cells mixed with quail mesenchyme cells. Lung tumour cells (arrowed) are in wing ectoderm. Scale bar 50 μm.

Fig. 22. Adjacent section to that in Fig. 21. Feulgen-stained. In addition to lung tumour cells in ectoderm, shows quail cells (darkly staining nucleoli) in the underlying mesenchyme. All the ectoderm cells are chick. Scale bar 50 μm.

Fig. 23. Piece of solid mouse lung tumour grafted. Lung tumour cells positioned along common basement membrane (arrowed) with chick ectoderm cells. Scale bar 25 μm.

Fig. 24. Rat bladder tumour grafted. 2 tumour cells positioned on the underside of the basement membrane in the mesenchyme. Scale bar 50 μm.

Fig. 25. Benign human breast tumour graft with 1 tumour cell (arrowed) away in mesenchyme. Note non-adhesion between limb mesenchyme and apical surfaces of tumour cells. Graft left 1 day. Scale bar 50 μm.

Fig. 26. Malignant human breast tumour graft showing a smooth border with the mesenchyme and no invasion. Graft left 1 day. Scale bar 50 μm.

Fig. 27. Malignant human breast tumour graft with tumour cell (arrowed) within the chick ectoderm. Graft left 1 day. Scale bar 50 μm.

Fig. 28. Pigmented retina (p) interrupting wing ectoderm. Shows close approach of blood vessels to graft, compare with standard distance (at *) between ectoderm and nearest blood vessel. Graft left for 1 day. Scale bar 50 μm.
Bladder adjacent to tumours. Most of the grafts of bladder adjacent to the tumours consisted of stroma. Two grafts were made that contained cells. No cells were found away from the grafts (except for 2 that were separated by one chick cell from the main graft) and no cells had penetrated into the ectoderm.

Human breast tumours

Small samples of 4 human breast tumours were obtained from the Pathology Department of the Middlesex Hospital and successfully grafted; 2 of these tumours were malignant and 2 were benign.

The majority of the cells in both malignant and benign grafts did not invade. In 3 out of 6 grafts of malignant tumour, 1 cell only was found away from the graft. In the other 3 cases no cells had invaded. Benign tumour grafts showed, in one case, no cells away from the graft, and in the other case, 3 cells had invaded (Fig. 25). We have not been able to detect any difference in the number of single cells away from the grafts of either malignant or benign breast tumours. The border of the graft with the limb was often quite smooth (Fig. 26).

All breast tumour grafts were adjacent to the chick wing ectoderm. Cells from grafts of malignant tumours were positioned within the ectoderm in each case (Fig. 27) (see also mouse lung tumour and Bladder tumour). In contrast, although the benign tumour was adjacent to the ectoderm in 1 graft we have examined there was no penetration of the cells from the graft into the ectoderm. In this case the breast epithelium fused with the chick ectoderm.

It should be noted that the cells from the breast tumours were of several different types and were not a homogeneous population. The different kinds of cells will be described elsewhere (Tickle, Crawley & Goodman, in preparation). However many mitoses were observed and the cells in the grafts were healthy.

Embryonic quail pigmented retina

The pigmented retina cells were organized into a single epithelium with a smooth border along the chick mesenchyme. In some cases the grafts consisted of folds of the single layer of cells. A layer of pigmented cells frequently came to lie alongside a rod of developing cartilage. In another case the pigmented epithelium surrounded a well defined lumen. This type of structure occurs in aggregates formed from suspensions of pigmented retina cells.

One striking feature of these grafts was that in most cases they were very well vascularized (see also heart ventricle and to a lesser extent neuroblastoma). In one case the blood vessels appeared to penetrate right into the graft. Blood vessels were also seen running right up to the base of the pigmented epithelium. This was particularly noticeable in a graft that had been pinned to the outside of the limb. A short length of pigmented retina interrupted the wing ectoderm. Blood vessels of the wing normally are positioned a standard distance under the ectoderm. In the region of the pigmented retina ‘insert’, the blood vessels came right up to the graft (Fig. 28). The pigmented retina that was grafted to the outside of the limb buckled so that the apical surfaces of the cells faced outermost.
Comments on the interpretation of the results

We have already pointed out that the developing chick limb bud does not provide an ideal site in which to assay invasive behaviour, in that it is a rapidly growing organ. We have stressed that some of the separation of the cells from the graft will be due to the growth of the intervening tissue. However, we do not wish to give the impression, by stressing this difficulty, that the distribution of grafted cells which we have called invasion could have arisen solely by this means. We can see both by the distances involved and the location of the grafted cells that movement of the cells must also have taken place. For instance, it is clear that cells grafted to the centre of the dorsal surface of the limb bud could not be displaced by growth alone to come to lie under the apical ectodermal ridge.

We want to mention here, also, that the form of the graft, whether piece of tissue or centrifuged pellet, does not appear to determine the distribution of cells. If the distribution of cells throughout the limb occurred only by cells being displaced from the graft followed by intervening growth of the limb, it might be expected that centrifuged pellets of cells of loose consistency would always appear to be invasive. This is clearly not the case because cells in pellets of both mouse lung tumour cell line and cells teased from breast tumours do not invade the limb: the cells behave in the same way whether they are grafted as a pellet or a piece of tissue cut from the solid tumour. It has also been our experience with pellets of cells disaggregated from a range of normal embryonic tissues, such as limb mesenchyme, mesonephros and liver, that cells from these do not invade the mesenchyme. In addition, we have found that cells can readily invade the limb from the solid tissue grafts of sarcoma 180 and trophoblast. However, we would not wish to suggest that the tightness with which cells are held together is not a factor involved in the invasion of cells in these experiments and, of course, of tumours in vivo.

DISCUSSION

A striking finding to emerge from our survey of cell behaviour in the chick wing bud is that, although many different kinds of cells, trophoblast, sarcoma 180, BHK, PyBHK, Nil 8, HSV Nil 8 and neuroblastoma, all move into the mesenchyme, cells from 3 different carcinomas, rat bladder tumour, mouse lung tumour and human breast tumour, do not. These tumour cells of epithelial origin appear reluctant to move into the chick wing although, clinically, the process of invasion by carcinomas involves penetration of underlying mesenchymal tissues. Substantially the same results have been obtained with guinea-pig hepatoma cells (Easty & Easty, 1974) and human breast carcinoma cells (Ambrose & Easty, 1976) when these cells are placed on to the chick chorioallantoic membrane (CAM) in culture. Individual cells show little invasion into the interior of the membrane. These results suggest that carcinomas may not invade by single cells becoming detached from the main tumour and actively moving into the surrounding tissues. This casts a rather different light on the invasiveness of epithelial tumours, which we will discuss in detail later on, and suggests that
not all tumours invade by active cell movement. Our finding, however, that fibroblast-like cells and some other cells such as neuroblastoma cells do move into the chick wing mesenchyme supports the idea that cell movement may be important in the invasiveness of sarcomas and some other tumours.

A number of different kinds of cell did move into the wing mesenchyme and this should provide insights into the different mechanisms of invasion of these cells.

**Contact inhibition and invasiveness**

We can examine the role of contact inhibition of movement in our assay for invasiveness in 3 dimensions. We have compared the behaviour of embryonic heart fibroblasts and sarcoma 180 tumour cells when implanted into the wing because Abercrombie et al. (1957) used these cell types in their original studies on contact inhibition in tissue culture. Although the behaviour of embryonic heart cells has subsequently been studied in 3 dimensions in fused fragments of heart with heart and with other embryonic tissues (Weston & Abercrombie, 1967), the behaviour of cells in the combination of heart and sarcoma 180 tumour surprisingly has been only briefly mentioned (Wolff & Schneider, 1957). The behaviour of these cells in the 3-dimensional tissue environment of the wing bud is what would be predicted from the behaviour of the cells in culture. The cells that show contact inhibition of movement in culture, the embryonic heart fibroblasts, do not move into the limb, whereas the cells that are not contact-inhibited, the sarcoma 180 cells, do indeed invade into the limb mesenchyme. It is interesting to contrast this clear difference in the behaviour of the 2 types of cells when implanted as small pieces of tissue into the chick limb bud, with what happens when the pieces are instead placed in tissue culture. In culture the cells emigrate out of both tumour and embryonic tissue on to the substratum. The difference in the behaviour of the 2 types of cell in the chick wing supports the idea that lack of contact inhibition of movement may lead cells to become invasive. If we accept that both normal and transformed fibroblastic cells are contact-inhibited in their movement, we should expect that neither type of cell would move into the chick mesenchyme. However, we have found that PyBHK, BHK, Nil 8 and HSV Nil 8 cells all invade the limb mesenchyme. This taken at face value does not appear consistent with the hypothesis that it is lack of contact inhibition that allows invasion by freeing cells from the constraints of their neighbours. However, as we have already pointed out, contact inhibition of movement is a complex phenomenon and depends on events that occur locally on the cell perimeter. We can imagine circumstances in which a cell might still be able to move through a tissue even if the surface was paralysed wherever it was in contact with adjacent cells. One such case would be if the tissue being penetrated was a loose meshwork of cells. The chick wing bud has this structure and there is plenty of space into which moving cells could extend processes without immediately contacting another cell. A contact-inhibited cell would also be able to invade if the tissue adjacent to the infiltrating cells was broken down and thus space created. In this way contact would be avoided between the invading cells and the cells of the tissue being penetrated. However, if, indeed, the chick limb mesenchyme is readily invaded even by contact-inhibited cells because of its loose-knit
Cell movement and mechanism of invasiveness

arrangement of cells, it is not clear why normal embryonic cells also do not move into
the limb. We know there are cells in embryonic heart tissue, for example, that have
the intrinsic ability to move because as we have already mentioned that if explants
are plated out in culture, fibroblasts will emigrate out. This suggests that the individual
cells are constrained to remain in place in normal embryonic tissues by factors other
than contact-inhibition of movement such as, for instance, adhesion to adjacent cells.

The morphology of the invading cells may be significant. BHK, PyBHK, Nil 8
and HSV Nil 8, cells all appear to be very elongated. In contrast, the invading
sarcoma 180 cells look much more rounded. Thus, the capacity of the cell to change
shape may be important in determining whether a contract-inhibited cell can invade.
We should add here that the morphology of these cells in the limb mimics their
appearance in tissue culture.

Trophoblast and neuroblastoma cells were also found to invade the limb. We know
of no investigations into the contact behaviour of these cells. However, these may fall
into the category of cells that avoid contact inhibition by destroying adjacent tissue
(see later). The carcinoma cells, which do not invade the mesenchyme may be contact-
inhibited in their movement. Normal epithelial cells (Middleton, 1973; Di Pasquale,
1975) and some transformed epithelial cells form monolayers (Vasiliev & Gelfand,
1976) and cease ruffling activity on contact (Wilbanks & Richart, 1966). The important
question, however, for consideration of invasiveness, is what happens when carcinoma
cells contact fibroblasts and there are few data available. The only recent experiments
we know of, are those of Wilbanks & Richart (1966) who found that cells from intra-
epithelial neoplasms did not invade cultures of fibroblasts.

Lack of contact inhibition was found to be correlated with the invasiveness of
sarcoma 180 cells. However, other cells invaded despite being contact-inhibited in
their movement and this places emphasis on the tissue that is being invaded. We
conclude that lack of contact inhibition of movement is not a necessary feature of
invading cells.

Tissue breakdown

We have already mentioned that tissues adjacent to the tumour may be broken down
and thus create space into which the tumour cells can move. We have found that
trophoblast, neuroblastoma and transformed Nil 8 cells show signs of eroding the
surrounding wing mesenchyme. This erosion was particularly striking in the grafts
of trophoblast and it has been recently shown that trophoblast produces plasminogen
activator (Strickland et al. 1977). However, other invading cells, such as sarcoma 180,
appeared to do no damage to the chick tissue and this has frequently been noticed in
the invasion of other tumour cells in vivo (for example, Locker, Goldblatt & Leighton,
different cells on the CAM, similarly found that while some invading cells caused local
damage, other cells invaded without associated destruction. Breakdown of adjacent
tissues may be one way in which invasion by cells that are contact-inhibited in their
movement, can take place.
Normal and transformed cells

We have found that both normal and transformed BHK and Nil 8 fibroblasts invade the wing mesenchyme although we have only compared the invasion qualitatively.

In view of the similar contact behaviour and movement of BHK and PyBHK cells in culture, this result does not seem very surprising. Easty & Easty (1974) also found that normal BHK cells invaded the CAM in culture but not as extensively as their transformed counterparts. These results support the idea that transformed cells are not intrinsically different from normal cells, but some cellular parameters may vary in degree. Scher et al. (1976), however, have found a difference in invasive behaviour between a normal 3T3 line and its transformed counterpart when cultured on the CAM. It appears that the transformed fibroblasts penetrated the intact epithelium and moved into the underlying mesenchyme to form a tumour. The normal 3T3 cells did not invade the intact epithelium. We have found that the normal and transformed cells we have tested, will move into mesenchyme if implanted there. It seems possible that in the experiments of Easty & Easty (1974) the CAM was damaged and this may account for the normal BHK cells gaining access to the mesenchyme of the CAM.

Cell survival

It appeared that normal cells survive less well in the limb than cells of their transformed derivative. This result also emerges from the work of Scher et al. (1976), although in this case it is not clear whether the enhanced growth of the transformed cells is due to their location in the interior of the CAM. The capacity for survival of the transformed cells in the chick wing is very striking. It is commonly said that tumour cells are very hardy and it is mentioned as an example that cells stuck to the surgeon's knife may give rise to tumours at the edges of incisions made when removing a main tumour (Walter & Israel 1974). This ability to survive may be an important factor in the establishment of secondary tumours.

Patterns of invasion

Our survey has shown that there are several different patterns of invasion in the chick limb. We have found that cells from the trophoblast and fibroblast cell lines, both normal and transformed, tend to become aligned along the basal lamina of the ectoderm. Such behaviour has been termed contact guidance (Weiss, 1941) and is reminiscent of the classical observations of Harrison (1914) who grew cells on spider webs and fish scales and showed that the cells became aligned along fibres or in the grooves. In the light of a new hypothesis for contact guidance (Dunn & Heath, 1976) it is not surprising that it is the fibroblast cells that move along the interface of the mesenchyme and ectoderm. We found that the cells were frequently aligned parallel to the long axis of the limb. This suggests that the basal lamina may be intrinsically structured along this axis or may become structured, as fibres in a plasma clot can become aligned, by being stretched in one direction. The trophoblast and fibroblast
cells may collect at the basal lamina for another reason: that they are more adhesive to this than to the surfaces of the chick wing cells. Cells have been shown to accumulate on substrata to which they are more adhesive when there is a choice of substratum available (Carter, 1965; Harris, 1973). Movement of cells along a basal lamina occurs during migration of neural crest cells during normal development (Noden, 1975). Wolff & Schneider (1957) also noticed that sarcoma cells invaded fragments of chick embryonic organs in vitro along preferred routes. Finally pathologists note that cancer cells invade along paths of least resistance, for example, along connective-tissue septa (Walter & Israel, 1974).

Another striking pattern of invasion was the clustering of sarcoma 180 and neuroblastoma cells around nerves. We do not know how this association arises: whether one partner is active or whether there is mutual attraction. For instance, it is known that sarcoma 180 tumour produces nerve growth factor (Levi-Montalcini, 1952) and this could divert the ingrowing nerves towards the graft. Alternatively, the sarcoma and neuroblastoma cells may migrate along the edge of the nerves due to contact guidance. Many types of carcinoma show perineural invasion. This has been ascribed to lymphatic spread (Walter & Israel, 1974) but it now seems likely that the tumour cells invade along the nerve itself (Rodin, Larson & Roberts, 1967).

The cartilage of the chick limb was rarely invaded by cells from the graft. PyBHK cells were present more frequently in the cartilage than any other cell type. When the odd cell was found in the cartilage it was not clear whether these cells had actively moved into the cartilage or whether they were stranded when the cartilage differentiated around them. Other pathological (Walter & Israel, 1974) and experimental (Wolff & Schneider, 1957; Sorgente, Knettner, Sobel & Eisenstein, 1975) data also show that cartilage is relatively resistant to invasion by cancer cells.

Several different kinds of grafted cells were found associated with blood vessels. HSV Nil 8 and PyBHK cells were found flattened under the endothelium of blood vessels. This localization may be due to the presence of a basal lamina here as at the interface between the wing ectoderm and the mesenchyme. More specifically the cells may be adhering to the collagen present in both regions. It has been shown that fibroblasts from the developing avian cornea colonize collagen matrix (Bard & Hay, 1975). Trophoblast cells similarly showed affinity for both the border between the ectoderm and mesenchyme and the vicinity of blood vessels, and sometimes made up part of the vessel wall.

Clumps of neuroblastoma cells were often associated with blood vessels although the individual cells were not flattened along the base of the endothelium. This association may have arisen due to production of angiogenesis factor (Folkman, 1974) by the neuroblastoma cells. It has been suggested that the acquisition of angiogenic capacity by cells may be one of the first signs of neoplastic change (Brem, Gullino, & Medina, 1977). However, the bulk of extensive grafts of PyBHK and HSV Nil 8 cells remain apparently avascular. In contrast, other cultured cells, a transformed 3T3 cell line (Scher et al., 1976) do produce new blood vessel growth when cultured on the CAM.

We found that the most vascular grafts were those of the embryonic tissues, heart and pigmented retina. The blood supply of the heart grafts was probably established
by a link-up between the host blood vessels and those of the graft. This mechanism is believed to occur in vascularization of a variety of embryonic tissues on the CAM (Ausprunk, Knighton & Folkman, 1975). However, in the case of the pigmented retina, the vascular pattern of the limb appeared to be distorted locally and blood vessels came to lie very close to the graft. We do not know in this case whether cell division of the endothelial cells is involved.

Carcinomas and ectoderm

Carcinoma cells showed a specific interaction with the ectoderm. In grafts of all 3 kinds of carcinoma, cells from the graft were found in the ectoderm in register with the adjacent chick cells. The basement lamina seems to be very important in the positioning of the carcinoma cells within the ectoderm. Cells from grafts of the other tissues tested were rarely, if ever, found in the ectoderm.

There have been few studies, as far as we know, on the interaction between carcinoma cells and normal epithelial cells. Wilbanks & Richart (1966) found that cervical intraepithelial neoplastic cells did not invade normal epithelial sheets spreading in tissue culture. However, in organ culture human breast carcinoma cells were found to penetrate into the intact epithelium of the chorioallantoic membrane (Ambrose & Easty, 1976). Tumour cells that have metastasized to epithelial sites such as the lung take up similar positions (Brooks, 1970), as do tumour cells that implant into the mesothelium following intraperitoneal injection (Birbeck & Wheatley, 1965; Buck, 1973). This interaction between carcinoma cells and other epithelial cells may be important in determining sites of metastasis.

We are aware that the developing chick wing bud is an artificial site in which to assay invasiveness, but it does provide a test of cell behaviour in a 3-dimensional tissue. Our survey has resulted in highlighting 2 main points, which bear on the way we think about invasiveness. Firstly, it seems that different cells may move into a loose mesenchyme in different ways. There may not be just one factor that leads to invasion. Also, within a single population of tumour cells there is considerable variability and only some cells invade. This means that any tests of cell behaviour which average the results over the population as a whole will not provide relevant information about malignant cell behaviour. In metastasis the behaviour of a small minority of the tumour cell population could be important (Fidler, 1975).

The other major finding is that cells from carcinomas, the most common type of human tumour, do not move into a loose mesenchyme. We are thus faced with the problem of how carcinomas invade. It seems likely from our results that infiltration of cells, as individuals, is not involved. Indeed, the pathology of many tumours shows that invasion has involved tongues or sheets of cells that are continuous with the main tumour mass. It seems, therefore, in considering the invasiveness of carcinomas, the emphasis should shift to other mechanisms to deal with questions such as how expansion of a cell sheet occurs without a free edge. The force for invasion could be generated by cell division and the mechanism of penetration will depend on the interaction of the carcinoma cells and the underlying tissue. These ideas are not new and it has been believed for a long time that the pressure of a growing tumour could
Cell movement and mechanism of invasiveness lead to invasion (see for example, Willis, 1973). In this light, the invasiveness of carcinomas appears to involve the same mechanisms as the epithelial invasion of the mesenchyme that occurs during gland development. Furthermore, it thus seems possible that the first step in metastasis, the entry of the invading tumour into the blood or lymph vessels, need not be due to the active movement of the individual cells into the stroma, but to the fusion of growing epithelia with the blood or lymph vessels. This process would be accelerated if the blood vessels came into close proximity with the tumour and there is evidence that many tumours produce substances that lead to the growth of blood vessels towards them (Folkman, 1974).

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Cell movement and mechanism of invasiveness


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