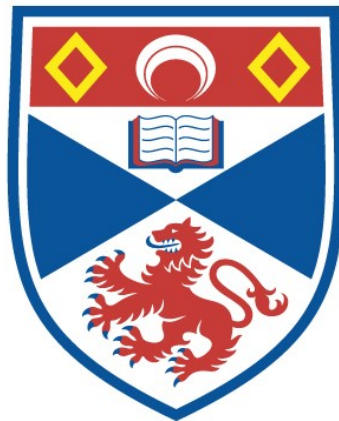# THE CONTROL OF MORPHOGENESIS IN 'DROSOPHILA' IMAGINAL DISC CELL LINES 'IN VITRO'

Andrew S. Miller

A Thesis Submitted for the Degree of PhD
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The control of morphogenesis in *Drosophila* imaginal disc cell lines *in vitro*

by

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Submitted in candidature for the degree of Ph.D. in the University of St.

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Abstract

The experimental component of this thesis represents the continuation of studies carried out in the Milner laboratory to characterise the biology of *Drosophila* imaginal disc cell lines growing *in vitro*. The bulk of this study was concerned with the morphological and molecular characterisation of imaginal cell interactions *in vitro* and contrasting this with what is known of imaginal cell biology *in vivo*. The imaginal cell lines were thus seen as both an amenable model for the detailed analysis of a cells interaction with its environment and, more broadly, as an assessment of the effects of isolating and maintaining animal cells *in vitro*.

The reaggregation of single imaginal cells when in suspension is rapid and extensive when in the presence of the divalent cations, Ca^{2+} and Mg^{2+}, but is not entirely dependent on them suggesting the existence of separate systems of adhesion and the presence of a variety of cell adhesion molecules (CAMs). During the course of growth *in vitro* there appears to be a shift in strategy between cell-substrate and cell-cell adhesion as cells move from monolayers into aggregates. A new set of leg cell lines were cloned, some of which had a radically reduced capacity to reaggregate in suspension suggesting that CAMs can be selectively lost from the imaginal cell surface.

PS integrins are widely expressed in imaginal cells *in vitro*, as *in vivo*, and seem to be similarly involved in various cell adhesion events here. PS integrins are expressed at all stages of imaginal cell growth and function both in mediating cell-substrate adhesion, notably to human fibronectin, and in cell-cell adhesion. PS integrins appear to mediate a 'higher order' cell-cell adhesion via an unidentified component of the extracellular matrix (ECM) which again can be substituted using vertebrate molecules. PS integrins may direct aggregation by forming tension transmitting junctions with other cells,

evidence for which is provided by F-actin and tubulin expression in aggregating cells.

The *in vivo* epithelial phenotype, characterised by apical-basal polarity, can be re-established in part by growing cells on fibronectin-coated membranes in the presence of unidirectional nutrient uptake and a feeder layer of cells. Cells *in vitro* lack such polarity signified by the absence of specialised intercellular junctions and a loss of restriction of expression of the putative CAM and signalling molecule, CRUMBS.

Another CAM which appears to be expressed during imaginal cell growth *in vitro* is the immunoglobulin, fasciclin III, or a variant, which is restricted in expression to single migratory cells and during cell-cell adhesion.

Some evidence is also provided that these cell lines express, and can endocytose, proteins known as 'larval serum proteins'. The nature of this uptake remains obscure, but does not seem to be enhanced by the presence of the moulting hormone, 20-hydroxyecdysone (20-HE).

Abbreviations used

20-HE	20-hydroxy ecdysone
AP	After pupariation
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
cpm	Counts per minute
CSM	Complete sterile medium (after Cullen and Milner, 1991)
D=	PBS minus Ca^{2+} and Mg^{2+} (Dulbecco's formula)
DAB	3, 3'-diaminobenzidine
DNA	Deoxyribosenucleic acid
DMSO	Dimethyl sulphoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic Acid
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GRGDSP	Synthetic peptide containing the sequence glycine-arginine-aspartic acid (RGD).
GRGESP	Synthetic peptide containing the sequence arginine-glycine-glutathione (RGE).
IBMX	3-isobutyl-1-methylxanthine
IU	International units
LSP	Larval serum protein
MTOC	Microtubule organising centre
OD	Optical density
PBS	Phosphate buffered saline
PMSF	Phenylmethysulphonylfluoride

RNA	Ribonucleic acid
S2	Schneider's Line 2
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SPIT	Solid phase immunoisolation technique
TE	Tris/EDTA based buffer
TEM	Transmission electron microscopy
TCA	Trichloroacetic acid
TTBS	Tris buffered saline

Acknowledgements

1.1   Overview

The fruit fly *Drosophila* has, for most of this century, been the subject of intense scientific scrutiny and interest to a large and diverse community of biological researchers. The primary reason for this enormous interest in *Drosophila* is the potential to utilise sophisticated genetic analyses of function *in situ*.

In this thesis, I have chosen to study an *in vitro* based aspect of *Drosophila* biology. At present, genetics is widely accepted as being a successful method for studying many aspects of *Drosophila* development, and is utilised by many researchers, however, *in vitro* studies still have a great potential for experimental application in this field. The value of *in vitro* tools to the researcher has been significant. The culture of a particular cell type *in vitro*, which traditionally has been of vertebrate origin but more recently includes those from invertebrates, allows the researcher to study cellular behaviour within a precisely controlled environment.

Cells in culture are, by their nature, highly accessible, homogeneous and amenable for a wide range of analyses. The isolation of cells from the original tissue, as a primary culture, and their subsequent maintenance as established cell lines can also give an insight into the behaviour and function of these cells which may not be immediately apparent by observing them *in situ*.

Drosophila melanogaster, known more commonly as the vinegar fly, is a dipteran insect that has a holometabolous mode of development in that it must undergo a complete metamorphosis as a larva to form the imago or adult fly. Thus, the lifestyle of *Drosophila*, unlike some lower dipterans where the youngest larva appears very similar to the adult insect, can be sharply defined into three distinct stages: the larva, the pupa and the imago.

The *Drosophila* egg hatches into the larva, which is physically distinct from the imago, both having body plans specialised for development at that particular stage. The lifestyle of the *Drosophila* larva, which undergoes three successive moults as it grows, is centred on burrowing and feeding in food and therefore it lacks any well developed external appendages. The adult fly, on the other hand, is very mobile, and has well developed legs, wings and sensory organs. As these distinct forms are concurrently organised in each animal, the adult primordia, which have been determined during embryogenesis, are secluded from the other cells and exist as discrete structures known as the imaginal discs and histoblast nests (see Figure A.1). During metamorphosis at the pupal stage these precursors undergo a dramatic morphogenesis to form most of the adult integument; the discs forming the components of the head, thorax and genitalia and the histoblasts forming the entire abdominal integument, save for that part formed by the genital disc.

Each imaginal disc has a specific shape, size and distribution within the larval haemocoel (see Figure A.2). At the end of the third larval instar, the mature imaginal discs appear as a discrete pouch of cells, composed on one side of a simple folded columnar epithelium, known as the disc proper, which then downgrades to a squamous epithelium on the other side, which is known as the peripodial membrane. Cells from both of these tissues contribute to the formation of the adult epidermis. The cells of the disc proper exhibit apical-basal polarity. The basal side of each cell is outermost in the disc, facing the haemocoel and is in contact with the basal lamina while the apical side is studded with microvilli and faces inward to the disc lumen (Poodry and Schneiderman, 1970). The *Drosophila* basement membrane is known to contain laminin, collagen type IV, glutactin, the proteoglycan papilin plus some other glycoproteins (Fessler and Fessler, 1989). Although the discs consist mostly of epithelial cells, there also exist populations of

haemocytes and muscle cell precursors which are known as adepithelial cells. Larval neurones are also found and serve to connect the larger discs to the brain (Tix *et al.*, 1989).

The discs themselves arise in the embryo at the dorsal ventral compartment boundaries as invaginations of the epidermis and increase in size by cell division throughout development while remaining confluent with the larval epidermis (for review, see: Cohen, 1993). Disc morphogenesis, which for the appendage forming regions is a process of elongation and evagination to form the presumptive structure, occurs in response to the presence of the steroid hormone 20-hydroxyecdysone (20-HE) or, occasionally, its naturally occurring homolog, makisterone A (Riddiford, 1993).

The morphogenesis of each disc is stereotyped, following a distinct sequence of events which can be divided into prepupal and pupal morphogenesis. For development of the leg and wing imaginal discs, from which most established imaginal cell lines have been isolated (Currie *et al.*, 1988), prepupal morphogenesis involves the elongation and shaping of appendages, the eversion of these appendages and the spreading and fusion of the peripheral tissues of the disc to form a contiguous adult epidermis. The pupal stage involves the completion and apolysis (moulting) of the pupal cuticle, some further cell division, differentiation of hairs and sensory structures and the laying down of the adult cuticle. Together, elongation and eversion of the imaginal disc appendage are refferred to as evagination.

Throughout these stages of metamorphosis, imaginal disc cells are observed to undergo pronounced changes (Fristrom, 1989; Condic *et al.*, 1990). During the process of elongation and the shaping of appendages, disc cells are known to change shape first from columnar to cuboidal and finally to squamous, which is especially evident in the leg disc, such that they contribute to the marked increase in appendage length at this stage. These

cells also undergo two stages of rearrangement, once during the late third larval instar and then again during the prepupal period, where the apical ends of cells repack to join new neighbours, and the basal ends then re-align with the apical ends (Fristrom and Fristrom, 1975; Fristrom, 1976; Fristrom and Rickoll, 1982; Fristrom and Fristrom, 1993). However, the converse is true for cells of the peripodial membrane, where the squamous cells contract to become columnar, the area of the peripodial membrane thus being greatly reduced. It is this rapid reduction in peripodial membrane area which is believed to drive the actual eversion of the disc through the peripodial stalk (Milner *et al.*, 1984a; Fristrom and Fristrom, 1993).

In wing discs at this stage, at around 2 to 6 hours after pupariation (AP), changes in cell shape are believed to bring the basal surfaces of the putative dorsal and ventral wing surfaces together at the distal end. However, intercellular junctions and transalar microtubule arrays (Tucker *et al.*, 1986; Milner and Muir, 1987) do not form until just prior to pupation (Fristrom and Fristrom, 1993). Therefore, from this example, it seems that cell shape change itself does not provide the sole impetus for complete morphogenesis, such as the formation of established cell-junctions.

During this stage in the lengthening wing disc, and as the two putative epithelia come into contact, cells are seen to disconnect from the basal lamina (Brower *et al.*, 1987; Fristrom and Fristrom, 1993). The basal lamina also disappears at a similar stage in some parts of the leg discs. This phenomenon suggests that imaginal disc morphogenesis can proceed in the absence of a distinct, basally situated, ECM. Here, the cells seem to come into direct contact with cells from the opposite epithelium. The reason for this apparent loss of the basal lamina from certain areas here seems to be illustrated by the radical cell movements that are occurring during evagination.

During the shape change of epithelial cells from columnar to cuboidal, the basal and apical surface area is greatly increased but the basal lamina

itself appears to be relatively inextendible. Brower *et al.*, (1987) identified an extracellular matrix (ECM) component that remains associated with the basal surface of the epithelia throughout disc evagination. This molecule, a proteoglycan, seems to belong to a pliable, or perhaps even slick, matrix which is present at the basal end throughout this extensive cell movement. This is quite distinct from vertebrate systems where tracts of substantial basilar material are found supporting developing epithelia.

The absence of a need for a basal lamina during morphogenesis is further indicated by studies utilising proteases. At a slightly later stage, trypsin is known to accelerate disc evagination (Poodry and Schneiderman, 1971); although whether this is due to the removal of extracellular constraints or whether it induces some signal, by proteolysis of a precursor, which leads to cell shape change is unknown.

An apical surface glycoprotein known as gp125 which is believed to be involved in imaginal disc morphogenesis, is known to appear on imaginal disc cells in response to 20-HE but can be prematurely produced by treating the discs with trypsin (Birr *et al.*, 1990). It was later found that this gp125 was a product of the 20-HE directed proteolysis of basement membrane collagen type VI at a specific site (Fessler *et al.*, 1993). Thus, it seems that basement membrane breakdown, either to form a more pliable substrate or to create signalling molecules, is important in bringing about imaginal disc cell morphogenesis.

There then follows a period of imaginal disc cell spreading and fusion to form a continuous adult epidermis, which is complete by 10-11 hours AP (Fristrom and Fristrom, 1993). In the eye-antennal discs, fusion leads to the formation of a single head capsule, which has been the subject of detailed morphological studies *in vitro* (Milner *et al.*, 1984b; Milner and Bleasby, 1985). At the area of contact between the two eye anlagen, the basal lamina is again seen to break down and the basal surfaces come into contact via sets of cell

processes, or filopodia, that span the gap between them which may serve to properly align the two tissues correctly. The epithelial surfaces then become closely apposed, but do not intermingle (Milner *et al.*, 1984b). Head eversion marks the end of the prepupal period and the start of pupal morphogenesis (Fristrom and Fristrom, 1993).

During pupation the final shaping of the appendages occurs. This is largely influenced by a rise in hydrostatic pressure caused by abdominal muscular contractions that, as well as everting the pupal head, inflate the pupal legs and wings to extended, bloated, sacs. This inflation causes the epithelia of the appendages to straighten and flatten and to undergo significant extension (Waddington, 1941; Fristrom and Fristrom, 1993). During apolysis, as the pupal cuticle is shed, the leg discs are known to narrow but nevertheless stay the same length. This is due to a further imaginal cell shape change, from flattened back to cuboidal (Fristrom and Fristrom, 1993).

There has been something of a resurgence of interest in the past decade or so for the examination of pupal wing morphogenesis both *in vitro* and *in vivo* (Milner and Muir, 1987; Fristrom *et al.*, 1993) considering that the last major examination of this subject, prior to these, was written well over 50 years ago (Waddington, 1941). During wing inflation the opposing dorsal and ventral epithelia are far removed but the bilayer is reformed by 30 hours AP. By 60 hours AP, however, the epithelia separate again during adult cuticle deposition. By the end of metamorphosis, the wing is devoid of cells and is entirely cuticular in structure (Johnson and Milner, 1987). During the stages when the bilayers are physically removed, there exist numerous cell extensions that span the basal extracellular gap, connecting the two surfaces (Waddington, 1941; Fristrom and Fristrom, 1975; Milner and Muir, 1987). These basal projections contain specialised cytoskeletal units known as transalar arrays. These are large complexes of microtubules and actin

filaments which are nucleated at the apical surfaces of the epithelial cells which run down the cell body into the basally extended processes and terminate as convoluted desmosomal junctions at a site of contact with the opposing process midway in the wing cavity. These structures are believed to function in maintaining the structural integrity of the wing bilayer during metamorphosis (Fristrom and Fristrom, 1975; Milner and Muir, 1987; Mogensen and Tucker, 1987; Mogensen and Tucker, 1988).The role of the transalar arrays in pupal wing morphogenesis is reviewed in greater detail, and in the context of PS integrin expression, in Chapter 4 of this thesis.

By the late third instar larva, the imaginal discs have ceased to proliferate and exist as large discrete pouches of undifferentiated tissue. For this reason, imaginal discs have been widely used to study aspects of determination and pattern formation in the adult epithelium. Because the metamorphosed adult integument is covered with hairs and bristles that can be used as very specific spatial markers, the state of determination of the imaginal, progenitor, cells can be accurately established.

The phenomenon of transdetermination, when a cell undergoes a change in developmental fate, were discovered through the *in vivo* culture of *Drosophila* imaginal discs (for review, see: Hadorn, 1978). Whole or fragmented imaginal discs which were implanted into the abdomen of an adult fly will continue to proliferate and grow. This method was developed, by Hadorn, into a continuous *in vivo* culture by transferring the proliferating imaginal tissue from older flies to younger ones. At any time, the state of determination of the cells within each blastema can be identified by removing a piece of the tissue and implanting it into the haemocoel of a third instar larva and allowing it to pass through metamorphosis, the cuticular pattern that arises being used as a specific marker for which part of the adult integument it represents. Transdetermination, where groups of cells develop into adult tissue which is distinct from tissue they would be expected to

metamorphose into, can be observed in imaginal tissues which have been grown *in vivo* as a common spontaneous event, often occurring during early stages of culture (Hadorn, 1978).

Another set of experiments utilising this *in vivo* culture technique of manipulating imaginal tissue was designed to test the pattern forming ability of these cells (for review, see: Bryant, 1978). Imaginal disc fragments which have been implanted into the haemocoel of young larvae, rather than adult flies, often undergo regeneration of the missing tissue or the duplication of the existing structure. Such a culture technique is potentially useful, therefore, for experiments designed to determine the regulation of cell fate and pattern formation (including sorting-out) of imaginal cells. However, some problems with this system can occur (and are addressed in more detail in Chapter 3). As soon as the imaginal tissue is transplanted to its host, any changes within it cannot be directly monitored until it is removed at the end of the culture period; an interval of proliferation, for instance, may go unnoticed.

Experiments have been designed to test the sorting out capabilities of imaginal cells by dissociating imaginal discs into single cells and observing their interactions *in vitro* (for review, see: Fehon and Schubiger, 1987). This approach eliminates the problem of not being able to observe the test cells during such an assay which is certainly true with *in vivo* culture, but the dissociation techniques , by their very nature, can be damaging to the cells and this creates different problems. The development of an *in vitro* system which supports the growth of imaginal disc cells, where the cell interactions can be closely observed and where the environmental conditions can be precisely controlled, would be favourable for use in such experimental analyses.

The interest in, and limited employment of, *Drosophila* tissue and cell culture over the past few decades belies the potential benefits which such

systems may have for use in developmental studies, but reflects the practical difficulties implicit in their establishment. These problems of tissue and cell culture seemed to originate with the inadequacies of culture medium formulation.

The first *Drosophila* embryonic cell lines were established by Kakpakov *et al.* (1969) and Echalier and Ohanessian (1970) from 6-12 hour embryos. Homogenised *Drosophila* embryos undergo complex changes when placed in primary culture, attaching either singly or as groups of cells on the culture surface. Identifiable cells in these cultures are mostly muscle and nerve derived although some epithelial-like, macrophage-like, chitin-secreting, fat body and imaginal cells were also found (Shields and Sang, 1970; Seecof and Donady, 1972; Shields *et al.*, 1975; Dübendorfer *et al.*, 1975). Thus a potential problem becomes evident in that the specific identity of each cell line, established by such means, is somewhat uncertain and could therefore limit subsequent applications.

After some initial cell death and alternate stages of growth and quiescence, for culture periods as long as 10 months, it was possible to successfully subculture some of these embryonic cells. The widely used K_C cell line was established using such a technique (Echalier and Ohanessian, 1970). Many other embryonic cell lines that are still in use today, numbering at least over 30 (Currie *et al.*, 1988), such as Schneider's Line 2 (S2) cell line, were derived using similar techniques (For review see: Schneider and Blumenthal, 1978; Sang, 1981). However, it was not until much later that a corresponding set of continuous *Drosophila* imaginal disc cell lines were successfully established.

Some imaginal cells had originally been isolated along with a whole range of other cells from homogenised embryonic tissue grown *in vitro* (Schneider, 1972; Dübendorfer *et al.*, 1975). These cells grow as large vesicles and were identified as being imaginal in origin as they undergo complete

metamorphosis into fragments of differentiated adult cuticle when transplanted to third instar larvae. Transplanted imaginal cells do not seem to undergo any change in fate, or transdetermination (Hadorn, 1978), even after 10 months of primary culture (Schnieder, 1972). However, these transplanted tissues only represented adult structures after a minimum *in vivo* culture period of 48 hours. Imaginal vesicles were also known to differentiate *in vitro*, in liquid media, when co-cultured with 20-HE.(Dübendorfer *et al.*, 1975). Although these vesicles could be subcultured for extended periods of time, and the cells did seem to be imaginal in origin, attempts at subculturing continuous cell lines were unsuccessful (Sang, 1981).

Whilst problems existed for the generation of cell lines in *Drosophila*, imaginal cell lines were being derived from other species. Imaginal epithelial cells from the lepidopteran *Trichoplusia ni* were successfully isolated *in vitro* and maintained as a continuous cell line (Lynn *et al.*, 1982). These cells, which were derived through the primary culture of imaginal disc fragments, grow as fluid filled vesicles suspended in the medium. These cells were then subcultured by pipetting the vesicles, causing them to rupture. This method was again successfully utilised for the isolation and establishment of cell lines from the imaginal discs of two other Lepidopterans: *Spodoptera frugiperda* and *Plodia interpunctella* (Lynn and Oberlander, 1983). Therefore, it seemed that the growth requirements for *Drosophila* imaginal cell culture *in vitro* might prove to be more exacting.

Nevertheless, by the mid 1980s, the requirements of *Drosophila* tissue culture were beginning to be more accurately realised. Some groups had been devoting studies to observe the development of imaginal tissues *in vitro* (Fristrom *et al.*, 1973; Milner and Muir, 1987). Projects such as this, which tended to emphasise the importance of devising suitable growth media, paved the way for subsequent attempts at imaginal cell culture. An attempt was made at subculturing imaginal disc cells by Wyss in 1982. This particular

study utilised a culture medium which had been supplemented with insulin, a hormone present in *Drosophila* which is known to have a mitogenic effect on cells in embryonic primary culture (Seecof and Dewhurst, 1974) and an extract made from homogenised adult flies, which also is known to have a beneficial effect on cell survival (Grace, 1962). Wyss managed to induce limited colony formation, each containing around 100 cells, but failed to establish continuous cell lines. This may be a reflection not of culture medium inadequacy but of a damaging dissociation technique, which employed the use of protease VIII followed by vigorous pipetting (Wyss, 1982).

Subsequent imaginal cell isolation studies experimented with different dissociation techniques. Fausto-Sterling and Hsieh (1983) settled on the use of trypsin in a Ca^{2+} and Mg^{2+} free solution, followed by a period of stirring and centrifugation. This dissociation method produced an 80% cell viability as determined by trypan blue exclusion. Another method, used by Fehon and Schubiger (1985) and Fausto-Sterling and Hsieh (1987) centred on the use of collagenase, followed by a brief exposure of the cells to a solution of citric acid. This particular technique successfully dissociated cells of which around 90% were viable.

In 1987 Ui *et al.*, finally managed to establish a set of continuous cell lines from dissociated *Drosophila* imaginal discs (Ui *et al.*, 1987). In this study, imaginal cells were dissociated using a similar technique to that used by Wyss (1982) of protease treatment followed by vigorous pipetting. These cells were then suspended and subsequently maintained in a culture medium consisting of Cross and Sangs M3 (BF) medium which had been supplemented with 10% heat inactivated foetal bovine serum (FBS), insulin and either supernatant taken from primary embryonic cell cultures or larval haemolymph. In this study, 20-HE was observed to enhance cell aggregation and insulin was found to markedly enhance proliferation. Cell aggregates taken from established cell lines were observed to differentiate when

transplanted to metamorphosing larvae, although such adult features tended to be lost after prolonged culture *in vitro*.

This ability of isolated imaginal cells to differentiate to form adult structures was later found to be linked to the efficiency with which cell lines were originally established, the wing disc seemingly being the easiest to initiate and the most likely to produce adult features (Ui *et al.*, 1988). In this study, Ui and co-workers did not manage to successfully initiate continuous cell lines from eye and leg discs and attributed this to inappropriate culture conditions; maintaining that a medium which enhances cell proliferation also enhances the differentiation of adult structures.

However, at approximately the same time, a different group (Currie *et al.*, 1988) were succeeding in establishing continuous cell lines from both *Drosophila* wing and leg imaginal discs. Here, again, fragments of discs were dissociated through the use of an enzyme, which in this case was protease VIII (dispase) dissolved in Ca^{2+} and Mg^{2+} free saline (Dulbecco's formula PBS, known as D=: Dulbecco and Vogt, 1954). These fragments were then further dissociated by treatment with 1% trypsin, ethylene diaminetetraacetic acid (EDTA), centrifugation and pipetting to give a suspension of single cells mixed with multicellular clumps. These cells were then incubated in 96 well plates in a new medium based on Shields and Sangs M3 medium (Shields and Sang, 1977). M3 medium had already been modified by the addition of a low amount of foetal bovine serum (FBS), at a concentration of 2% (Edwards *et al.*, 1978) which was then referred to as MM3. To this, Currie *et al.*, (1988) added insulin, a small titre (1 ng/ml), of 20-HE and a fly haemolymph extract, known as FE2, which was made by the homogenisation of adult flies.

Under these conditions, the primary cultures of partially dissociated disc tissue proceeded to bleb and grow into multicellular vesicles, similar to those found in earlier studies (Schneider, 1972; Dübendorfer *et al.*, 1975); those cells which had been completely dissociated tending to die off. These vesicles,

and successive cell lines, could be induced to differentiate under the influence of 20-HE to form cuticular structures, suggesting that the isolated cells here were of an epithelial origin.

From the fragments of partially dissociated imaginal disc tissue, sheets of cells grew and spread onto the tissue culture surface. These cells could, after a sufficient phase of proliferation, be subcultured and were eventually deemed cell lines: the loose definition of an established cell line being that one which can be subcultured indefinitely (Currie *et al.*, 1988; Cullen and Milner, 1991; Schneider and Blumenthal, 1978). In each of these cell lines, there existed a broad variety of cell morphology, with epithelial, fibroblastic-like, lamellocyte-like and sickle-shaped cells all existing in the same cultures. It was unknown at this stage if each of these cells represented a range of cell types, or whether cells of a single type could display this diversity of morphology. It certainly seems feasible that some adepithelial cells, rather than the intended epithelial cells, could also have been selected during the isolation of imaginal cells for primary culture and these could account for the variety in the cell shapes that are seen. Another group of cells which are possible candidates for 'contamination' of these uncloned cell lines are the haemocytes which are known to adhere to the exterior of the imaginal discs (Milner and Muir, 1987). To test this, some imaginal cell lines were successfully cloned to provide homogenous populations of cells (Peel and Milner, 1990). The resulting cloned cell lines were found to exhibit a similar range of morphology to that seen in the uncloned lines, with the fibroblastic-like and epithelial like cells predominating. It was therefore concluded that variance in cell shape was not related to a specific parent tissue but was instead the result of local changes in their micro-environment.

These established leg and wing cell lines have been demonstrated to respond, morphologicaly and biochemically to the moulting hormone 20-HE (Peel and Milner, 1992b). In the presence of this hormone, cells stop dividing

and there is a degree of cell death. The remaining cells tend to aggregate, a phenomenon also reported by Ui *et al.*, (1987), and send out hugely elongated cell processes which connect with neighbouring aggregates. Putative pupal cuticle also seems to be expressed as a result of exposure of these cells to 20-HE, as confirmed by chitin synthesis analysis (Peel and Milner, 1992b). However, some cells were known to be insensitive to the growth-inhibiting effect of 20-E, and instead continued to proliferate.

During routine cell culture *in vitro*, established *Drosophila* imaginal disc cell lines, both cloned and uncloned, undergo a characteristic and stereotyped sequence of morphogenetic events, beginning with cell-substrate adhesion and ending with aggregation (Peel *et al.*, 1990). After the plating out of a seeding population onto a tissue culture surface, cells grow as a monolayer and reach confluency within approximately 2 days. The cells then begin to aggregate which, by around 7 days of growth, results in the formation of numerous multicellular foci dotted on the tissue culture substrate, with large areas between them left denuded of cells.

Cells within the fluid-filled vesicles which form during the primary culture of imaginal disc fragments (Currie *et al.*, 1988) are known to exhibit apical-basal polarity much the same as is seen in cells of the disc proper (Poodry and Schneiderman, 1970) and, as mentioned before, are known to differentiate after treatment with 20-HE. In these vesicles, the apical, microvilli covered, surface faces inwards to the fluid filled lumen and the basal side, which lacks any obvious basal lamina, faces outwards (Peel *et al.*, 1990). A full array of cell-cell junctions, as one might expect to find *in vivo*, are also found here. The morphology of established cell line cells, however, is quite different to that of cells found in the vesicles of the primary cultures. Here the aggregating cells lack any obvious apical basal polarity, and there are apparently no intercellular junctions between neighbouring cells. Some cell processes, which are packed with microtubules, are seen to extend from

cells between aggregates, presumably contacting other cells, but do not appear to terminate in any specialised junctions (Peel *et al.*, 1990).

During the course of one passage *in vitro*, from the time cells are plated out as a monolayer on day 1 to the time most cells are found in huge multicellular aggregates on the tissue culture surface at around day 7, imaginal cells most likely undergo changes in their adhesive specificities. One such period of imaginal cell growth would entail a series of movements, presumably beginning with cell-substrate adhesion, followed by some form of migration into aggregates and ending with cell-cell adhesion once the cells were within the aggregates. Peel *et al.*, (1990) suggested that this aggregation was due to the contraction of the cell processes that were seen to extend between neighbouring aggregates. This is reflected *in vivo* by the elongation and shortening of basal processes, or feet, which are known to extend to neighbouring cells, between the basal cell surface and the basal lamina and are believed to play a role in directing cell shape change and rearrangement (Locke and Huie, 1981; Fristrom 1989). An example of such a process which is known to occur *in vivo* is seen in the *Drosophila* peripodial membrane. During the extensive prepupal contraction of the peripodial membrane as the driving force behind imaginal disc eversion, the cells change shape from squamous to columnar (Milner *et al.*, 1983; Milner *et al.*, 1984a). At the basal surface of these cells during this process, numerous basal cell processes are seen and these may mediate the cell shape change from cuboidal to columnar.

The cells of the imaginal disc proper are sometimes pseudostratified in appearance, the nuclei being found at various positions relative to the apical and basal surfaces (Poodry, 1980). The cells of the peripodial membrane are also known to undergo pseudostratification during disc eversion, further reducing the membrane's surface area (Milner *et al.*, 1984a). This phenomenon may reflect what is seen *in vitro* when imaginal cells pile up on each other during aggregate formation. Imaginal cells are also known to grow and

migrate on top of each other in the elongated tracts and fascicles of cells that are often seen linking the aggregates (Peel *et al.*, 1990).

The greater aim of the experimental work that I have carried out, and which comprises much of this thesis, was to further elucidate the modes and mechanisms by which *Drosophila* imaginal cells underwent movement *in vitro*, and to contrast this with what is known about imaginal cell morphogenesis *in vivo*. Movement of imaginal cells *in vitro* encompasses the broad series of events observed during routine cell culture that begins in monolayer formation and ends in aggregation. It appears that this *in vitro* 'morphogenesis' may initially require cell-substrate adhesion which would then, perhaps following a period of migration, be supplanted by cell-cell adhesion.

The interpretation of adhesion, both cell-cell and cell-substrate, have changed over the years, tending to move away from the concept of adhesiveness as merely being some sort of intercellular cement to a more defined view of a dynamic interaction at the cell surface, mediated by receptor ligand interactions which are intimately linked to the cytoskeleton and the behaviour of the cell.

J. P. Trinkaus realised that cellular adhesion was both complex and dynamic (see Chapters 4-7 in: Trinkaus, 1969), and of a central importance in biology. He illustrated the latter point by paraphrasing Warren Lewis, who wrote in 1922, "Were the various types of cells to lose their stickiness for one another and for the supporting extracellular white fibers, reticuli, etc., our bodies would at once disintegrate and flow off into the ground in a mixed stream of ectodermal, muscle, mesenchyme, endothelial, liver, pancreatic, and many other types of cells" (Trinkaus, 1969).

Vertebrate cell adhesion proteins have often been identified through the use of adhesion assays and antibody blocking whereas *Drosophila* adhesion proteins have been identified through other means. Some strategies

have centred on the identification and examination of an interesting mutant phenotype and the subsequent identification, by various molecular methods, of the related gene and the encoded protein, which is then tested for homology with known vertebrate molecules. A more likely scenario, however, is that *Drosophila* CAMs have been discovered through the use of randomly generated monoclonal antibodies in an immunocytological screen. Here, instead of an interesting phenotype being selected, an interesting distribution of antigen is searched for. It was through such screens that the PS integrins and fasciclin III (a member of the Ig superfamily) were originally discovered.

Three of the experimental chapters in this thesis (3, 4 and 6) are devoted to defining the expression and functional role of certain cell adhesion molecules (CAMs) each of which represent one of the three major families of CAMs that are known to exist in *Drosophila*. These families comprise the cadherins, the PS integrins and the immunoglobulin (Ig) superfamily (for respective reviews, see: Takeichi, 1988; Brown, 1993; Grenningloh *et al.*, 1990).

Acknowledging that defining cell adhesion may be problematic, some of the assays that are used in this study have been adapted and designed to measure specific types of cell to cell and cell to substrate interactions. The reaggregation assays which are used extensively in this study (adapted from: Fausto-Sterling and Hsieh, 1987) are simple methods for measuring the extent of physical connections between cells in a single cell suspension over a fixed time period.

Antibody blocking studies have also been used extensively in the past, to disrupt the correct function of certain molecules in cell lines and tissue explants *in vitro* and for tissues *in vivo* and to assign putative roles for them. Masatoshi Takeichi and co-workers have carried out many assays centred around the use of antibodies to successfully determine the role of cadherins in various vertebrate systems (Yoshida-Noro *et al.*, 1984; Matsunaga *et al.*, 1988;

Hirai *et al.*, 1989). Antibody blocking assays such as these were also employed throughout this study, in tandem with various immunochemical analyses.

Figure A.

Figure A.   Figure **A.1** details a schematic sagittal section of a leg imaginal

disc. Imaginal discs are infoldings of the larval epidermis composed of

thickened columnar epithelial cells (ce), also known as the disc proper,

with microvilli at their apical end facing into the disc lumen (l).

Opposite the columnar epithelium is the peripodial membrane (pm).

The basement membrane (bm) encloses the entire disc. Lying between

the basement membrane and the columnar epithelium are the muscle

precursors, known as the adepithelial cells (ad), nerve (n) and

tracheolar cells (not shown). A short stalk (s) connects the disc to the

larval hypodermis. (After Poodry and Schneiderman, 1970.)

Figure **A.2** represents the location of each imaginal disc within the

third larval instar and the corresponding part of the adult fly that they

would form following metamorphosis. (From Walbot and Holder,

1987.)

A.1.

A.2.

Labium
Clypeolabrum
Femur
Eye-Antenna
Wing-Thorax
First leg
Second leg
Third leg
Haltere
Abdomen
Genital apparatus
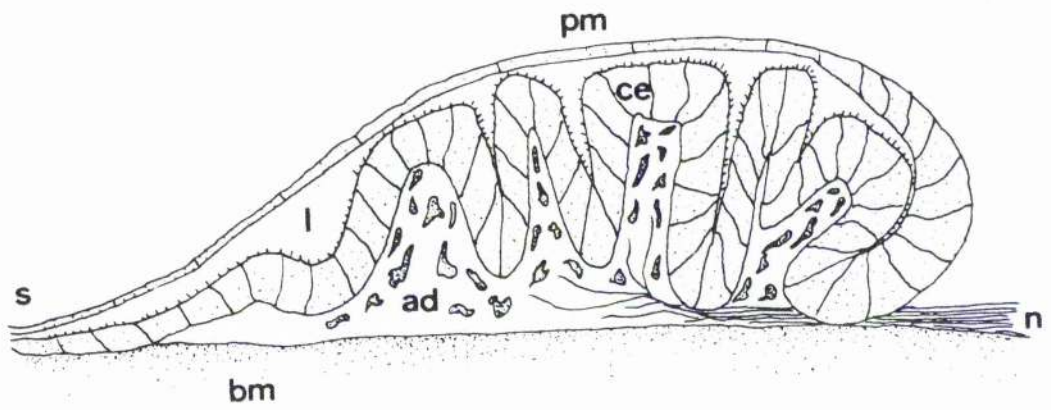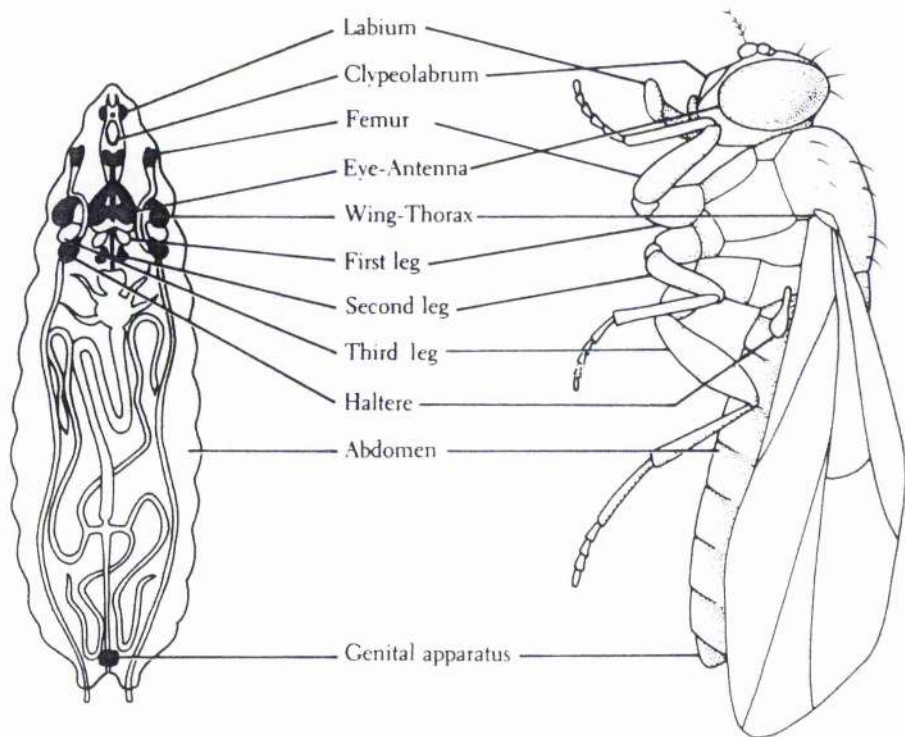
Table 1.   Existing cell line nomenclature and history

The cell lines listed in this Table are the ones which have been used in the course of this study, and do not represent all available lines. The numbers entered in the column for passage number denote the numbers of passage since cloning for that cell line. In most cases the primary culture was carried out by Currie *et al.*, 1988, and the subseqent cloning (denoted by the prefix 'Cl.' before a name) was carried out by Peel and Milner (1990). The lines L. 4, L.13, L. 14 and L. 15 were cloned from the leg cell line, L127D6. Each cell line was derived from late third instar imaginal disc epithelia from *Drosophila* larvae, unless otherwise stated.

Name	Tissue of Origin	Characteristics	Current passage no.*
Cl. 8+	Wing	20-HE responsive	30 (100)
Cl. 8R	Wing	20-HE unresponsive	30 (33)
Cl. 9	Wing	20-HE responsive	30 (10)
Cl. C9	Wing	20-HE unresponsive	30 (24)
Cl. 13	Wing	20-HE unresponsive	30 (18)
W20C6	Wing	20-HE responsive	30
L127D6	Leg	20-HE responsive	30
L. 4	Leg	Reduced adhesion	30 (6)
L. 13	Leg	Reduced adhesion	30 (6)
L. 14	Leg	Reduced adhesion	30 (6)
L. 15	Leg	Reduced adhesion	30 (6)

Table 2. **Shields and Sang's MM3 Medium, (Shields and Sang, 1977).**

(composition as mg/100 ml)

Salts		Amino acids (*cont.*)	
$MgSO_4.7H_2O$	400	alpha-alanine	150
$CaCl_2.2H_2O$	932	Valine	40
KCl	260	Methionine	25
$NaH_2PO_4.2H_2O$	88	iso-leucine	25
		Leucine	40
		Tyrosine	25

Amino acids			
Monosodium glutamate	786	**Sugar**	
Aspartic acid	30		
Threonine	50	Glucose	1000
Serine	35		
Asparagine	34		
Glutamine	60	**Vitamins, etc**	
Phenylalanine	25		
beta-alanine	25	T.C. Yeastolate (Difco)	100
Histidine	55	Choline. Cl	5
Tryptophan	10	Oxaloacetic	25
Arginine	50	BIS-TRIS buffer	105
Cysteine.HCl	20		
Lysine.HCl	85	Penicilin G.Na	3
Proline	40	Streptomycin Sulphate	10
Glycine	50		

Made up in double-distilled H_2O and pH brought to 6.8 with 1% NaOH.

Table 3. **Complete sterile medium (CSM), for use in routine subculture**

Complete medium is based on previous modifications of Shields and Sang's MM3 medium, (Currie *et al.*, 1988; Cullen and Milner, 1991).

2% foetal bovine serum (FBS)

2.5% Fly extract (FE2)

1 ng/ml 20-hydroxy ecdysone (20-HE)

0.125 I.U./ml Insulin

Reagents were added to Shields and Sangs MM3 medium and sterile filtered through a 0.22 µm Millipore filter.

Table 4. **Saline buffers used for routine cell culture**

Phosphate buffered saline (PBS):

Using Dulbecco's formula (Dulbecco and Vogt, 1954)
(Units represent mg/l)

NaCl	8000
KCl	200
Na_2HPO_4	1150
$CaCl_2.2H_2O$	132
KH_2PO_4	200
$MgCl_2.6H_2O$	100

pH 7.2, filter sterilised.

Table 4. (contd).

D= (Ca^{2+} and Mg^{2+} free PBS)

Using Dulbecco's formula, (Dulbecco and Vogt, 1954)
(Units represent mg/l)

NaCl	8000
KCl	200
Na$_2$HPO$_4$	2000
KH$_2$PO$_4$	400

pH 7.2, filter sterilised.

2.1   Introduction

The reason for writing this short section was to detail and unify some of the general methods that were employed throughout the course of the entire study, placing emphasis on the more routine techniques such as imaginal cell line subculture. Those techniques which were deemed more specific in their applications are detailed separately in the relevant experimental chapters. Some of the protocols used for the general maintenance of the imaginal disc cell lines can also be found in the following papers: Currie *et al.*, (1988); Peel *et al.*, (1990); Peel and Milner (1990) and Cullen and Milner (1991).

Imaginal disc cell culture

Imaginal disc cell lines had been originally established according to Currie *et al.*, (1988) from dissociated imaginal disc fragments. Some of these cell lines were subsequently cloned as detailed in Peel and Milner (1991). A selected list of current cell line nomenclature and tissues of derivation is contained in Table 1.

These cells were grown in Shields and Sangs modified MM3 medium (Shields and Sang, 1977) the constituents of which are listed in Table 2. This medium had been later modified for imaginal disc growth (see Table 3) through the addition of foetal bovine serum (FBS), insulin, fly extract and 20-hydroxyecdysone (20-HE) and is referred to as complete sterile medium (CSM). Due to a problem encountered with a bacterial contamination early on in this project, and in light of the relatively insignificant effect it has on increasing cell yield (Cullen and Milner, 1991), 20-HE was later omitted from all batches of CSM.

Fly extract (FE2)

The fly extract used as an additive to CSM, referred to as FE2 (Cullen and Milner, 1991), was prepared by homogenising 200 well nourished, egg laying, flies in 1.5 mls of MM3 medium. These flies are not sexed, although the fly extract which was devised for use in primary culture, FE1, used a ratio of 3:1 female to male flies. The extract was then centrifuged at 2,600 rpm (1500g) for 15 minutes at 4°C. The orange supernatant was then removed along with the oily film which forms at the surface, which is believed to consist primarily of the fat body. This supernatant was then incubated in a waterbath at 60°C for 5 minutes, to heat inacitvate the enzyme tyrosinase, the activity of which tends to turn the extract black. The extract was then centrifuged again at 2600 rpm for 90 minutes at 4°C. The supernatant was then removed and pipetted through a 0.22 µm Millipore filter and was stored as individual aliquots at -20°C.

Foetal Bovine Serum (FBS)

The foetal bovine serum used as an additive in the CSM throughout this study was supplied by Globepharm which was aliquoted as 2 ml amounts in sterile 5 ml vials and stored at -20°C.

Insulin

The insulin used in this study was supplied by Sigma (25 I.U./mg. Cat. No: I-6634, derived from bovine pancreas), and was dissolved as 10 mg in 500 µl of hydrochloric acid (HCl). This was then added to Ca^{2+} and Mg^{2+} free phosphate buffered saline (D=), to give a working concentration of 12.5

I.U./ml and was filter sterilised by pipetting through a 0.22 µm Millipore filter.

Routine subculture of imaginal disc cell lines

Imaginal cells were passaged once every 7 days when in culture. The culture medium from a plate of cells would be removed and the cells would be rinsed in 1 ml of D=, to minimise the inhibitory effects that certain constituents of CSM have on trypsin action. A 0.1% solution of trypsin (Difco Bacto) in 2mM ethylenediaminetetraacetic acid (EDTA) in D= was then added to the cells for 5 minutes at 20°C before being inactivated with 2 mls of CSM. Latterly, as established imaginal disc cells reached high passage numbers (around 80-100) the cells were more easily removed from the tissue culture surface, and this stage was often omitted. The cells were then pipetted off of the tissue culture surface and were centrifuged at 1500 rpm (150 g) for 5 minutes. The supernatant was then pipetted off and the cell pellets were resuspended in 1 ml of CSM. An aliquot was taken, diluted 1:100, and used to determine the total cell count using a Neubauer haemocytometer. Cells were then plated out at a seeding density of 3×10^6 cells/5ml CSM onto a 50 mm Petri dish (Nunc). Dishes were then dated and marked for cell line name and passage number and were placed in humidified incubator with a 5% CO_2 atmosphere and set at 25°C.

Cryogenic storage of cell line stocks

For long term storage, cell lines were preserved in a frozen state. Cells to be frozen were prepared at 3 days growth, to ensure they were in a proliferative stage of growth. The cells were then harvested and counted, using the routine passage method detailed above. This sample was then

diluted in CSM to give a concentration of 1×10^7 cells/ml and, to this, 10% sterile dimethyl sulphoxide (DMSO) was added. This mixture was then aliquoted out as 1 ml amounts to individual marked cryogenic storage vials and immediately placed in a freezing container. This container is a polystyrene box which contains a vial rack which overlays a liquid nitrogen soaked sponge. The vials are left to freeze in this container for 2 hours before being removed to a liquid nitrogen freezer.

Microscopy

During routine passaging and maintenance, imaginal cells were monitored using a Leitz Diavert photomicroscope set for phase contrast microscopy. Cells treated for visualisation by immunofluorescence were observed using a Biorad laser scanning confocal microscope. Cells treated for visualisation by transmission electron microscopy (TEM) were viewed using a Phillips 301 electron microscope.

Bradford method for protein determination

This is a commonly used assay, based on a method devised by Bradford (1976), for determining an unknown protein concentration in test samples based on a colometric change which can be monitored using a spectrophotometer. The method operates by comparing the results obtained for the unknown samples with that of samples of a known concentration. the readings from the known samples are presented as a standard curve, from which the concentration values for the unknown samples are estimated.

Bovine serum albumin (BSA) was used as a protein standard and was diluted to a concentration of 1 mg/ml. This stock solution was then aliquoted

out as duplicates, using a Hamilton syringe, as the following amounts: 0 μl, 2 μl, 4 μl, 6 μl, 8 μl, 10 μl, 15 μl and 20 μl.

Two aliquots were then taken from the unknown samples, usually of 5 and 10 μl, taking care to completely dissolve all the protein which were then diluted in 0.1 M sodium hydroxide (NaOH). These samples were then brought to the same volume with 0.2 M NaOH, to which 200 μl of Bradford reagent (50 mg Coomassie Blue (Brilliant Blue G), 25 mls 95% alcohol, 50 ml 85% phosphoric acid (brought up to 100 ml with H_2O and stored at 20°C) and 800 μl of distilled H_2O was added. Each reaction tube was mixed thoroughly and left to stand for 15 minutes. Each solution was then added for reading to the spectrophotometer, set at 595 nm. Optical Density (OD) values were read for each duplicated sample of known protein concentration and were used to plot a standard curve, from which the protein content of the unknown samples were determined.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Some cell samples prepared during the course of this study were analysed using SDS-PAGE, whereby samples were dissociated into their constituent polypeptide units by passing them through a polyacrylamide gel, driven by electrophoresis which were then bound to a membrane where they could be immunochemically probed for the presence of specific proteins. The protocols used in this thesis were based on those found in Sambrook *et al.*, (1982).

The concentration of acrylamide within the gel is directly related to the range of resolution, depending on how 'loose' the polymerised gel is which is determined by pore size. For the purposes of this study, three concentrations of acrylamide were generally used (see Table 5 (1)): either 5%, which

separates species between 57 and 212 kDa; 7.5%, which separates species between 36 and 94 kDa; or 10%, which separates lighter species, of around 16 to 68 kDa. A looser, stacking gel (Table 5 (2)) would then be added to the top of the polymerised gel prior to the samples being loaded.

Table 5 (1): **Solutions for preparing resolving gels for SDS-PAGE electrophoresis**

Solution components	Volumes (mls) for 15 ml gel		
	5%	7.5%	10%
H_2O	7.9	6.9	5.9
30% acrylamide mix	3.0	4.0	5.0
1.5 mM Tris (pH 8.8)	3.8	3.8	3.8
10% SDS	0.15	0.15	0.15
10% ammonium persulphate	0.15	0.15	0.15
TEMED	0.012	0.009	0.006

Table 5 (2) **Solutions for preparing a 5% stacking gel for SDS-PAGE electrophoresis**

Solution components	Volume (mls) for 5ml gel
H_2O	3.4
30% acrylamide mix	0.83
1.0M Tris (pH 6.8)	0.63
10% SDS	0.05
10% ammonium persulphate	0.05
TEMED	0.005

Prior to the samples being loaded to the gel they were first solubilised for 3 minutes at 100°C, in SDS loading buffer: 50 mM Tris.Cl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol. The gel was then placed upright between upper and lower reservoirs of running buffer (25 mM Tris, 250 mM glycine and 0.1% SDS and brought to a pH of 8.3.) and was resolved for 1 hour at 250 v (using a Mini-Protean II dual slab gel kit, Biorad).

The gel plus resolved proteins were then transferred to a nitrocellulose membrane (Sigma) using the Mini-Trans Blot part of the apparatus powered for 1 hour at a current of 100 mA in a reservoir of transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol). The next stage entailed the nitrocellulose membrane being probed using an unlabelled antibody against the protein of interest which is then incubated with a secondary reagent, which was either an anti-immunoglobulin or protein A which is coupled to horse radish peroxidase.

After transfer the membrane was removed and washed in a Tris buffered saline solution, known as TTBS (100 mM Tris, 0.9% NaCl, pH 7.5) and then 'blocked' in a solution of 1% non-fat dried milk in TTBS for 1 hour at 25°C. The membrane was then given three 10 minute washes in TTBS and incubated with a solution containing the primary antibody of interest. The nitrocellulose membrane was washed and blocked before a secondary or tertiary antibody was applied. After a final set of washes the membrane was stained using the horseradish peroxidase/3, 3' -daminobenzidine (DAB) substrate reaction as follows: 6 mg of diaminobenzidine tetrahydrochloride was dissolved in 9 ml of 0.01 M Tris.Cl (pH 7.6) to which 1 ml of 0.3% (w/v) $NiCl_2$ was added. 10 μl of 30% H_2O_2 was added to this solution and the solution was then added to the washed and blocked membrane. As soon as banding could clearly be seen on the nitrocellulose, the reaction was stopped by rinsing in water and transferring to PBS.

3.1   Introduction

In recent years much has been discovered about the molecular basis of adhesion in animal systems. As mentioned in the Overview, in Chapter 1, the cell adhesion molecules (CAMs) that are known of in *Drosophila* fall into three major categories: the immunoglobulin-like molecules and the cadherins, which behave as monomers, and the PS integrins, which are dependent on ligands for their function (Grenningloh *et al.*, 1990; Oda *et al.*, 1994; Hynes, 1992). The expression and proposed function of representatives of the immunoglobulin (Ig) and integrin classes are addressed in more detail elsewhere in this thesis. This chapter outlines some assays that were carried out to determine the gross adhesive capabilities of cultured imaginal disc cells, and provides evidence that implicate the existence of molecules that belong to the third subclass, the cadherins. This chapter also details the cloning of a leg disc cell-line with reduced cell-cell adhesive capabilities.

The cadherins are a family of transmembranal Ca^{2+} dependent glycoproteins (for reviews, see: Takeichi, 1989; Takeichi, 1991; Takeichi, 1993) which exist as members of specific subclasses, four of which have been well characterised in vertebrates. These are known as E-cadherin (epithelial cadherin), P-cadherin (placental cadherin), N-cadherin (neural cadherin) and L-CAM (liver cell adhesion molecule). The first known *Drosophila* cadherin, DE-cadherin, has recently been identified and is known to be structurally and functionally homologous to the vertebrate cadherins (Oda *et al.*, 1994).

If cadherins are targeted using specific antibodies, normal cell-cell adhesion and morphogenesis is sometimes disrupted. The cadherins were named as such in a study which used anti-cadherin antibodies to prevent cell-cell adhesion in mouse teratocarcinoma and embryonic cell monolayers *in vitro* (Yoshida-Noro *et al.*, 1984), thus indicating a potential role in cell-cell adhesion. Cadherins bind via homophillic interactions, selectively adhering to

molecules of their own type. This suggests that, beyond merely functioning in mediating cell-cell adhesion and in the formation of tight junctions, cadherins may be involved in selective cell-cell adhesion and sorting out, which certainly seems to be the case in vertebrates (for review, see: Takeichi, 1991). L cells, which normally have little cadherin activity and which have been transformed to express either E- or P-cadherin and are observed to adhere together and with a specificity related to the particular cadherin they are expressing (for review, see: Takeichi, 1989). This and similar assays confirm the involvement of cadherins in preferential cell-cell adhesion in vertebrate development.

It was known that a protein with cadherin-like structure existed prior to the discovery of a DE-cadherin.This is the product of the tumour suppressor gene *fat*, but the encoded protein is believed to be too large to be considered as a true cadherin and has not yet been demonstrated to be involved in cell-cell adhesion (Mahoney et al., 1991). Oda et al., (1994) managed to copurify a 150 kDa glycoprotein along with *Drosophila* α-catenin, which was also known to associate with Armadillo (β-catenin). The cDNA for this molecule was then cloned and the encoded molecule was found to have a significant sequence homolgy (ranging from 24-37%) with the vertebrate cadherins. The cDNA of this molecule was then transfected to the normally non-reaggregative Schnieder's line 2 (S2) cells and was found to promote cadherin-like adhesion. DE-cadherin is known to localise to the apical end of cell-cell contact zones in embryonic epithelia and is believed to function in the construction and maintenance of these tissues (Oda et al., 1994). During the larval stages, DE-cadherin is ubiquitously expressed again at the apical pole of the lateral surfaces of imaginal disc epthelial cells and is again believed to be involved in the setting up and maintenance of the epithelia (Oda., pers. comm.).

Takeichi reported that cell-cell adhesion could be functionally categorised into two systems; either Ca^{2+} dependent or Ca^{2+} independent (Takeichi, 1977). These two systems can co-exist on single cells, and can be removed by trypsin under different conditions. The Ca^{2+} dependent system (CADs) is very sensitive to trypsin but can be protected from proteolysis by the presence of calcium ions. The Ca^{2+} independent system (CIDs), on the other hand, requires high levels of trypsin to be inactivated but is not protected by the presence of calcium ions. Cadherins are believed to form the major component of CADs (Takeichi, 1988).

Such selective cell adhesion systems also seem to exist in *Drosophila*. Dissociated cells from different imaginal discs are known to sort-out and form homotypic aggregates (Nöthiger, 1964; Garcia-Bellido 1966; Fehon and Schubiger, 1985; Fausto-Sterling and Hsieh, 1987; Peel, 1991). These results indicate that cells from different discs, and even different regions of the same disc, have recognition properties and are able to sort-out. It certainly seems possible that the ability of imaginal cells to sort out may depend on the expression of cadherins.

The potential benefit of using *Drosophila* imaginal disc cells in reaggregation and sorting-out studies is clear. In the past, experiments designed to test the sorting-out capacity of imaginal disc cells employed the use of potentially damaging techniques to procure a population of single cells; such as by mechanical shear, enzymatic action or a combination of both (Garcia-Bellido, 1966; Fehon and Schubiger, 1985). Single cells from different discs, or different areas of one disc, would then be mixed in aggregates followed by a period of culture in the abdomens of early- to mid-third instar larvae. This period of *in vivo* culture ostensibly allowed for cell growth and sorting. After metamorphosis, which provided the correct environment for cuticular differentiation in both the host and the implanted aggregate, the aggregate would be removed as collections of cuticular vesicles. Cuticular

markers, such as multiple wing hairs, ebony and yellow, were then used to determine the identity of the cells in these vesicles.

These techniques, as one might imagine, can be problematic. The dissociation techniques used may be too damaging and a period of imaginal cell proliferation *in vivo* may give the appearance of sorting-out (Poodry *et al.*, 1971; Fehon and Schubiger, 1987). It is known that in primary cell culture that a large proportion of isolated single cells die (Currie *et al.*, 1988; Cullen and Milner, 1991). Extensive cell death in such a system would tend to favour the growth of clumps rather than single imaginal cells. There also exists the possibility that an imaginal cell's fate within an aggregate may be altered by the process of pattern respecification cause by local interactions with neighbouring cells (Bryant, 1978).

Fehon and Schubiger (1985) avoided prolonged *in vivo* culture, and used very specific cell markers in their assays whilst Fausto-Sterling and Hsieh (1987) observed sorting out of imaginal cells *in vitro*, in roller culture, again with specific cell markers. Use of imaginal disc cell lines has the advantage that the cells do not have to be dissociated prior to use in reaggregation and sorting assays *in vitro*. Some earler studies utilised collagenase to dissociate the cells followed by exposure to citric acid, almost certainly damaging the cell surface (Fehon and Schubiger, 1985; Fehon and Schubiger 1987). Peel carried out some experiments using imaginal disc cells *in vitro*, the results of which suggested that some sorting out between cells of different disc types, such as wing and leg, did occur (Peel, 1991).

Reaggregation assays, based on protocols used by Fausto-Sterling and Hsieh (1987) and Peel (1991) were used to determine the cell-cell adhesive properties of imaginal disc cell lines *in vitro* and to what extent this was calcium ion dependent. Similar assays are used elsewhere in this thesis. The ability of these cells to form aggregates in the presence and absence of trypsin was also considered.

Another short assay that was performed was designed to compare the reaggregative capacity of cells at early and late stages of morphogenesis. Imaginal disc cells early in growth, *in vitro*, are known to adhere strongly to the culture substrate. As morphogenesis proceeds, and the monolayer of cells begins to move into aggregates, this cell-substrate adhesion seems to be replaced by cell-cell adhesion, which is believed to drive aggregation (Peel *et al.*, 1990). If this was the case, then one would expect cell-cell adhesion to be strongest later in morphogenesis and that this may be reflected in an adhesion assay.

Cloning of a cell line with reduced reaggregative properties

As an adjunct to this study, a new group of leg cell lines were clonally derived from the existing L127D6 leg cell line. These cell lines were selectively cloned on the basis of their reduced capacity for cell-cell adhesion.

The protocol for this cloning study was adapted from a method devised by Peel and Milner (Peel and Milner, 1990; Peel, 1991) from a method originally used to clone vertebrate cell lines. The reason for creating a cloned cell line with a reduced reaggregative capacity was to determine if CAMs could be selectively lost from the imaginal cell surface, and what effect this would have on normal morphogenesis. If so, this would have certain implications for the long-term culture and use of imaginal disc cells, especially as models for use in adhesion studies.

3.2   Materials and Methods

3.2.1   Reaggregation studies

The protocol for measuring the rate of cell-cell adhesion in these cell lines was similar to those found elsewhere in this thesis, and was originally adapted from Fausto-Sterling and Hsieh (1985). This particular approach defines the rate of cell-cell adhesion within a population of cells, which are suspended in a test solution within a rotating cylinder, by recording the reduction of the number of single cells over a fixed time period.

Imaginal disc cell lines are believed to be very amenable for use as models in reaggregation and sorting-out studies. Peel found that established imaginal disc cell lines dissociated very easily, not requiring the mechanical shear or enzymatic treatment usually associated with the dissociation of cells from imaginal discs. Indeed, because less severe methods are needed to dissociate imaginal disc cell lines, the viability of these cells is very high (Peel, 1991). The reaggregation of *in vitro* derived imaginal disc cells is known to be comparable to, if not greater than, that of freshly dissociated imaginal disc cells and some existing *Drosophila* embryonic cell lines.

Cell lines to be used in these assays were grown to confluence, for 3 days, in Petri dishes to ensure an active state of growth. The cells were then washed with 1 ml of D= and were removed from the dish by vigorous pipetting using a Pasteur pipette. Next, each sample of cells was centrifuged for 5 minutes at 500 g and the resulting pellet was resuspended in 1 ml of D=. Aliquots of 4×10^6 cells were taken from each sample of cells and were added to 1 ml of the appropriate test solution and were centrifuged as before. Each cell pellet was then resuspended in the appropriate test solution in a 15 ml siliconised tube (Corning) in replicates of four. All tubes were rotated at 20 rpm about an axis of 8°C from the horizontal on a Spiramix roller (Denley).

Each imaginal disc cell line used in these studies was found to dissociate very well *in vitro*. This viability of these dissociated cells is believed to be very high. Older cell lines have a viability of up to and around 97% when dissociated, whilst the younger cell lines, of lower passage number, tend to be more difficult to dissociate and of these cells around 70% are viable (Peel, 1991). It is possible that this ease of dissociation and high viability of established imaginal disc cell lines reflects a reduced level of cell-cell adhesivity.

The rate of reaggregation in each sample was presented by plotting the percent decrease in single cell number as the Y axis, with the number of cells at time zero being taken as representing 100%, against the elapsed time of incubation, as the X axis. This method, thus, provided a visual representation of the rate of reaggregation in each sample. In some assays, the number of cells in each aggregate at different stages of incubation was measured, as an alternative to the measurement in single cell number reduction. Prior to counting, an aliquot was taken from each sample and was placed on a glass slide which was then coverslipped. In this way, any aggregates were gently squashed, and cell numbers could be recorded. For each sample at each time point, cell counts were recorded for 20 randomly chosen aggregates which were then normalised.

In this particular assay, the test solutions within which the cells were to be reaggregated were either phosphate buffered saline (PBS) or a solution of D= containing 2mM ethylenediaminetetra-acetic acid (EDTA). PBS contains the divalent cations, Ca^{2+} and Mg^{2+} whilst EDTA is a chelating agent and is known to remove these ions from the buffer. The formulae for both PBS and D= can be found in the General Methods section, in Chapter 2. The effect of adding trypsin, at a concentration of 0.01%, to these solutions was also tested. In these experiments cells were removed from dishes without the use of enzymes, trypsin only being added at the initiation of the assay, at time zero.

Cells that were at different stages of growth were also used in reaggregation assays to determine if there was any increase or decrease in calcium and magnesium ion dependent adhesion during morphogenesis. For this study, aliquots containing 1×10^6 Cl. 8R cells were taken at the day of plating and 4, 7 and 11 days after that. These were then suspended, as detailed above, in PBS and single cell numbers were counted at 0, 30, 60 and 120 minutes of roller culture.

The rate of cell-substrate attachment of imaginal disc cells, either in the presence or absence of divalent cations was also tested. For this assay, 3 day old Cl. 8+ cells were isolated, as for the previous reaggregaton assays, by pipetting and centrifugation. Aliquots of 1×10^6 cells were measured out and centrifuged down in 1 ml of either PBS or D= plus 2 mM EDTA. These cells were then plated out into individual wells of a 24 well plate (Nunc) as a single cell suspension in 1 ml of each test solution. and were incubated at 25°C. Four replicate samples were taken for cell counting at 0, 30 and 120 minutes for each test solution, by completely removing the medium in each well and leaving any adherent cells.

3.2.2   Cloning

The technique used for the cloning of a leg cell line was based on a protocol used by Peel and Milner (1990; Peel, 1991). This technique utilised a customised cloning plate which was made by adhering a 3 cm Petri dish base to the underside of a 5 cm Petri dish lid. The 3 cm plate was adhered to the underside of the larger dish by partially dissolving the rim in propylene oxide. The design of this dish allows singly plated cells on the 3 cm dish surface to grow in medium which is conditioned by a feeder layer of cells

immediately adjacent to them, on the 5 cm dish surface, whilst preventing any possible cross-contamination between these populations.

L127D6 cells for cloning were selected from populations of cells that displayed low reaggregative capacities. To isolate these cells, 4 tubes, each containing 1×10^7 cells which had been harvested after 6 days growth, were rotated on a Spiramix roller as before and left to reaggregate in MM3 for 2 hours at 25°C. After this period, the tubes were centrifuged at low speed (200 rpm for 15 minutes) to separate the multicellular aggregates from any single cells. After removal of any aggregates, single cells in the supernatant were incubated for a further 2 hours and centrifuged as before. The number of single cells in the supernatant was taken and an aliquot was then removed from each sample and plated at a low density (approxroximately 500 cells/dish) onto the 3 cm Petri dish base in 1 ml of CSM and left to adhere overnight at 25°C. In this way, a very sparse seeding of single L127D6 cells was created, each cell being physically unable to establish contact with the next.

Each feeder layer of L127D6 cells had been grown for 2 days, at a seeding density of 3×10^6 cells/dish, in a 5 cm Petri dish prior to use. The 3 cm dish bases from each of the four samples were then inverted onto the 5 cm dishes, in which the medium was presumably sufficiently conditioned, and incubated at 25°C. After 1 weeks growth the feeder layer was replaced, and this was repeated for 4 weeks.

After 4 weeks of growth, some colonies had grown to a sufficient size to be transferred to dishes containing only complete sterile medium (CSM), without the feeder layer. After 1 week of growth, each suitably sized clone, now containing hundreds of cells, was removed by careful scraping with a sterile silicone 'policeman' to a 96 well plate and were incubated in CSM. Again, after continued incubation, the cells from each well were transferred to a 24 well plate and then onto a six well plate and then to a 5cm Petri dish (all

plastics used were supplied by Nunc). These cells were then routinely passaged for the next 8 weeks until they had reached a sufficient state of proliferation. Thus 4 new cell lines representing each original sample were created, and were named: L4, L13, L14 and L15 (named after well noumbers in the 24 well plates they were taken from), and were then frozen down as established stocks.

These cell lines were subsequently used in reaggregation assays, as detailed above, to determine the rate of cell-cell adhesion in each line when in the presence of calcium ions (PBS). The reaggregative capacity of these lines was then compared to that of the parent cell line, L127D6.

3.3   Results

3.3.1   Reaggregation studies

Figure 3.A illustrates the typical reaggregation response of an imaginal disc cell line when incubated in PBS and D= with 2 mM EDTA. The subject for this particular assay was the cloned wing cell line, Cl. 8R. This graph illustrates well a marked difference in response of the cell line to incubation in either of the test solutions. Cells that were suspended in PBS, and were thus exposed to the metal ions Ca^{2+} and Mg^{2+}, had reaggregated to a great extent, with only around 20% of the cells failing to aggregate after 120 minutes of suspension. Most of this aggregation was achieved within a short space of time (30 minutes of incubation). Cl. 8R cells which had been incubated in D= with EDTA had not reaggregated to the same extent. In this environment cells seemed to adhere more slowly and less efficiently. After 120 minutes of assay, only around 60% of the cells had failed to reaggregate.

Figure 3.B details the rate of reaggregation of Cl. 8R cells in PBS and D= plus 2 mM EDTA, but this time trypsin (Gibco), at a concentration of 0.01%, had been added at the start of the assay. In both samples of cells there seemed to be a reduction in the extent of total reaggregation by the end of the assay, as compared to cells which had not been exposed to trypsin (Figure 3.A). However, some reaggregation was observed in both samples, the greatest adhesion being seen with the PBS treated cells. Cells in this sample, after 1 hour of culture, had achieved approximately 60% reaggregation. In each of the treatments, this early spurt of reaggregation was followed by a rise in the number of single cells and a return to a state found at time zero, suggesting that the aggregates which had formed in the first hour had then begun to dissociate.

Figure 3.C represents the rate and extent of reaggregation of another cloned wing cell, Cl. C9. This time, however, the rate of reaggregation has been recorded by counting the number of cells in each aggregate within a given field of the haemocytometer, after 120 minutes of incubation, and calculating the average number of cells in an aggregate for that treatment. Those cells which have reaggregated in PBS can again be seen to have formed aggregates more quickly and to a greater extent than those cells which have been incubated in D= with EDTA. What can also be seen from this graph is that there is a large variance in the number of cells found in aggregates which had been formed in PBS; with cell numbers ranging from a few cells to around fifty in different clumps.

In Figure 3.D, the average number of cells at various stages of reaggregation has been calculated for trypsin-treated cells in both PBS and D= with EDTA. Again, trypsin has been used at a concentration of 0.01%. Cells in each treatment here tend to form aggregates of varying sizes; the larger aggregates being found, perhaps predictably, in the PBS treated samples. However, as is also seen when monitoring the decrease in single cells (Figure 3.B), this initial period of adhesivity apparently only existed during the first 60 minutes of culture, which was then followed by a decline in aggregate-cell number over the remaining 60 minutes. The largest cell aggregates, thus, were found in the PBS treated samples midway through the assay, with the average aggregate size dropping by the end of 120 minutes.

A comparison of average aggregate sizes in the different assays suggests that aggregates can be equally large in both PBS and D= with EDTA either with or without trypsin. Therefore, from this assay, it seems that the calcium ion independent adhesion system (CIDs) in these imaginal cells is not degraded by trypsin, as one might expect it to be. The calcium ion dependent system (CADs) on the other hand, does behave as expected, with protection from trypsin proteolysis being seen during the first 60 minutes of culture.

Therefore, one can conclude that trypsin seems to have little effect on the early phase of reaggregation in both test solutions. However, by the end of the assay, the presence of trypsin does seem to have a deleterious effect on average aggregate size, dropping to numbers similar to those found at 30 minutes of culture.

In imaginal cells at different stages of morphogenesis, one might expect there to be a variance in cell-cell adhesivity, depending on the type of CAM expressed at that particular stage. Figure 3.E illustrates the rate of reaggregation of Cl. 8R cells at different stages of growth in culture. These results suggest that Cl. 8R cells are at their most adhesive at around 7 days of growth, i.e. during aggregate formation, although the adhesivity of each sample appears to be fairly similar to the next. These results also indicate that the cells are at their least cell-cell adhesive when either very early on in morphogenesis, as the cells form a monolayer on day 1 with little or no cell-cell adhesion, or when very late on in morphogenesis, on day 11 by which time the cells will have been in aggregates for a number of days.

These results provide some evidence that imaginal disc cells may change their modes of adhesion during morphogenesis depending on whether or not they have established contact with other cells. The normal sequence of *in vitro* morphogenetic events may be characterised by interactions and adhesion with the cell substrate during the early phase of growth, as the cells plate out as a monolayer and migrate or extend cell processes (day 0) followed by a period of cell-cell adhesion, during the intermediate stages of aggregation (days 4-7). Later stages (day 11) may, again, favour a lack of cell-cell adhesivity due to the cells already being found in aggregates thus suppressing the need for such adhesion.

This initial period of cell-substrate adhesion seems to depend very strongly on the presence of divalent cations. Figure 3.F illustrates the rate of cell-substrate adhesion for Cl. 8+ cells to tissue culture plastic, either in the

presence or absence of the divalent cations Ca^{2+} and Mg^{2+}, over a period of 2 hours. In the PBS sample, cell-substrate adhesion appears to be very rapid, with around 90% of all cells adhering to the plastic by 30 minutes of culture. The converse is true of cell-substrate adhesion in the D= plus EDTA samples, where, by the end of the assay only around 20% of all cells have adhered to the substrate.

3.3.2 Cloning

Within a few weeks of culture, some of the single cells plated to the underside of the cloning-plate had begun to proliferate and form isolated patches similar to those seen in Figures 3.G and 3.H, with fibroblast-shaped cells gradually growing outwards from a few aggregates at the centre. The same clonal group photographed in Figure 3.G is also pictured in Figure 3.I, by which time 14 days have elapsed and the feeder layer of cells has been removed: these cells have noticeably proliferated in the intervening period.

Figures 3.J, 3.K and 3.L represent subsequent stages in the process of deriving a new leg cell line, L. 14. Cells are removed from the original clone and moved to a 96 well plate. Following periods of proliferation, the cells were moved into 24 well plates (Figure 3.J), 6 well plates (Figure 3.K) and finally onto 50 mm Petri dishes (Figure 3.L) where they were routinely maintained. The leg cells pictured at the 24 well stage (Figure 3.J) seem to be growing sparsely, and have formed loose aggregates. During the 6 well stage and during growth in Petri dishes, as an established cell line, the L. 14 cells are epithelial in appearance and do not seem able to form definite aggregates (Figures 3.K and 3.L). Figure 3.M details cells from the parent line photographed at the same stage of passage as the L. 14 cells picture in Figure 3.L (5 days), which are at a more advanced stage of aggregation.

Figure 3.N illustrates the rates of reaggregation, in PBS, of four cell lines cloned from the parent line, L127D6. These results indicate that there does not seem to be a common trend of adhesion and that each cloned line has a different potential to reaggregate compared to the next. This reflects the clonal derivation of these lines and suggests that individual cells within an uncloned cell line, such as L127D6, may have different adhesive specificities.

From this assay, L. 14 was selected as a candidate line which exhibits a reduced capacity for short term cell-cell adhesion in roller culture. In Figure 3.N, 20% of all L. 14 cells have reaggregated by 60 minutes of culture, but this number has dropped by 120 minutes of culture to around 5%. Any aggregates that have formed are presumably very weakly cohesive, subsequently dissociating to single cells again.

Figure 3.O is a comparison of the rate of reaggregation of the cloned daughter line, L. 14, to that of the parent cell line, L127D6, when suspended as single cells in PBS. At the time of this assay, L. 14 had become an established line, the cells of which had been passaged routinely for 3 months. Therefore, it seems likely that the cells of this line could be considered sufficiently viable for use in adhesion assays such as these. This figure shows that the daughter cell line, L. 14, has a radically reduced capacity to reaggregate compared to that of the parent cell-line L127D6. L127D6, as one might expect from previous reaggregation assays, aggregates very rapidly and by 60 minutes of roller culture, approximately 60% of all single cells have formed aggregates. L. 14 cells, on the other hand, do not reaggregate very well at all and by the end of the assay, at 120 minutes, only around 10% of all cells seem to have reaggregated; an even lower estimate than that provided by the earlier assay designed to select cell lines on their reduced reaggregative capacity (Figure 3.N).

Figure 3.A   The rate of reaggregation of Cl. 8R imaginal cells in PBS and D=
plus EDTA. This graph clearly illustrates that the divalent cations, Ca^{2+}
and Mg^{2+}, are important, but not essential, for cell-cell adhesion in roller
culture. The indication here is that there may be two different
populations of cell adhesion molecules present: one that is cation
dependent and one that is cation independent.

Figure 3.B   The rate of reaggregation of Cl. 8R cells in PBS and D= plus
EDTA after being treated with trypsin. In this graph, some cell-cell
adhesion does occur during the early stages of the assay but these
aggregates seem to be partially dissociated, in both samples, by the end
of the assay

Fig. 3.A. Cl. 8R reaggregation: PBS and D= + EDTA
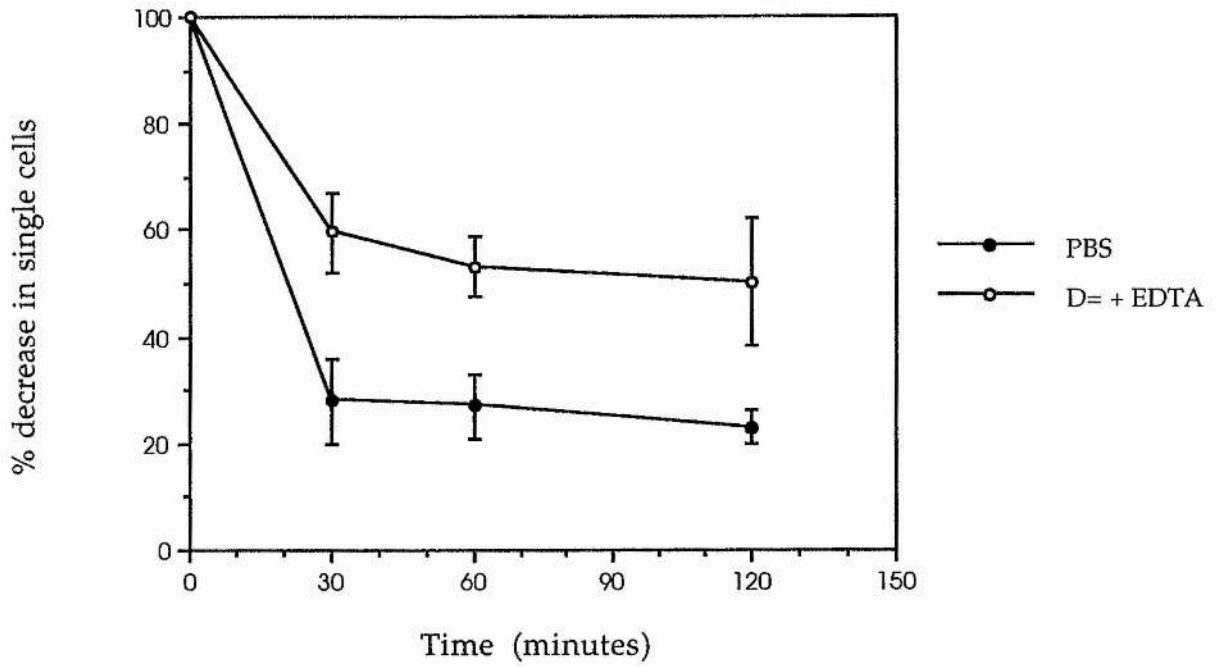
Fig. 3.B. Cl. 8R reaggregation (trypsin): PBS and D= + EDTA
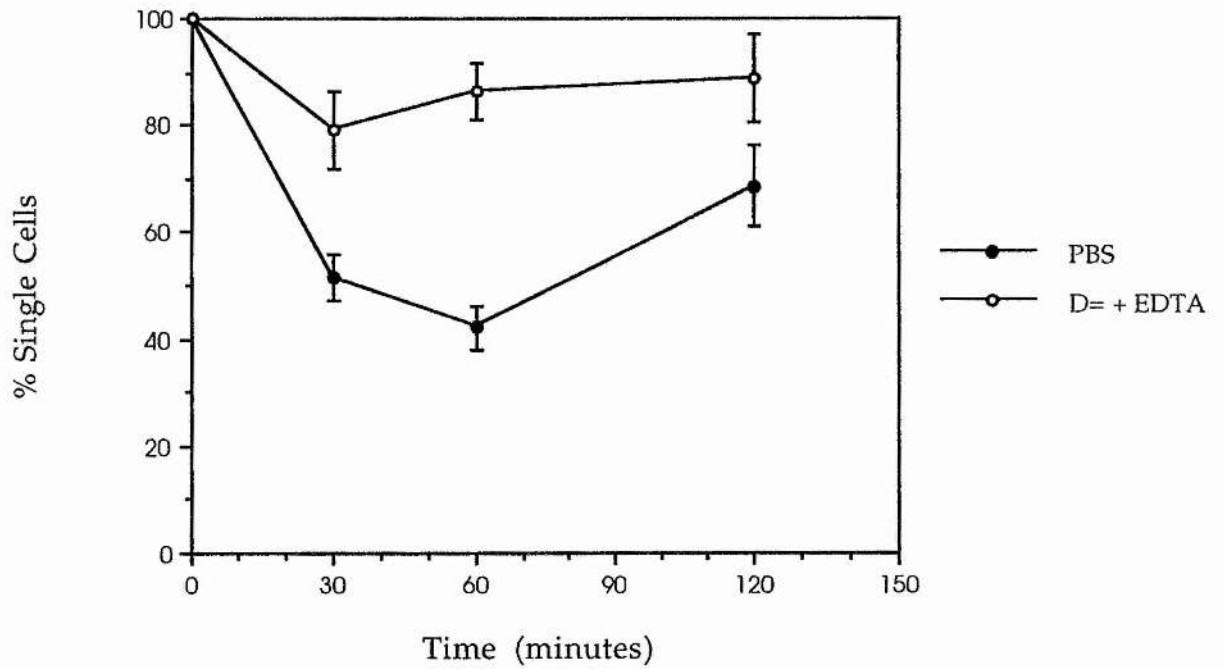
Figure 3.C The reaggregation of Cl. C9 cells in PBS and D= plus EDTA as determined by aggregate size. Again, over a period of 120 minutes, the presence of divalent cations is seen to enhance cell-cell adhesion, this time reflected in aggregate size. Here the average aggregate size by the end of the assay is larger in the PBS samples than in the EDTA containing ones. There is also seen to be a large variation in aggregate size in the PBS samples.

Figure 3.D Average aggregate size of Cl. C9 cells in PBS and D= plus EDTA after being treated with trypsin. As before (Figure 3.B) a period of initial cell-cell adhesion in both sets of samples is then reversed, so tat by the end of the assay any aggregates that may have formed are beginning to dissociate again. However, in the interim, at around 60 minutes after the start of the assay, the cells in both samples manage to aggregate as well as their counterparts in samples without trypsin, indeed perhaps even more so (D= plus EDTA).

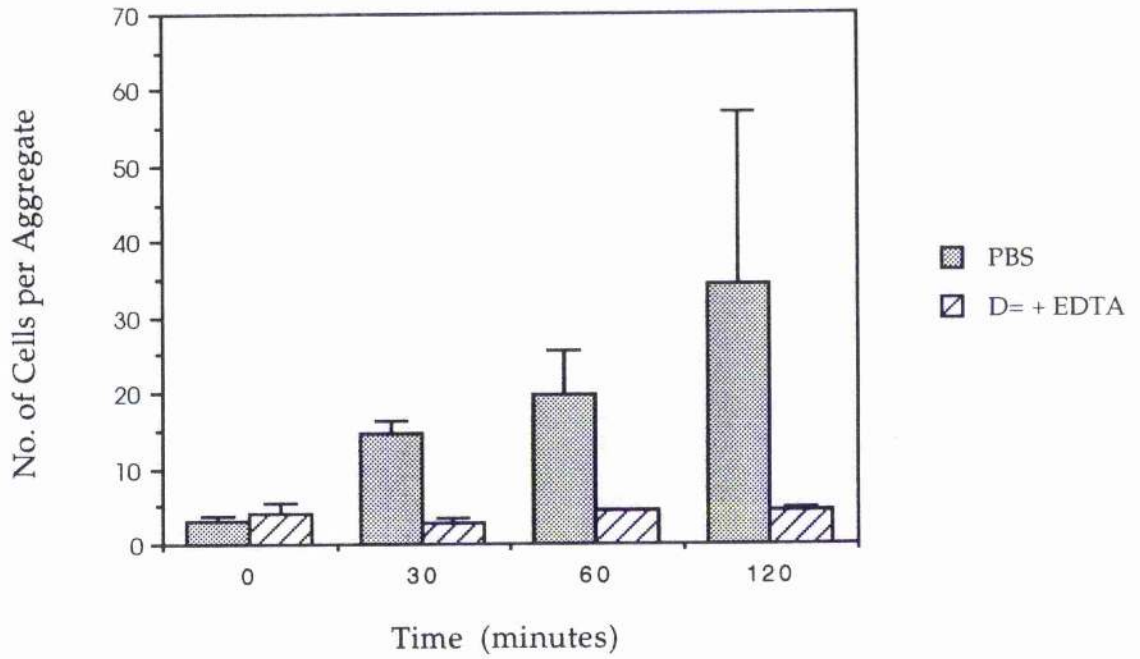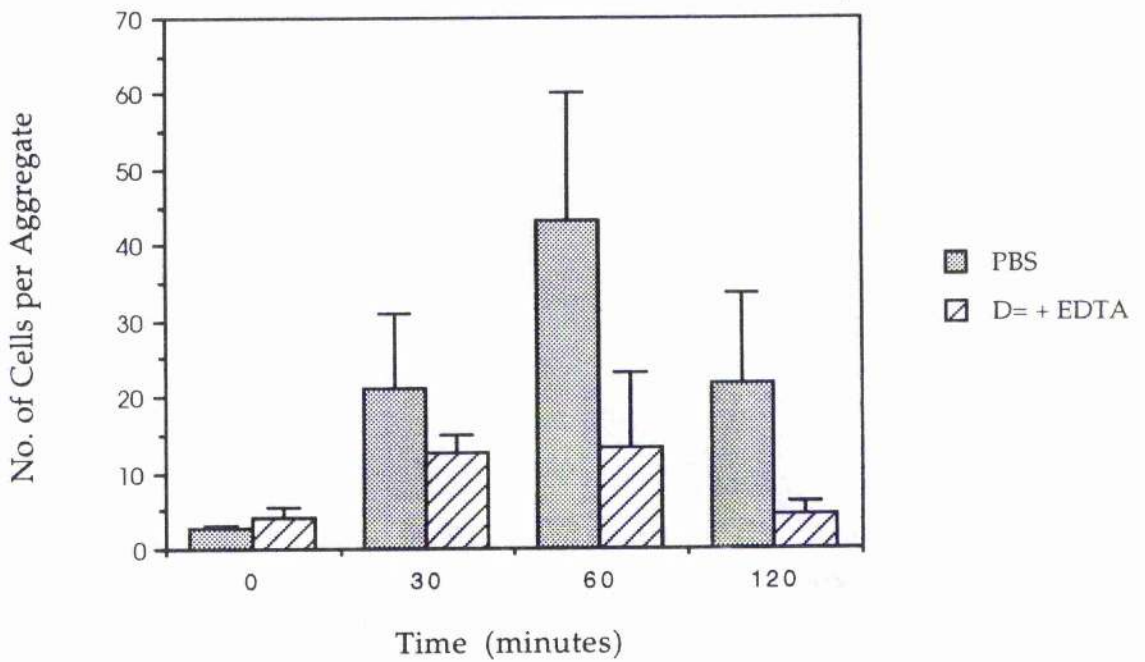**Fig. 3.C. Cl. C9 reaggregation: PBS and D= + EDTA**

Fig. 3.D. Cl. C9 reaggregation (trypsin): PBS and D= + EDTA

**Rate of reaggregation of Cl. 8R cells
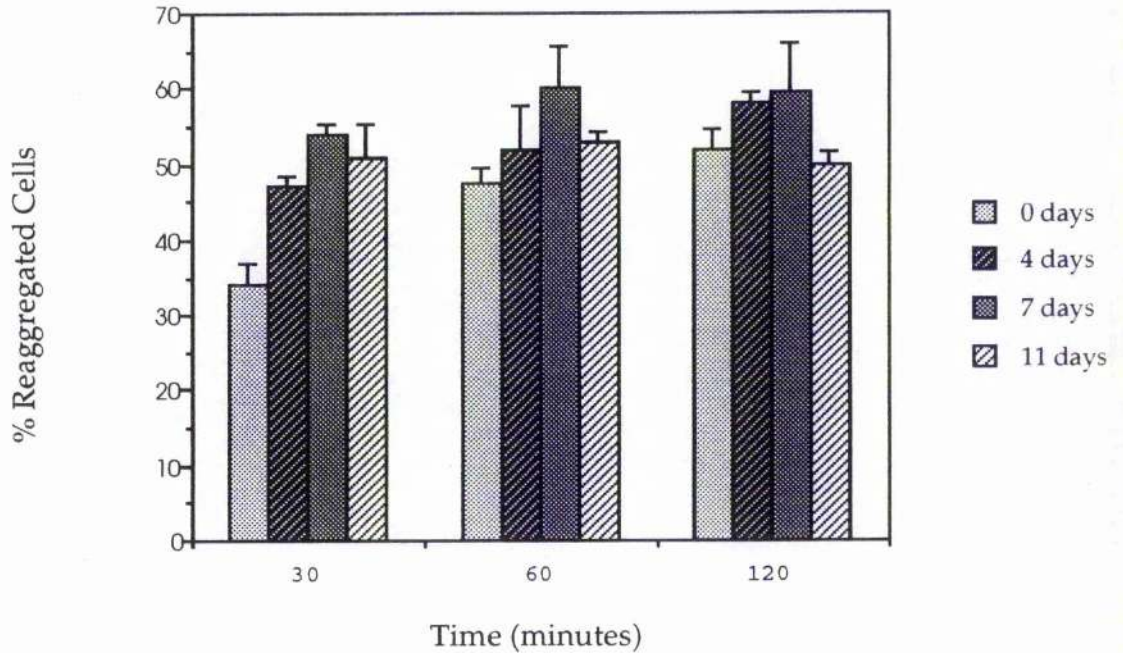at four different stages of morphogenesis**

Figure 3.E.   The rate of reaggregation of Cl. 8R cells at four different

stages of one passage *in vitro* (at 0, 4, 7 and 11 days after plating onto

tissue culture plastic). These results suggest that Cl. 8R cells have a

relatively low capacity for cell-cell adhesion very early on in growth.

As the cells progress in culture and begin to aggregate, cell-cell

adhesion seems to be enhanced, with strongest adhesion occurring at

around day 7. by day 11, the reaggregation potential has begun to

decrease.

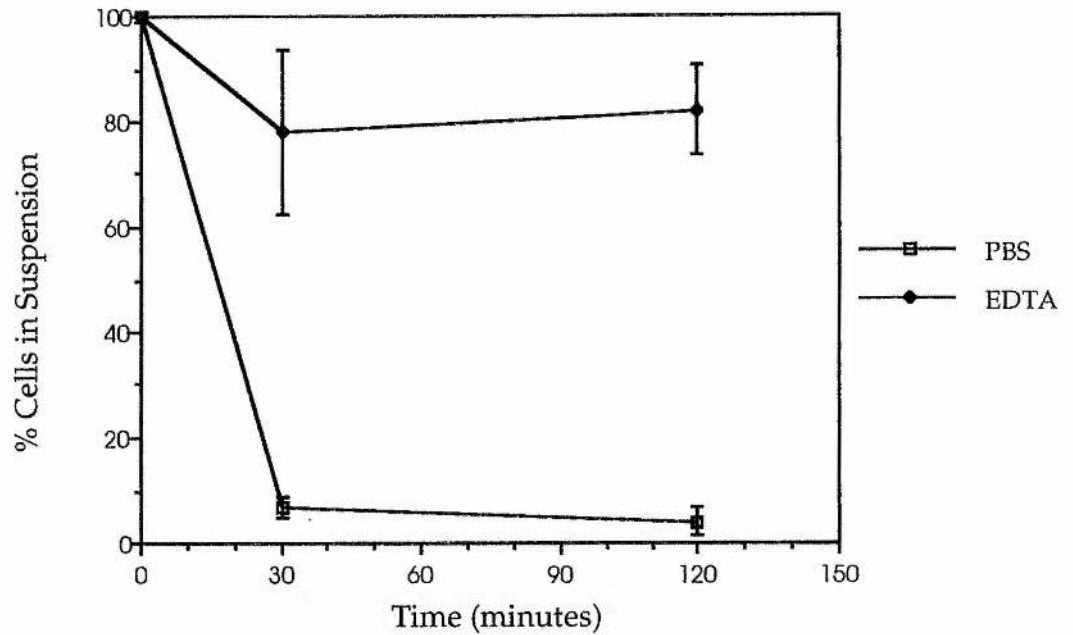**Rate of substrate adhesion of Cl. 8+ cells in PBS and D= with 2 mM EDTA**

Figure 3.F. The rate of adhesion of Cl. 8+ cells to a plastic substrate when incubated in media allowing for either the presence or absence of Ca^{2+} and Mg^{2+}. The adhesion of cells to the substrate when in PBS is very rapid and extensive, with almost all cells adhering wthin 30 minutes of culture. The opposite is the case when cells are cultured in D= plus EDTA: only around 20% of cells have adhered to the tissue culture plastic by the end of the 2 hour assay.

Figure 3.G   A light micrograph of a single clone derived from the unlconed leg disc line, L127D6, growing on the underside of a cloning dish, above a feeder-layer of cells (not visible). fibroblast-like cells (f) are seen spreading out from a group of aggregates at the centre of the micrograph.

Scale bar represents 100 μm

Figure 3.H   A phase-contrast micrograph of the edge of the clone featured in Figure 3.A. This image shows in greater detail the darkened, fibroblast-like (F) nature of the cells as they grow away from the centre of the clone.
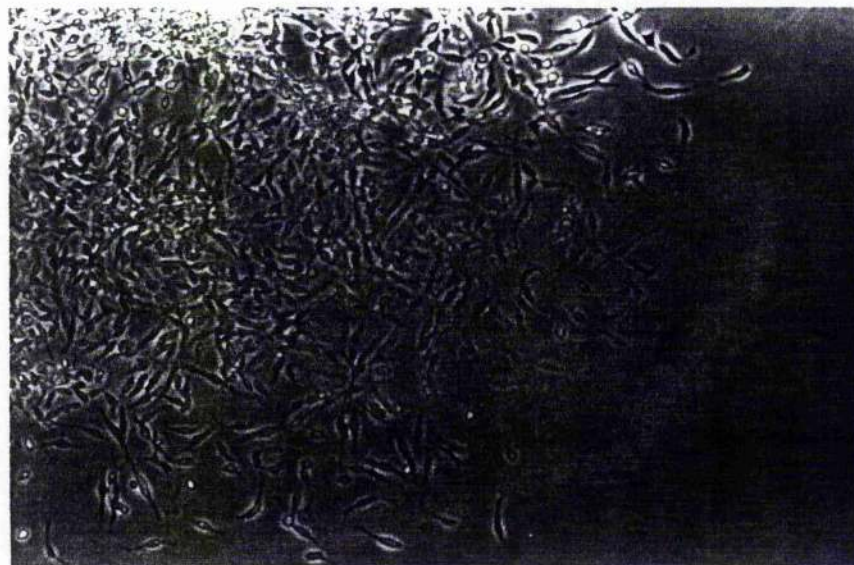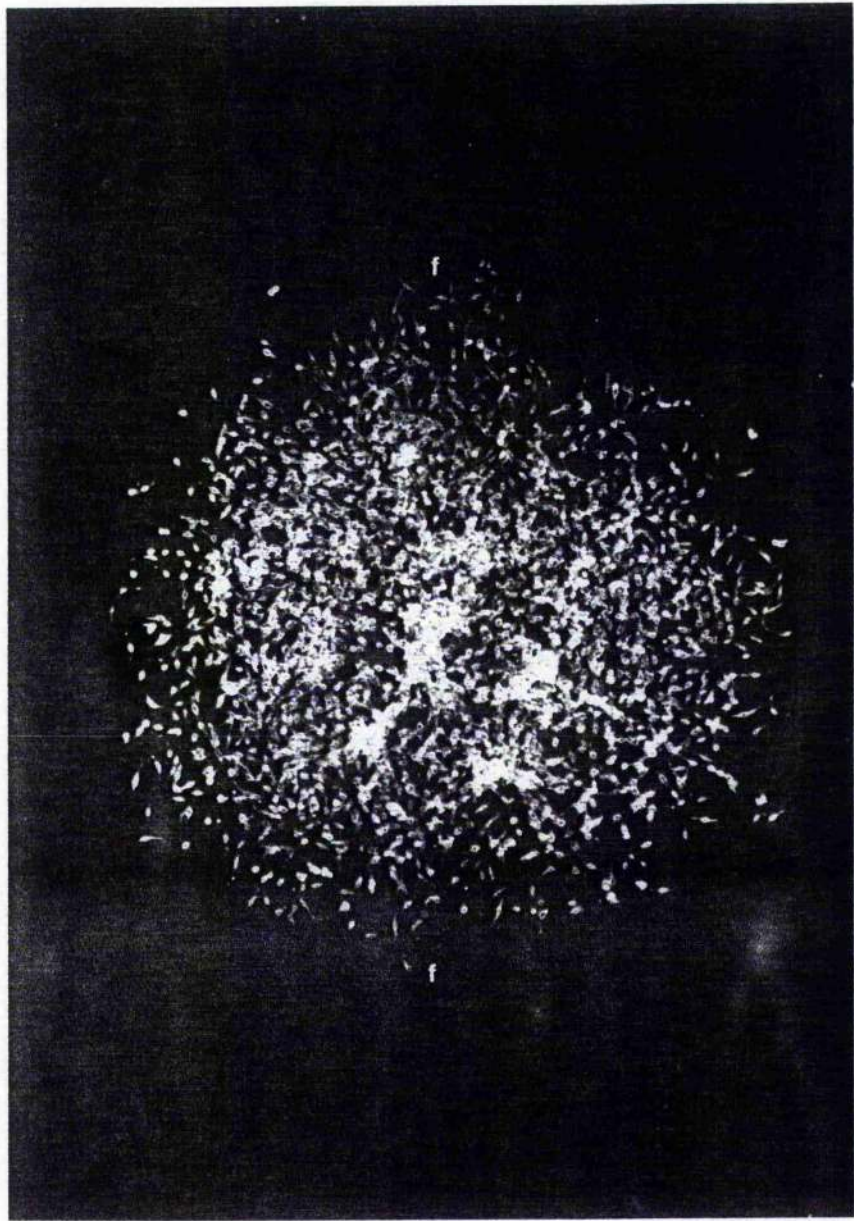
Scale bar represents 50 μm

Figure 3.I A light micrograph of the same clone seen in Figure 3.G after a further 14 days of growth. During that intervening period, the cells within the clone have noticeably proliferated and have become denser, but do not seem to have migrated much further from the periphery of the clone when compared to Figure 3.G.

Scale bar represents 100μm

Figure 3.J A detail of a clone which has been transferred to, and is growing in, a 24-well plate. These cells have already been passed through a 96 well plate and have now reached an advanced level competence of growth, proliferating well *in vitro*.
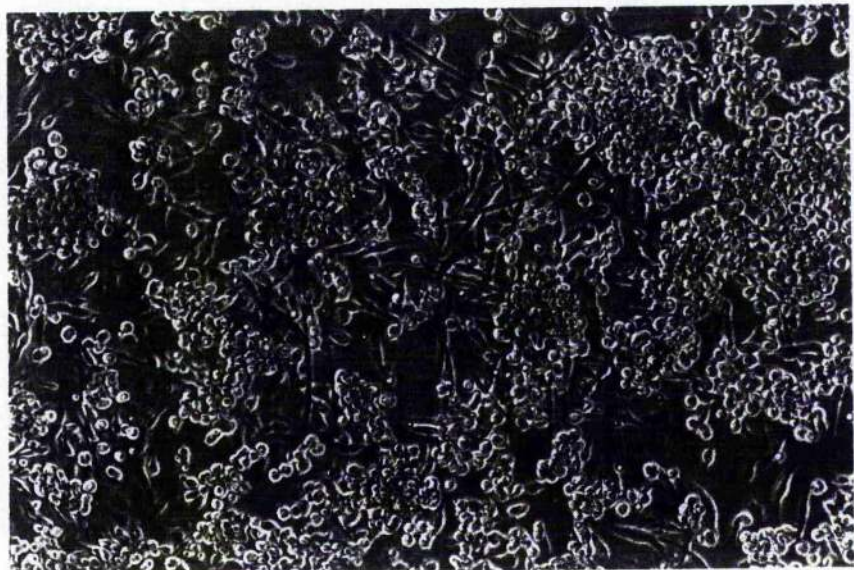
Scale bar represents 50 μm

Figure 3.K Cells of the clone L. 14 growing to confluence in a six well plate. These cells are by this stage responding to conditions *in vitro* much as an established cell line would. Cells in this culture do not seem to be forming dense aggregates and are mostly rounded in appearance.

Scale bar represents 50 μm

Figures 3.L and 3.M The established cell line, L. 14, growing in a 50 mm Petri dish 3 months after the initial isolation from the parent cell line, L127D6. Many of the cells in this picture are rounded and epithelial (e) in shape. These cells are pictured at day 5 of a passage, and have failed to form dense aggregates, which are evident in cultures of the parent cell line, L127D6, at a similar stage, in Figure 3.M below.
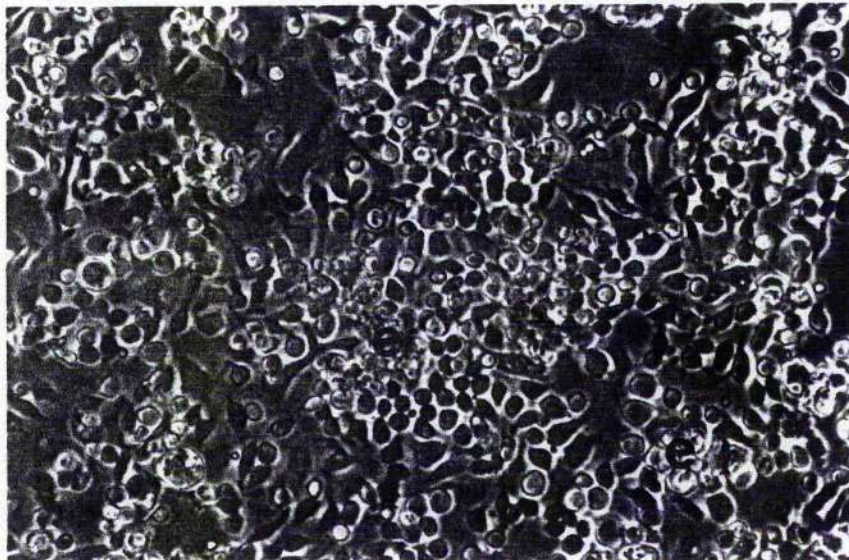
Scale bars represent 25 μm
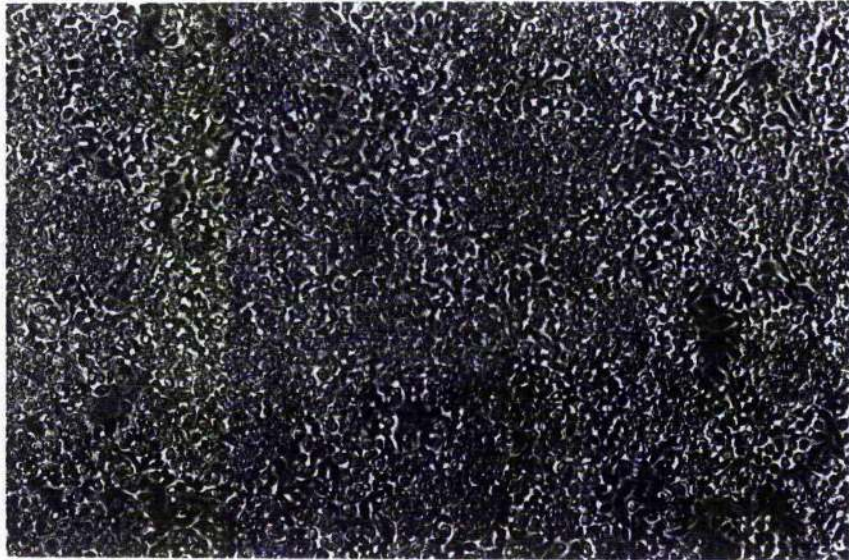
Figure 3.N   The rate of reaggregation, in PBS, of four cell lines cloned from
the existing uncloned leg disc cell line, L127D6. Each cell line has a
slightly different rate of reaggregation compared to the next. This
reaggregation ranges from the extensive, such as with the line L. 4, to the
minimal, as with the line L. 14.

Figure 3.O   A comparison of the rate of cell-cell adhesion of the daughter
cell line, L. 14, to that of the parent cell line, L127D6 (both in PBS). The
differences of ability of each cell line to reaggregate, as depicted here, is
radical. L. 14 has a significantly diminished capacity to reaggregate, with
only around 10% of single cells having aggregated by the end of the
assay, compared to 70% in the L127D6 samples.

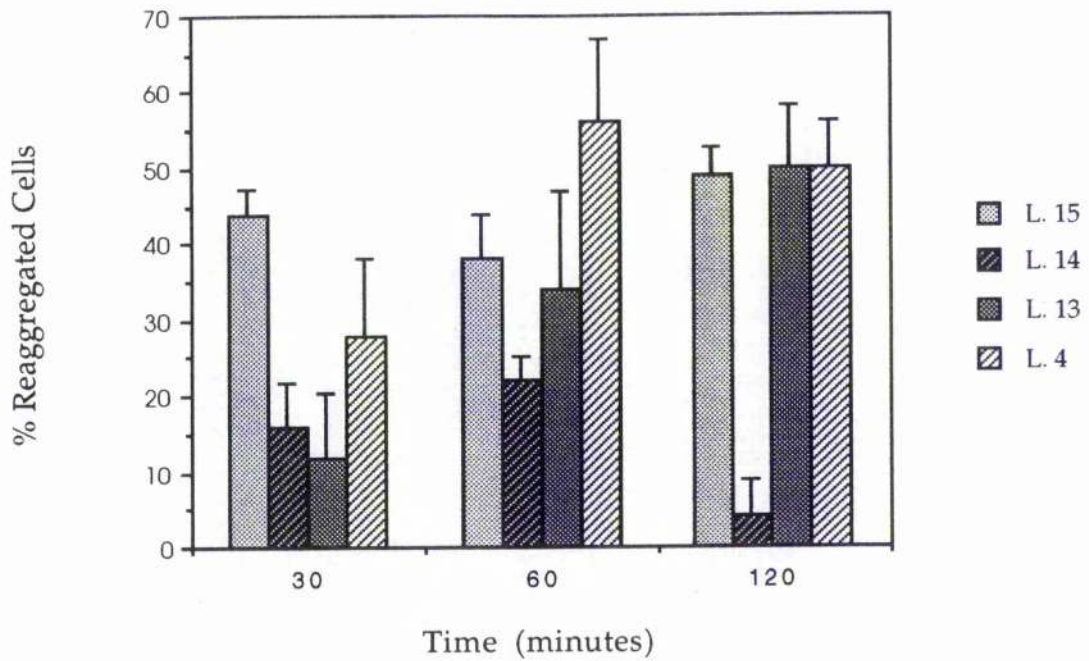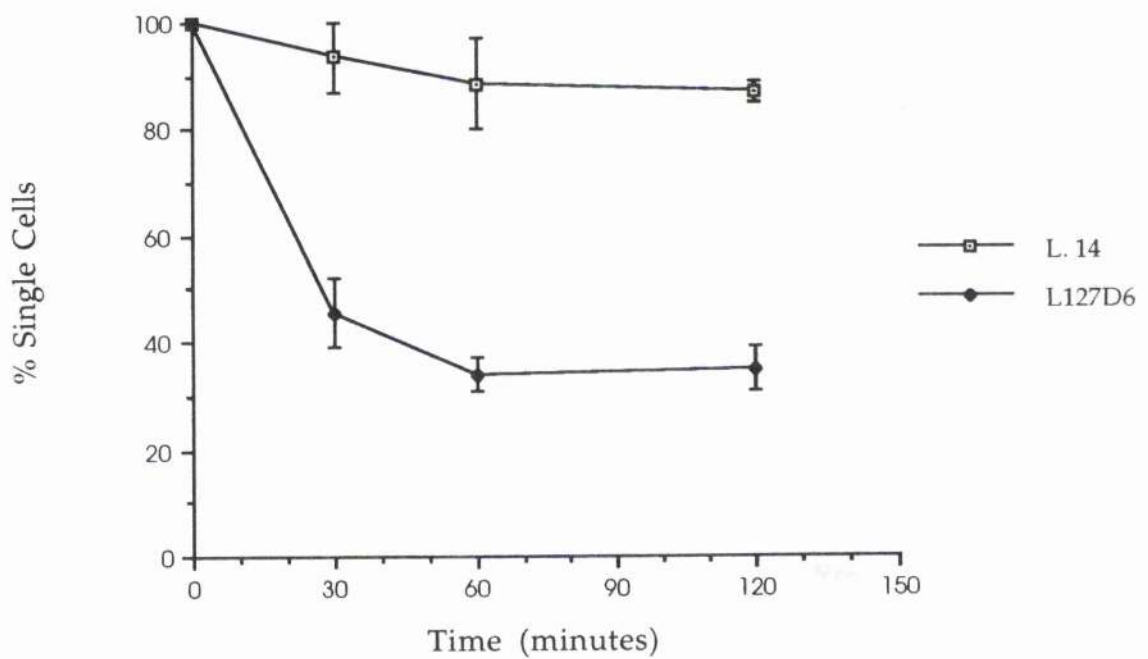# Fig. 3.N. Rate of reaggregation of four cloned leg cell lines

Fig. 3.O. Rate of reaggregation of parent (L127D6) and daughter (L. 14) cell lines.

3.4   Discussion

3.4.1   Reaggregation

From the results of the reaggregation assays covered in this chapter, some general conclusions can be made about certain modes of adhesion of *Drosophila* imaginal disc cells *in vitro*. The movement of imaginal disc cells from a monolayer into sharply defined aggregates *in vitro* has already been well documented (Peel *et al.*, 1990; Peel., 1991). Over a period of 7 to 10 days, a small seeding population of imaginal disc cells plated onto tissue culture plastic in complete sterile medium (CSM) will proliferate (Cullen and Milner, 1991) and migrate into multicellular foci which are evenly spread on the substrate surface. This process of morphogenesis would, therefore, necessitate a clearly defined system of cell-substrate followed by cell-cell adhesion mechanisms. The primary purpose of this chapter was to assess the short term cell-cell adhesion of imaginal disc cells *in vitro* and to postulate possible schemes for such a system.

It is also known that established imaginal disc cells can reaggregate very quickly and extensively when in single cell suspension in either PBS or MM3 medium (Peel, 1991). In that study, Peel made the observation that established imaginal disc cells reaggregated to a greater extent than *Drosophila* embryonic cells *in vitro*, instead more closely resembling the cell-cell adhesion of freshly dissociated imaginal disc cells (Gratecos *et al.*, 1990; Fehon *et al.*, 1987).

Danielle Gratecos and co-workers measured the reaggregation, in suspension, of cells derived by the mechanical disruption of gastrula-stage *Drosophila* embryos (Gratecos *et al.*, 1990). This aggregation was observed to be completely dependent on the presence of Ca^{2+} in the medium. They also observed that the reaggregation could be inhibited by trypsin, and that this

proteolysis could be blocked by the presence of free Ca^{2+}. Antibodies raised against the surface of these cells were also found to inhibit cell-cell adhesion in a trypsin-like manner. From this study, Gratecos *et al* proposed that a cell adhesion molecule (CAM) or molecules, similar to the vertebrate cadherins in function, could be expressed on the cell-surface and is/are involved in mediating cell-cell adhesion and the formation of intercellular junctions.

Oda *et al.*, (1994) found that a monoclonal antibody directed against the calcium-dependent *D*E-cadherin led to the blocking of normal reaggregation of cells freshly dissociated from 3- to 5-hour *Drosophila* embryos. It, thus seems possible that *D*E-cadherin could be contributing to the calcium-dependent reaggregation response in the embryonic cells as originally reported by Gratecos *et al.*, (1990).

In two earlier studies, Anne Fausto-Sterling and colleagues (1983; 1985), assayed for the adhesiveness of dissociated imaginal disc cells and cells from the established *Drosophila* embryonic cell-line, Schneiders Line 2 (S2), using assays similar to the one employed in this chapter. Freshly dissociated eye-antennal imaginal disc cells were observed to undergo an approximate 40% reduction in the number of single cell units, although the authors emphasised the potential problems manifest in the dissociation techniques, which relied upon trypsinisation and mechanical disruption (Fausto-Sterling and Hsieh, 1983). This strong reaggregation phenomenon was later used in a sorting-out assay by the same group (Fausto-Sterling and Hsieh, 1987). In the later study, Fausto-Sterling *et al* discovered that S2 cells, which tend to have a low adhesiveness, depended on the presence of calcium ions but not magnesium ions for aggregation. Trypsin was also found to reduce the extent of aggregation, but this could be blocked by the presence of a high concentration of calcium ions (Fausto-Sterling and Hsieh, 1985). This particular study benefited from the lack of a need to disperse a tissue to a

population of single cells using potentially disruptive means, as S2 cells already exist in a single, dissociated, cell state.

The reaggregation of all imaginal disc cell lines tested in this study was rapid and extensive. The rate of reaggregation of Cl. 8R, reported in this chapter is typical of the reaggregation of all tested imaginal disc cell lines. These findings reflect the reports of Peel (1991) in that imaginal disc reaggregation *in vitro* tends to be extensive and fast, with most of the cells being found in aggregates within the first 60 minutes of roller culture. Established imaginal disc cell line cells reaggregate to a significantly greater extent than cells which have been freshly dissociated. Approximately 80% of all suspended Cl. 8R cells in PBS have reaggregated by the end of the assay (Figure 3.A), compared to the value of around 40% for dissociated imaginal disc cells (Fausto-Sterling and Hsieh, 1983). Cells from lines derived from embryos and cells from dissociated embryonic tissues seem to reaggregate to levels similar to those for imaginal disc lines; reaggregating to around 80% in each instance (Fausto-Sterling *et al.*, 1990; Gratecos *et al.*, 1990).

Imaginal disc cell reaggregation, to a large extent, seems to depend on the presence of divalent cations in the suspension medium. However, imaginal disc cell reaggregation is not exclusively dependent on calcium and magnesium ions, as some adhesion can occur in their absence (Figure 3.A). It therefore seems possible that two separate cell adhesion systems reminiscent of the calcium dependent (CADS) and calcium independent system (CIDS), as reviewed by Takeichi (1988), exist here.

In vertebrates, some of the identified cell adhesion molecules (CAMs) can be categorised as being either CADS or CIDS in function. Those proteins which are known to be CIDS-like in function are members of the immunoglobulin (Ig) superfamily such as N-CAM, which is expressed widely in developing and adult tissues and is proposed to be involved in adhering a variety of neural and non-neural cell types, and L1, expressed by postmitotic

neurons in the brain, is believed to mediate neuron-neuron interactions and fasciculation (for reviews see: Rutishauser, 1993; Schachner, 1993). CADS -like molecules which have been characterised in vertebrates include the integrins, heterodimeric transmembrane proteins which are mostly ligand dependent and mediate cell-substrate and cell-cell adhesion in many tissues, and the cadherins, which form the major component of known CADS molecules (Hynes 1991; Takeichi, 1988). Invertebrate CAMs that would fall into these categories have also been characterised. The fasciclin family of molecules, which are members of the IgG superfamily, are Ca^{2+} independent in function whereas the PS integrins, which are homologous in structure and function to the vertebrate integrins, are known to be Ca^{2+} dependent, as are the *DE*-cadherins (Patel *et al.*, 1987; Hynes, 1992; Oda, 1994).

Both the CIDS and CADS mechanisms for adhesion are further characterised by differing responses to trypsin, as mentioned in the introduction to this chapter. The CADS system is highly sensitive to trypsin, but is protected from proteolysis by the presence of Ca^{2+}, whereas the CIDS system needs a high concentration of trypsin to be inactivated but unlike CADS is unprotected by Ca^{2+}.

The presence of trypsin in roller-cultures of imaginal disc cells did cause a decrease in the extent of reaggregation, as might be expected but during the early stages of these assays some reaggregation was seen to occur (Figure 3.B). During the first 30 minutes of culture, reaggregation occurred almost as well as it did in cultures where trypsin was absent. Up until this point neither the calcium dependent (CADS) or independent (CIDS) systems seemed to be affected much by the presence of 0.01% trypsin in the medium. However, by the end of the assay, at 120 minutes, the aggregate size in both systems had dropped. Thus it seems that only a prolonged exposure to trypsin has a deleterious effect on reaggregation. CIDS adhesion is greatly reduced in the presence of trypsin, as one might expect, but is not completely

removed. This may be due to a concentration of trypsin which is too low to sufficiently inactivate the molecules on the cell surface, although the trypsin here was ten times more concentrated than that used by Fausto-Sterling and Hsieh (1985) to disrupt embryonic cell reaggregation.

These results were reiterated when the average aggregate size during roller culture was measured (Figure 3.C). The largest aggregates were found at 120 minutes of culture in the samples which contained Ca^{2+} and Mg^{2+} (PBS). Those aggregates formed in the absence of divalent cations (D= with EDTA) were very small in comparison, only consisting of, at most, a few cells. The average aggregate size in PBS was also seen to vary a great deal; from just a few cells to around 70. Peel (1991) reported that imaginal disc cell aggregate size in PBS tended to be small, when compared to cells that had been reaggregated in complete sterile medium (CSM), but this is not reflected in these findings. Aggregate size in PBS may indeed be smaller than those formed in CSM, but some large aggregates were observed under these conditions.

The assay designed to measure the effect of trypsin on average aggregate size in PBS and D= with EDTA yielded a result which reflected the findings of the original assay to measure the reduction of single cells when in suspension, with the greatest reaggregation being seen in the PBS samples. As before, the initial period of reaggregation was followed by a decline in aggregate size. Nevertheless, there was some reaggregative activity in each sample, which, until 60 minutes of culture, reflected the rate of reaggregation seen in samples in the absence of trypsin. In the PBS suspended samples, the aggregates contained as many as 60 cells, which was approximately the same as was seen in samples that do not have any trypsin, although the overall aggregation of cells in samples that contain trypsin was less than in samples that did contain it (Figure 3.D).

Again, in this assay, the presence of trypsin did not completely inactivate CIDS adhesiveness, as one might expect, and this, again, may reflect a concentration of trypsin that was too low. In both assays that had trypsin, CADS function also seemed to be degraded, but only in the latter stages of the assay. Again, only a prolonged exposure of the cells to trypsin seemed to have a deleterious effect on imaginal cell reaggregation.

Imaginal cell-substrate adhesion seems to be as dependent on the presence of Ca^{2+} and Mg^{2+} in the culture medium as imaginal cell-cell adhesion is, if not even more so. This dependency on divalent cations for adhesion to the substrate could be attributed to the requirements of the PS integrins (Brown, 1993), which are expressed during imaginal disc cell morphogenesis (Peel and Milner, 1992; Chapter 4, this thesis). The *Drosophila* PS integrins are known to mediate cell adhesion and spreading on certain substrates, with evidence being provided by studies using embryonic cells on extracellular matrix components (Hirano *et al.*, 1991; Gullberg *et al.*, 1993) and through the transfection of integrin genes to cells which do not normally adhere to the substrate (Bunch and Brower, 1992).

A switch in the expression of functional CAMs during morphogenesis?

Cells at very early and late stages of morphogenesis, when forming a monolayer or in defined aggregates, seem to have a reduced capacity to reaggregate when compared to cells which are at intermediate stages of growth and are actively involved in aggregate formation. These results suggest that there may be a shift in adhesion strategies throughout morphogenesis; the cells initially being engaged to a great extent in cell-substrate adhesion , followed by a period of cell-cell adhesion as the cells migrate into aggregates. Indeed, primary cultures using imaginal discs to yield new cell lines indicate that cells early after plating are very strongly

adherent to the tissue culture surface and are difficult to remove, either by trypsinisation or by shear. However, as these cells begin to aggregate, it becomes progressively easier to remove the cells from the plastic, suggesting that the original cell-substrate association has weakened (D. Cottam, pers. comm.)

Peel *et al.*, (1990) suggested that imaginal cell aggregation *in vitro* was more likely to be dependent on cell-cell interaction than on cell-substrate migration. Such a mode of aggregation would be different from the "traction-induced" models proposed by Harris *et al.* (1984) and discussed by Bard (1990) for mesenchymal cell condensation. The traction-induced models rely upon cell-substrate interaction for aggregation to occur. Peel *et al* made this assumption on the strength of an assay which involved the plating of cells onto a substrate of tissue culture plastic that had already supported the growth of imaginal cells, which had subsequently been flushed off, presumably leaving behind any extracellular matrix (ECM) material that had accumulated there during aggregation. They found that imaginal cells plated onto this surface did not aggregate any quicker than cells on control, non ECM-coated, dishes and concluded that a traction based mechanism was not functioning here.

However this does not sufficiently prove that a traction based model for aggregation is entirely absent; merely providing some evidence to contradict it. Indeed, the findings of Chapter 4 imply that, in some instances, the cell substrate can enhace imaginal cell aggregation. Here, W20C6 cells were observed to aggregate more quickly on a substrate of used tissue culture plastic than on an unused surface (see Figure 4.J). On a substrate of fibronectin, which was found to promote cell adhesion and growth, some imaginal cells were seen to form discrete, dense aggregates while control cells existed essentially as a monolayer (see Figure 4.P). Perhaps a future study could be directed at selectively blocking cell-cell adhesion, such as through

the use of antibodies against cell-cell adhesion molecules or junctional complex proteins, to determine more exactly the roles of cell-substrate and cell-cell interactions during aggregation.

What seems likely is that there is not a sharply defined switch during morphogenesis, from a mode of cell-substrate adhesion to one of purely cell-cell adhesion, as this study goes some way to indicate, but that an ensemble of CAMs, both cell-substrate and cell-cell, are expressed, with slight variations, throughout. Indeed, the differences between these two systems may actually be very subtle. A traction-induced system for morphogenesis identifies the interaction, and subsequent generation of force, between a cell and its substrate. It seems likely that the sort of adhesion-motifs that a cell uses to adhere to its substrate, such as the RGD pentapeptide sequence on the ECM protein tiggrin that is known to act as a ligand for the PS integrin $\alpha_{PS2}\beta_{PS}$ (Fogerty *et al.*, 1994), may also exist at sites of cell-cell contact, therefore the dichotomy between cell-substrate and cell-cell adhesion is becoming less clear.

Some evidence for a shift in strategies for adhesion during morphogenesis, albeit subtle in imaginal disc cells *in vitro*, is provided by the system of keratinocyte division and differentiation in the human epidermis. The epidermis is a stratified squamous epithelium where terminally differentiated cells are continually shed from the outermost layers of the tissue which are then replaced by the proliferation of stem cells in the basal layer.

Human keratinocytes are known to express several members of the integrin family and this expression is usually confined to the basal, proliferative, population of keratinocytes (Watt and Jones, 1993). These stem cells have been isolated, *in vitro*, and were found to have a high expression of $\beta1$ integrins and a rapid adhesion to ECM components. Cells with a high

expression of integrins were found to be more likely to proliferate, whilst cells with reduced integrin expression tended to be comitted to differentiation and cell stratification, and are referred to as transit amplifying cells (Jones and Watt, 1993). It seemed from these results that loss of integrin expression, in the basal layer, leads to commitment to terminal differentiation. However, it was also known that a functional downregulation of integrins in these cells could be reversed, but that the commitment to differentiation could not (Hotchin *et al.*, 1993).

Cadherins were implicated by such research as influencing the functional downregulation of integrins from the surface of keratinocytes, and subsequent differentiation as a low Ca^{2+} concentration was found to block cell stratification as did the presence of antibodies directed against both P- and E-Cadherin (Hodivala and Watt, 1994). The stratification of keratinocytes, whilst distinguished by the loss of basal integrin expression and the formation of functional cadherin complexes, is also characterised by a transient rise in intracellular Ca^{2+}, the assembly of adherens and desmosomal junctions and an expression of PS integrins at borders of cell-cell contact (Larjava *et al.*, 1990). It seems to be the case from such studies that cadherin complex formation is the cause for loss of integrin function (Hodivala and Watt, 1994).

Although the imaginal disc epidermis *in vivo* is not structurally homologus to the human epidermis, in that it is either a simple columnar or squamous epithelium (Poodry and Schneiderman, 1970), there may be a similarity between the variance in expression of CAMs in this system as compared to the morphogenesis of imaginal disc cells *in vitro*. Although imaginal cells *in vitro* do not routinely differentiate, they do seem to move from a mode of cell-substrate adhesion, when the cells are first plated out, followed by a mode of cell-cell adhesion as the cells move into aggregates (Peel *et al.*, 1990).

PS integrins appear to be expressed throughout imaginal cell morphogenesis *in vitro* and appear to be important in both cell-substrate and cell-cell adhesion (Chapter 4, this thesis). Imaginal cells in aggregates express integrin at cell-cell contact sites (Peel and Milner, 1992a), therefore, if the formation of functonal cadherin complexes was to downregulate PS integrin expression during aggregation it may only downregulate those integrins involved in cell-substrate adhesion. It certainly seems possible that there could be multiple functions for PS integrins during imaginal cell growth, with possible variation being provided by the alternative splicing of either the α or β subunits (for review see: Hynes, 1992; Brown, 1993). Splicing is known to occur in the ligand binding domain of the α_{PS2} subunit, which may affect the ligand specificity of this receptor. There has been some recent research to suggest that the cytoplamic tail of the β_{PS} subunit, which is now known to be essential for function, is developmentally regulated in *Drosophila* (Grinblat *et al.*, 1994). It is also possible that alternate, uncharacterised subunits exist with varying ligand specificities, which are preferentially expressed at different stages of growth. Therefore, as morphogenesis proceeds, PS integrins could become functionally altered and switch roles from that of cell-substrate to cell-cell adhesion perhaps through the influence of other CAMs such as *DE*-cadherin.

3.4.2 Cloning

The generation of cloned cell lines that have a reduced capacity to reaggregate in roller culture, such as the leg cell line L. 14, indicates that cell-cell adhesion molecules can themselves be functionally downregulated. L.14 was derived form the uncloned cell line, L127D6. The L127D6 line itself was derived from partly dissociated imaginal discs, according to the protocol communicated by Currie *et al.*, (1988), which therefore represents a potentially

heterogeneous population of cells. Uncloned cell lines such as this can feasibly include non-epithelial cells, such as the muscle precursors known as adepithelial cells. Milner (1975) noted the occasional emergence of muscle precursors from *in vitro* cultured imaginal discs that spread out onto the culture substrate and formed syncitia. However, whether L. 14 represents a founding cell which naturally lacked adhesivity, and corresponding cells could be found *in vivo*, or whether it represents a cell which has mutated is unclear.

Peel (1991) generated a cloned wing cell line, Cl. 8R, that was resistant to the influence of 20-hydroxyecdysone (20-HE). The selection of such a cell from the parent line was fairly simple in that the usual response to 20-HE in imaginal disc cell lines was widespread cell death. Thus, any cell resistant to the effects of this hormone was relatively easy to identify and isolate for cloning. The parent cell line, Cl. 8+, was itself derived from a clone therefore it is likely that the 20-HE resistant cell was the result of a spontaneous mutation. Inherent genetic variation in a cell may be a causative factor in the initial formation of a cell line (Freshney, 1987), therefore it is perhaps reasonable to consider that genetic instability is perpetuated in continuous cell lines. The reduced adhesive response of L. 14 could therefore be due to such a spontaneous mutation.

A potential future study would be to characterise why L.14, and similar lines, fail to reaggregate. Immunocytochemical studies, Western and Northern blotting studies would indicate if the appropriate CAMs were being expressed, and were being localised to the correct areas on the cell surface. Such a selective loss of function may have wider implications for the long-term use of established *Drosophila* imaginal disc cell lines.

Because *DE*-cadherin is also known to be expressed in the imaginal epidermis in a similar fashion to the expression seen in the embryo, (H. Oda,

pers. comm.), it seems possible that *D*E-cadherin could be contributing to the calcium dependent reaggregation that is seen in aggregating imaginal cells *in vitro*. Now that *D*E-cadherin has been identified, and probes have been raised against it, an assay to determine its expression in imaginal disc cell lines would be quite straightforward.

Oda *et al* also report that *D*E-cadherin is associated with two classes of vertebrate catenin homologs, Dα Catenin and Armadillo, which are reminiscent of vertebrate systems comprising the zonula adherens (ZA) complex which is formed at a defined site towards the apex of epithelial cells. Armadillo is known to be expressed in imaginal disc cells *in vitro* (van Leeuwen *et al.*, 1994), although in a non-restricted manner. There is a lack of apical basal polarity in these cells, indicated in part by the diffuse expression of the putative signalling protein, Crumbs (see Chapter 5, this thesis), which *in vivo* also has a spatially restricted pattern of expression towards the apical surface of imaginal cells (Tepaß *et al.*, 1990). This would suggest that *D*E-cadherin, if expressed, would lack any spatial restriction in imaginal cells *in vitro*.

It is possible that *D*E-cadherin and the PS integrins may not be the only Ca^{2+} dependent CAMs that are expressed in this system. Other cadherin-like molecules, which may belong to the cadherin superfamily, are known to be expressed during *Drosophila* development and may also be expressed during imaginal disc cell morphogenesis *in vitro*. Three such molecules encoded by the genes, *lethal (2) giant larva, fat* (mentioned briefly in the introduction) and *daschous* contain cadherin domains and may be involved in adhesion (for review, see Bunch and Brower, 1993; Fristrom and Fristrom, 1993). These proteins are known to be required for the control of proliferation, and are encoded by what are known as "tumour suppressor genes" (Bryant *et al.*, 1993), as mutations can lead to tumourous or hyperplastic disc overgrowth, suggesting a loss in cell-cell adhesion.

The tumour suppressor gene, *fat*, encodes a protein which is too large to be considered a classic cadherin, but may work in concert with other Ca²⁺ dependent CAMs in mediating cell-cell adhesion in imaginal cells both *in vivo* and *in* vitro (Bryant *et al.*, 1988; Mahoney *et al.*, 1991; Bryant *et al.*, 1993). However, there are some preliminary reports to suggest that imaginal cell lines derived from *fat* mutant discs undergo Ca²⁺ dependent adhesion identical to that of wild type lines (D. Peel, pers. comm.), which suggests that the *fat* gene product may be functionally insignificant in imaginal cell reaggregation, perhaps favouring the expression and function of DE-cadherin.

Recently, the *dachsous* gene has been cloned (Clark *et al.*, 1995) and is now known to encode a very large transmembrane protein similar to that encoded by *fat*. Both the Fat (Ft) and Dachsous (Ds) proteins contain large tandem arrays of cadherin domains, and both proteins, like the classic vertebrate and the *Drosophila* DE-cadherins, have cytoplasmic domains that connect with the cytoskeleton and may thus also have functions in signalling. It seems that Ft and Ds could mediate cell-cell adhesion, either homo- or heterophilically, and thereby influence disc cell morphogenesis and proliferation (Clark *et al.*, 1995).

Other CAMs that may be involved in imaginal cell morphogenesis *in vitro* are certain signalling molecules, two of which belong to the neurogenic group of genes, known as *Notch* and *Delta*, and another, *Serrate* (for review, see: Bunch and Brower, 1993). Cell transfection experiments, using S2 cells, indicate that these EGF-repeat proteins can function in mediating Ca²⁺ adhesion (Fehon *et al.*, 1990). These experiments indicated that *Notch* and *Delta* could interact in mediating adhesion, and that *Notch* could interact with *Serrate*. However, Notch expression seems largely restricted to neuronal lineages of cells in the embryo and imaginal discs, suggesting that this protein does not function in maintaining epithelial integrity (Fehon *et al.*, 1991). Other

such 'signalling' molecules expressed in larval epidermal tissues may yet prove to have adhesive functions, which could be identified in established imaginal disc cell lines.

4.1   Introduction

The integrins comprise a large family of heterodimeric glycoproteins that are known to be important in providing a transmembrane link between different cells which is usually mediated through the extracellular matrix (ECM). Each integrin consists of an α and a β-subunit; multiple α-subunits being able to associate with the same β-subunit. As a heterodimer, the integrins act as specific adhesion receptors for ligands either in the ECM itself or on the surface of other cells. Both subunits are important in determining ligand binding, as replacing either subunit is known to alter the specificity of the receptor (Hynes, 1987; Hynes, 1992).

The *Drosophila* position-specific (PS) integrins (see Figure B.1) were discovered on the surface of imaginal discs by a series of monoclonal antibody screens carried out by Michael Wilcox and co-workers, and were named as a consequence of their spatially restricted pattern of expression there. The original PS1 antibody that was used identified a molecule that was restricted in expression to the dorsal epithelium of the wing imaginal disc (Wilcox *et al.*, 1981). Hence the molecule was referred to as position specific as its expression was not restricted to a particular cell type but was determined by the position of those cells within the developing tissue. Other molecules within the PS family were subsequently identified, comprising three subunits in all: two α-subunits, α_{PS1} and α_{PS2}, each of which form a heterodimer with a common β-subunit, β_{PS} to form the integrins known respectively as PS1 ($\alpha_{PS1}\beta_{PS}$) and PS2 ($\alpha_{PS2}\beta_{PS}$) (Brower *et al.*, 1984; Wilcox *et al.*, 1984). The *Drosophila* PS integrins were later demonstrated to be homologous to the vertebrate integrins, both sharing an extensive sequence homology and similar structural features (Bogaert *et al.*, 1987; McKrell *et al.*, 1988; Leptin *et al.*, 1989).

Throughout development in *Drosophila*, the two integrin molecules, PS1 and PS2, are usually found in complementary positions on adjacent tissues or cells, and would seem to cooperate in their function. In the embryo the PS integrins function to attach the visceral and somatic musculature to the endoderm and the ectoderm respectively, PS2 usually being found on the mesodermal tissues and PS1 on the ectodermal and endodermal tissues (Bogaert *et al.*, 1987; Leptin *et al.*, 1989; Wilcox *et al.*, 1989).

In the developing wing, prior to metamorphosis in the late larva, there is also a striking complementary and restricted pattern of expression of the PS integrins (Brower *et al.*, 1984; Wilcox *et al.*, 1984). PS1 expression is confined to the presumptive dorsal surface of the wing whilst PS2 is confined to the presumptive ventral tissue. During metamorphosis these surfaces become intimately apposed and the PS integrins are seen to localise at basal foci between the dorsal and ventral cells; which are believed to be sites of functional significance (Fristrom *et al*, 1993). Wing metamorphosis involves a complex series of morphogenetic events comprising the apposition, adhesion, expansion and separation of the two opposing epidermal layers, each of which occurs twice during metamorphosis (see Figure C) (Waddington, 1941; Milner and Muir, 1987; Fristrom *et al.*, 1993). The basal junctions, which form as thin, process-like extensions between the cell layers early in morphogenesis, are either disassembled or pulled apart by the force of disc expansion at this stage. This disc expansion *in vivo*, seems to be due in part to the active pumping of haemolymph into the disc and the secretion of ECM proteins. These are then reformed and remain intact throughout the later stages of morphogenesis until wing maturation. PS integrin expression is concentrated at these sites of basal contact between the epidermal layers, thus these molecules are believed to be of functionally significant in this developmental process (Fristrom *et al.*, 1993).

Genetic analyses, which employ the use of viable mutations that only manifest themselves in the adult stages of development, strongly suggest that the PS integrins function in maintaining tension transmitting junctions at these basal contact sites, and at other analogous structures throughout development. Mutations of this sort have been found for both the α subunit, encoded by the *inflated* loci, and the β subunit, which is encoded by the *myospheroid* loci. Two such viable mutations of these integrin components were identified by Michael Wilcox and co-workers and were important in determining a role for integrins during larval morphogenesis. The mutation *non-jumper* (mys^{nj42}) in the β_{PS} subunit causes a wasting of the thoracic jump muscles in the adult and a blistering defect in the wings. The mutation in the gene which encodes α_{PS2}, *inflated (if)*, also leads to wing blistering. The combination of these two mutations leads to a pronounced enhancement of the mutant phenotype (Wilcox *et al.*, 1989). These results, along with similar studies in embryogenesis where loss of α_{PS2} and β_{PS} expression lead to defects in muscle attachment and embryonic lethality (Brabant and Brower, 1993), suggest that the PS integrins are of functional importance in forming tension transmitting junctions throughout development. Indeed, the findings of Brabant and Brower (Brabant and Brower, 1993) implied the importance of PS integrins at both the dorsal and ventral sides of the wing epithelium for typical wing morphogenesis.

Thus it seems that the PS integrins are important in mediating cell-cell adhesion throughout *Drosophila* development, the imaginal wing being only one example. The next task facing researchers was to determine exactly how these molecules function in mediating this adhesion.

As mentioned previously, the PS integrins are known to be homologous in sequence and structure to the vertebrate integrins thus it seems logical to suppose that they may function in a similar way, that is by binding to proteins of the ECM. It is also feasible that the PS integrins may

function in a homophillic manner; a phenomenon which also exists in vertebrates, most notably being the LFA-1 receptor and the $\alpha_2\beta_1/\alpha_3\beta_1$ receptors in lymphocyte and keratinocyte intercellular adhesion respectively (Springer, 1990; Symington *et al.*, 1993). The findings of Peel and Milner implied that such a system of homophillic PS integrin interaction may indeed exist during cell-cell adhesion in *Drosophila* imaginal cells *in vitro* (Peel and Milner, 1990). This theory was based on the expression pattern of the PS2 antigen, which was found only at sites of cell-cell contact. However, this study did not demonstrate that there was actually an absence of ECM proteins at these sites, and the possibility of a heterophillic interaction of PS integrins, by way of the ECM, during imaginal cell-cell adhesion has not been ruled out. Indeed the vertebrate molecules which PS integrins most closely resemble are those of the β_1 and β_3 class which are known to be receptors for the extracellular matrix molecules laminin, collagen, fibronectin and vitronectin (Hynes, 1992). Further evidence to suggest that the PS integrins function via the ECM is provided by the fact that the phenotypes of the β_{PS} and α_{PS2} differ; if PS1 was to bind directly to PS2, then one would expect the corresponding mutant phenotypes to be identical.

Candidate molecules in the *Drosophila* ECM, which may or may not interact with the PS integrins, are still relatively poorly understood. Those molecules which have been identified, and which also have vertebrate homologues, are collagen and laminin (for review see Fessler and Fessler, 1989). The amino acid sequence and structure of the *Drosophila* collagen have been studied and bear a resemblance to vertebrate collagen IV. *Drosophila* collagen also contains an RGD peptide sequence and this is known to be important in mediating cell attachment via integrins (Ruoslahti and Pierschbacher, 1987). *Drosophila* laminin is known to promote neurone growth, but is not blocked by RGD peptides. Thus if *Drosophila* laminin is a PS integrin ligand, it does not appear to be RGD dependent. There was also

evidence at one time to suggest the existence of a putative fibronectin in *Drosophila* haemolymph, however this has since remained unsubstantiated (Gratecos *et al.*, 1988).

In 1991, Shinji Hirano *et al* generated a monoclonal antibody against a cell-surface component of the MLDmBG-1 cell line, derived from *Drosophila* CNS (Ui *et al.*, 1994), that was observed to limit the extent of both cell-cell and cell-substrate adhesion (Hirano *et al.*, 1991). By immunoprecipitation, purification, and generation of further antibodies to this protein they were able to clone its cDNA. On sequencing this cDNA they found that the molecule they had been blocking in the earlier adhesion assays was in fact identical to the β_{PS} chain; the common subunit in both PS1 and PS2 in *Drosophila*. The monoclonal antibody raised against the β_{PS} subunit, which was named aBG-1, was then used in various blocking-assays by this group to try and determine a role for the PS integrins in this cell line *in vitro*, and specifically if they could find a provide a putative ligand for these receptors.

A different approach for the identification of possible ligands for the PS integrins was provided by Thomas Bunch and Danny Brower when they transfected the normally non-adherent, non-aggregating, Schneider's line 2 (S2) cells with a genes that expressed *Drosophila* PS2 integrin (Bunch and Brower, 1992). These transformed cells were then plated onto substrates of different matrix molecules, the adhesion to which directly reflected PS integrin function.

Hirano *et al* also tested for a role for PS integrins in cell-cell adhesion in MLDmBG1 cells. The antibody aBG-1, as well as inhibiting the extent of cell spread on certain substrates, was seen to inhibit clumping of cell aggregates. Immunolocalisation studies utilising aBG-1 also indicated the presence of antigen at sites of cell-cell contact. Both results imply the importance of PS integrins in cell-cell interactions *in vitro*, reflecting their role in forming

tendon cell-muscle and dorsal-ventral wing epithelium contact *in vivo* (Leptin *et al.*, 1989; Wilcox *et al.*, 1989).

Through the use of probes directed against the β_{PS} subunit, and PS2 integrin, I attempted to determine further the nature of PS integrin expression in *Drosophila* imaginal cells *in vitro*. I also performed a series of blocking assays, again by utilising the aBG-1, anti-β_{PS}, monoclonal antibody, to try and determine the role of PS integrins in both cell-cell and cell-substrate adhesive events. As an adjunct to this study, I assayed for the ability of various imaginal disc cell lines to adhere, spread and migrate on various vertebrate ECM molecule-coated substrates and whether this morphogenesis was mediated by PS integrins.
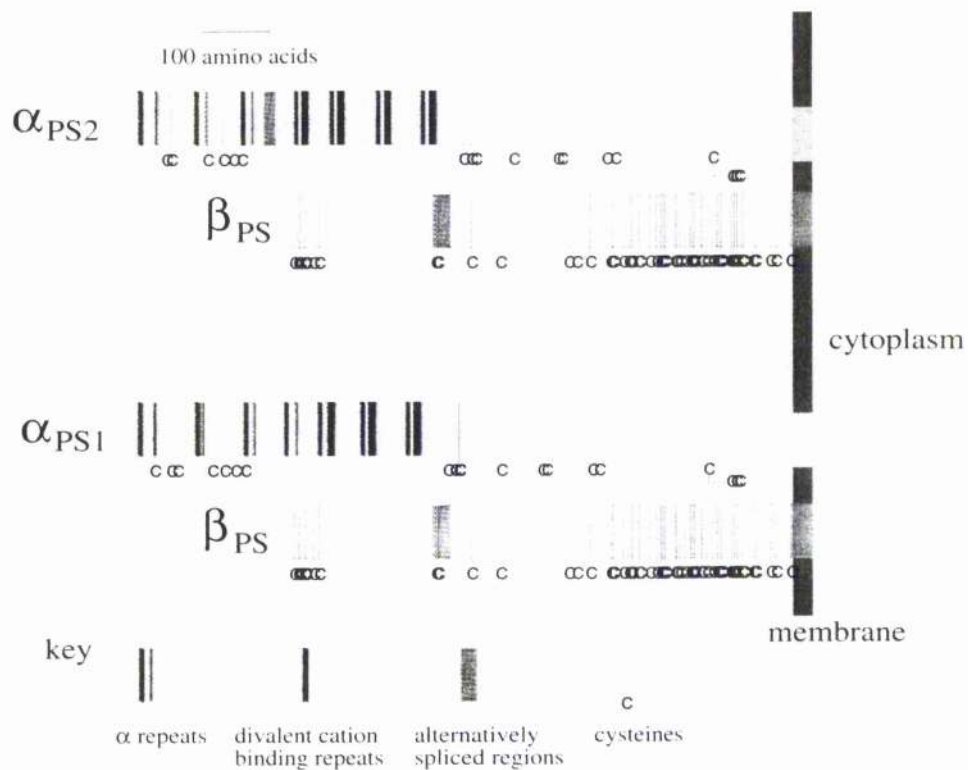
Figure B Schematic view of the primary sequences of the PS integrin molecules, PS2 (top) and PS1 (below). Each molecule is a heterodimer consisting of an α subunit, either α_{PS1} or α_{PS2}, which is linked to a common β subunit. (From Brown, 1993).
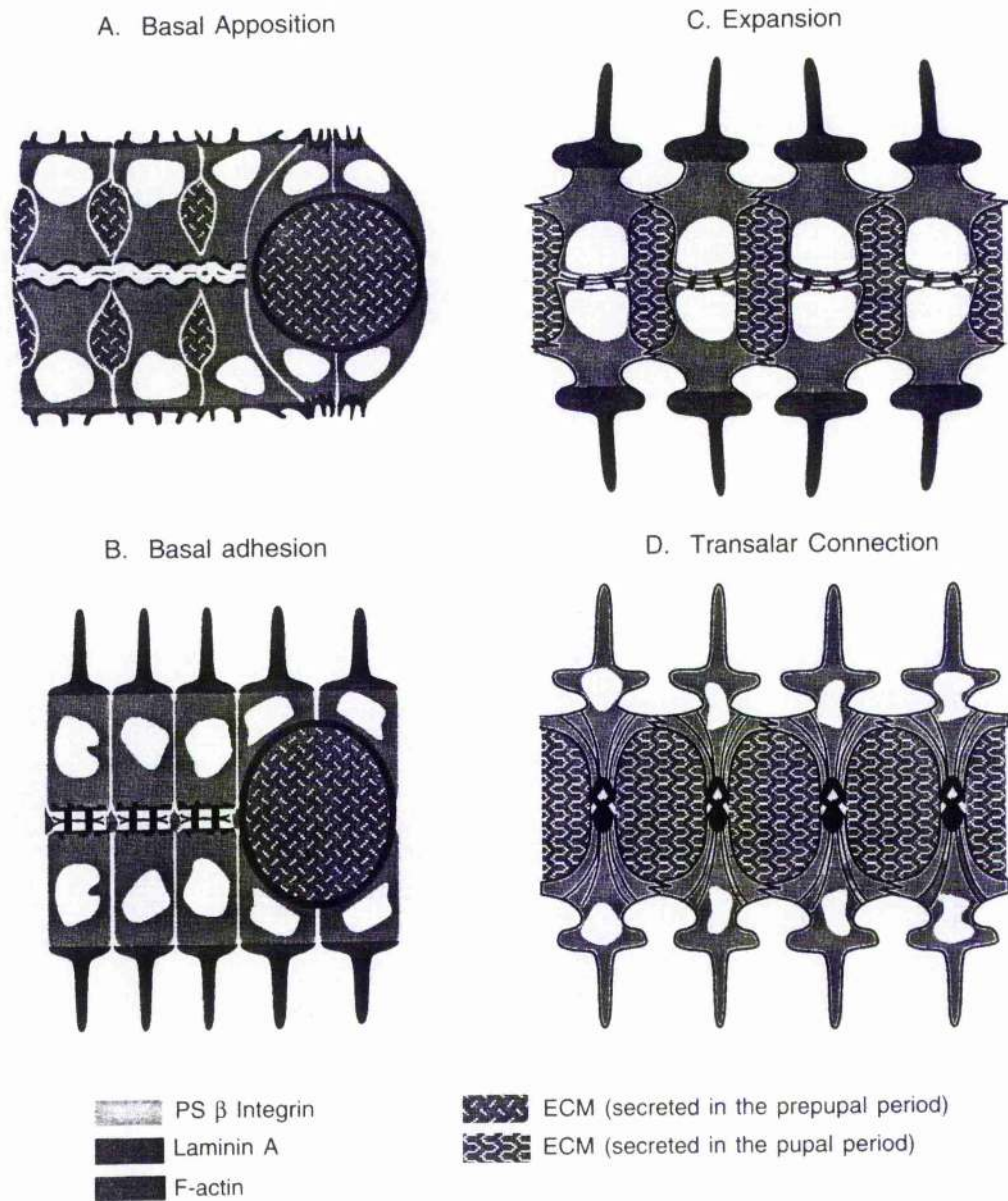
A. Basal Apposition

C. Expansion

B. Basal adhesion

D. Transalar Connection

PS β Integrin
Laminin A
F-actin

ECM (secreted in the prepupal period)
ECM (secreted in the pupal period)

Figure C   A schematic representation of the expression of the β_{PS} subunit,
laminin and F-actin at four key stages during the evagination and
differentiation of the pupal wing *in vivo*. White areas here represent
nuclei and black bars in B, C and D represent the basal, tension
transmitting, junctions. The extracellular spaces contain ECM in A, C
and D. Imaginal cell adhesion to the ECM, migration, and subsequent
adhesion to neighbouring cells *in vitro* can be considered homologous to
this series of events.

(From Fristrom *et al.*, 1993.)
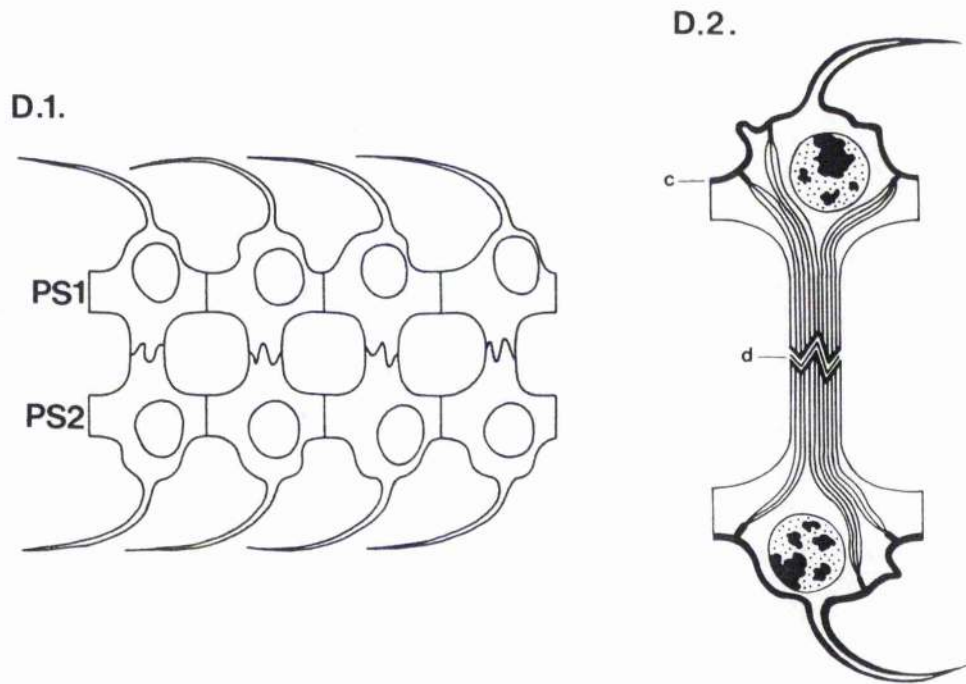
Figure D   Figure **D.1** is a schematic detail of a cross section of the
developing pupal wing blade. PS1 integrin expression is restricted to the
dorsal wing epithelium, whilst PS2 integrin expression is restricted to
the ventral wing epithelium. The two layers are connected by a series of
basal cell extensions which are seen to span the wing haemocoel. (From
Mogensen and Tucker, 1987). Transalar microtubule arrays, detailed in
Figure **D.2**, exist within these basal cell connections. These are
specialised cytoskeletal units that originate, and are anchored, at the
apical cell surface just beneath the cuticle (c) and which terminate,
midway in the wing haemocoel, as desmosomal junctions (d). (From
Tucker *et al.*, 1986).

4.2 Materials and Methods

4.2.1 Immunocytochemistry

Cells were plated out at a density of 2×10^5 per ml, in 1 ml of CSM into individual chambers of a LabTek slide (Nunc) and left to grow and aggregate for 3 days. The slides were washed for 5 minutes in PBS and were fixed in 4% paraformaldehyde, in PBS, for 10 minutes at 25°C. The slides were then washed for two 5 minute periods in PBS and were subsequently permeabilised, for 3 hours at 25°C, in PBT buffer: PBS with 0.01% BSA and 0.01% Triton X-100, (to which 0.02% horse serum was also added). After being given a further set of three 5 minute washes in PBT, the slides were incubated overnight at 4°C in a solution containing the primary antibody. Two antibodies were tested against the imaginal cell lines: the first was aBG-1, a monoclonal antibody directed against the β_{PS} subunit of the PS integrins (Hirano *et al.*, 1991), which was diluted 1:75 in PBS for staining purposes, and the second was CF2C7, an anti-α_{PS2} mouse IgG (Brower *et al.*, 1984), diluted 1:250 in PBS. The aBG-1 antibody was a gift from Shinji Hirano in Kyoto and the CF2C7 antibody was a gift from the late Michael Wilcox's laboratory in Cambridge. Next, the slides were washed in at least three changes of PBS plus 0.02% horse serum and were incubated for 1 hour at 25°C in a solution of either biotinylated anti-rat, for aBG-1, or biotinylated anti-mouse, for CF2C7 (both from Vector). Again, the slides were rinsed extensively in PBS plus horse serum and were then incubated for 1 hour at 25°C in a solution of avidin-FITC (Vector), diluted 1:250 in PBS. Finally, the slides were washed as before and were mounted in a UV-free mounting medium, containing 90% glycerol and 2.5% propyl-gallate, pH 7.9. Coverslips were added to the slides which were then sealed with dental wax. The cells were visualised using a Biorad laser scanning confocal microscope.

4.2.2   Cell-cell adhesion assays

A method for assessing the role played by PS integrins in short-term cell-cell adhesion was adapted from a roller drum method for studying the aggregation of freshly dissociated imaginal disc cells used by Fausto-Sterling and Hsieh (1983) and later modified by Peel for use with established imaginal disc cell lines (1991).

Cell lines to be tested were grown for 3 days in Petri dishes to ensure that they were in a proliferative state. The cells were then removed from the dishes by blasting with a Pasteur pipette, and spun down for 5 minutes at 500 g and resuspended in 1 ml of D=. Aliquots were then taken from this suspension for each individual sample, each containing 4×10^6 cells, which were then added to 1 ml of the specific test solution and were pelleted down once more. Next, each pellet of cells was resuspended in 1 ml of the appropriate test solution and was added to a siliconised 15 ml centrifuge tube (Corning). These tubes were placed on a Spiramix roller (Denley) which rotated the tubes at a speed of approximately 20 rpm about an axis of 8° from the horizontal. Samples of 10 µl were taken from each tube after 0, 30, 60 and 120 minutes of culture initiation. Each solution on test was replicated four times.

The rate of reaggregation in each test batch was presented by plotting the percent decrease in single cell units on the Y axis, with the number of cells at time 0 being taken as representing 100%, against the time of rotation on the X axis; providing a visual representation of the rate of cell-cell adhesion in each sample.

The test solutions in this particular assay utilised aBG-1, the monoclonal antibody directed against the β_{PS} subunit of the PS integrins (Hirano *et al.*, 1991) and CF2C7, the anti-PS2 mouse IgG (Brower *et al.*, 1984).

In these experiments aBG-1 was diluted 1:75 in PBS and CF2C7 was diluted 1:100 also in PBS. For the negative controls, cells were suspended in a solution of PBS containing an equivalent amount of rat and mouse pre-immune sera (supplied by Shinji Hirano and Michael Wilcox).

The soluble peptides GRGDSP and GRGESP (Telios) were also used in these assays. These synthetic peptides had originally been derived from fibronectin. The RGD sequence, contained in GRGDSP, is known to be important in mediating cell-matrix adhesion in vertebrate cell lines (Ruoslahti and Pierschbacher, 1987). The presence of RGD containing peptides was observed to cause the detachment of cells, representing a wide range of established cell lines, from fibronectin and vitronectin *in vitro* (Hayman *et al.*, 1985). Cl. 8+ cells were incubated in 1ml of MM3 plus 2% foetal calf serum with four different concentrations of each peptide: 10 μg/ml, 50 μg/ml, 100 μg/ml and 200 μg/ml, and were sampled and counted over a 2 hour time period as before.

Another reaggregation assay that was carried out was designed to measure the effect of introducing vertebrate serum (FBS) into the roller cultures prior to blocking PS integrin function. In this particular set of assays, Cl. 8+ cells were harvested as before but were thoroughly rinsed and centrifuged in three changes of MM3. A solution of 0.01% trypsin in MM3 was then added to the cells for 5 minutes at 25°C to help further remove any excess of ECM components that remained on the surface of the cells. The cells were then counted and aliquoted out to as three different treatments for assay. The experimental samples contained MM3 medium, one sample of which had a complement of 2% FBS, the other containing no serum at all. At the start of the assay, the integrin antibody aBG-1 was added to each to give a 1:75 concentration. The third sample, a control, contained MM3 plus 2% FBS and a 1:75 dilution of rat pre-immune serum. Single cell numbers were counted as before.

<u>4.2.3   Cell-substrate adhesion assays</u>

As a simple preliminary study to test if imaginal cells *in vitro* laid down any ECM components during aggregation, and as a rough indicator to determine if cell-substrate interactions were important in aggregation, imaginal cells were plated onto 50 mm Petri dishes which had already been used for cell growth. Dishes containing cultures of Cl. 8+ cells that had been allowed to grow under normal conditions for 7 days, by which time most cells were in aggregates, were rinsed in D= and the cells were flushed from the culture surface by vigorous pipetting. After the removal of the cells, the dishes were given 3 further rinses in D=. Aliquots containing 3×10^6 Cl. 8+ cells in 5 ml of CSM were then plated to these dishes and to control, unused dishes, and were photographed over a 7 day period.

<u>ECM protein substrate preparation</u>

Mouse laminin, calf collagen type III, human placental collagen type IV, bovine fibronectin and human fibronectin (all from Sigma) were used as substrata for cell adhesion, spreading and migration studies. These proteins were dissolved in sterile PBS at a concentration of 50 µg/ml; except for the collagens which were initially solubilised in 0.02 M acetic acid. These solutions were then used to coat individual wells of 24 well plates (Nunc): 200 µl of solution being added to each giving an equivalent coating of 10 µg protein/well (or 5.7 µg/cm^2). The dishes were then incubated with these proteins overnight at 4°C. Next, the dishes were rinsed with PBS and were incubated for 1 hour at 25°C in 1% BSA. The dishes were then washed three times in PBS followed by two washes in MM3 (imaginal cell culture medium minus any serum components).

Imaginal cell lines were grown in Petri dishes for 3 days prior to use in these studies. Cells were washed with D= and were removed from the dish surface by pipetting vigorously. The cells were centrifuged for 5 minutes at 500 g, washed in 1 ml of MM3 and a cell count was taken using a haemocytometer. The cell pellet was centrifuged again and then resuspended in either MM3 with no serum components or MM3 with 2% foetal bovine serum (FBS) to give a concentration of 2×10^5 cells/ml. Any antibodies to be tested in these assays were added at this point. The antibody aBG-1 was added at a concentration of 1/75, as was the control pre-immune serum, and were incubated with the cells for 10 minutes at 4°C. The cells, with or without antibodies, were then added to the wells coated with matrix proteins and were photographed at various stages of morphogenesis using a Leitz Diavert inverted microscope plus camera attachment, set up for phase contrast microscopy.

GRGDSP and GRGESP peptides (Telios), when used, were added to the cultures at a concentration of 500 µg/ml in MM3 plus 2% FBS. The cells in these cultures were then photographed as before.

4.3   Results

4.3.1   Immunocytochemistry

All cell lines tested seemed to stain well with the anti β_{PS} antibody, aBG-1, and showed little, or no, staining when incubated with the pre-immune serum. Figures 4.A and 4.B detail two groups of cloned wing (Cl. 8R) cells that have been stained using aBG-1: the image on the left of Figure 4.A is a phase contrast image and the image on the right is a fluorescent image of the same region. These cells had been left to grow and aggregate over a period of 3 days. At the centre of each image in Figure 4.A there is a short chain of cells adhering to each other. These cells exhibit a dense expression of PS integrins on their surface and especially at sites of cell-cell contact. Figure 4.C again illustrates PS integrin expression in Cl. 8R cells. Here, an elongated cell in the upper aggregate can be seen which is extending a process to the group of cells at lower right. PS integrins seem to be expressed uniformly on the surface of these cells and on the cell process itself. Similar patterns of expression are seen in other cell lines at all stages of morphogenesis.

Figure 4.D is a series of confocal micrographs which represent PS2 integrin expression at a central site of adhesion within a group of Cl. 8+ cells which were fixed and stained after 3 days of growth. This pattern of expression, first described by Peel and Milner (Peel and Milner, 1991), seems quite distinct from that of the β_{PS}-stained cells. Here, the PS2 receptor is only seen at cell-cell contact sites whilst β_{PS} integrin expression is found all over the surface of each cell. There appears to be little or no PS2 integrin expression in single, non-aggregated, imaginal cells *in vitro* (Peel and Milner, 1991), however, the β_{PS} subunit seems to be expressed to an equal extent and at similar sites in both aggregated and non-aggregated cells.

4.3.2   Cell-cell adhesion assays

Figures 4.E.1 and 4.E.2 detail the reaggregation of a cloned wing cell line (Cl. 8+) and a leg cell line (L127D6) respectively, which have been incubated in the presence and absence of a monoclonal antibody directed against the *Drosophila* β_{PS} subunit (aBG-1). There is an initial period of rapid reaggregation seen in both assays, which then plateaus out after about 30 minutes. In each case, the samples of cells which were incubated with the aBG-1 antibody do not reaggregate to the extent that cells incubated with the pre-immune serum do; the experimental sample of L127D6 cells in figure 4.E.2 exhibiting 20% less aggregation than their negative control counterparts by the end of the 2 hour assay. However, the blocking of reaggregation in these assays is not complete; a substantial proportion, about 40%, of both Cl. 8+ and L127D6 cells manage to reaggregate in the presence of the antibody. Other wing and leg cell lines displayed a similar response to this antibody in identical experiments.

The graphs in figures 4.F.1 and 4.F.2 detail the reaggregation of Cl. 8+ and L127D6 cells, respectively, in the presence and absence of an antibody directed against the *Drosophila* α_{PS2} integrin subunit (CF2C7). Here there seems to be less of a variation between the rates of reaggregation in the experimental and control samples as was seen in the assays which employed the use of aBG-1. CF2C7 treated cells reaggregate much to the same extent as control cells; indeed, antibody treated L127D6 cells even appear to reaggregate more quickly than the control cells over the first 30 minutes of culture.

Imaginal cells were also reaggregated in the presence of GRGDSP and GRGESP peptides to test whether these proteins are involved in imaginal cell-cell adhesion. Figures 4.G.1 and 4.G.2 illustrate the rate of cell-cell adhesion of

Cl. 8+ cells in the presence of differing levels of these peptides. Due to constraints imposed by the lack of available peptide, sample sizes were kept to a minimum; hence the absence of error bars. These assays were designed as a rough indicator of the effects of these peptides on imaginal cell-cell adhesion. In both there seemed to be no correlation between peptide effect and titre; higher concentrations of either GRGDSP or GRGESP had much the same effect as lower concentrations did. However, there does seem to be an overall difference seen between the two graphs, the samples containing GRGDSP tending to reaggregate to a greater extent than the contemporary GRGESP samples. However, neither GRGDSP nor GRGESP seemed to significantly affect the rate of imaginal cell-cell adhesion.

Figure 4.H represents the rate of cell-cell adhesion of Cl. 8+ cells where the function of PS integrins have been blocked, in the presence or absence of vertebrate serum (FBS). From this graph it can be seen that those cells which are in medium with serum tend to reaggregate more quickly than those in medium without. However, neither of the two samples, unsurprisingly, reaggregate to the same extent as the control. One interpretation for this variation in reaggregation rates is that the blocking effect of the PS integrin antibody is itself being blocked or out-competed by the serum constituents, which may themselves contain the RGD sequence. This provides good evidence to suggest that the PS integrins effect intercellular adhesion via intermediate ECM ligands. Because the serum was added prior to the addition of aBG-1, ECM ligands have already presumably bound to the integrins, effectively out-competing the antibody from binding and its blocking effect on cell-cell adhesion.

The micrographs in Figure 4.I shows W20C6 cells which have reaggregated after suspension in PBS (4.I.1), CSM (4.I.2) or D= (4.I.3) for 2 hours. Unsurprisingly, the aggregates formed in PBS and CSM, which contain Ca^{2+} and Mg^{2+} ions, are much larger and more numerous than those seen in

the Ca^{2+} and Mg^{2+}-free saline, D=, in accordance with the findings of Chapter 3. However, there also seems to be a variation in aggregate size between cells incubated in PBS and CSM, with larger aggregates tending to be found in the CSM samples. The overall rate of cell-cell adhesion, as determined by single cell-counts (data not shown), does not differ much between these samples, but aggregate size does, suggesting that the aggregates formed in CSM are formed by the clumping together of smaller aggregates, similar to those seen in the PBS samples.

4.3.2 Cell-substrate adhesion assays

The micrographs in Figure 4.J shows Cl. 8+ cells 2 days after plating onto either tissue culture plastic or onto 'pre-used' tissue culture plastic, that had already sustained 7 days growth of Cl. 8+ cells. There did seem to be a variation in the extent of aggregation seen between the two samples, with those cells plated on the pre-used tissue culture plastic (4.J.2) beginning to move into aggregates, leaving areas of plastic denuded of cells, whereas cells on fresh plastic (4.J.1) tended to remain in a monolayer, covering the entire substratum.

Next, the ability of imaginal cells to adhere, spread and migrate on substrates coated with vertebrate extracellular matrix molecules was studied . The response of imaginal cells when plated onto substrates coated with collagens type III and IV was negative. Figure 4.K details the response of Cl. C9 cells 2 days after plating onto a substrate of collagen III; cells fail to adhere to the substrate instead forming numerous clumps of cells. This is also seen with Cl. 8R cells plated to collagen III (Figure 4.L) cells forming aggregates and chains of cells rather than adhering to the substrate itself. Figure 4.M illustrates the response of Cl. 8+ cells when plated onto a substrate of collagen

IV which seems much the same as the response to collagen III, with little or no substrate adhesion and widespread cell-cell adhesion.

The consequence of plating imaginal cell lines onto an *in vitro* laminin substrate is similar to that of plating cells onto collagen substrates. Figure 4.N shows the response of plating Cl. 8+ cells onto a laminin substrate. Here, cells also form loosely aggregated clumps of cells rather than directly adhering to the substrate.

Human fibronectin elicits a different response to laminin and the collagens when used as a substrate for imaginal cell growth. Cells tend to adhere and spread well on this molecule and migrate into aggregates in much the same way as in the positive controls. The photographs in Figure 4.O demonstrate Cl. 8+ cells 1 day after plating onto a substrate of fibronectin. Here the cells display the variety of cell shapes characteristic of normal cell adhesion and growth, with epithelioid and fibroblast-like cells migrating into multicellular aggregates (Peel and Milner, 1990). These fibroblast-like cells are phase-dark in appearance, indicating that they have spread on the substrate and can be seen to extend numerous processes and growth cone-like extensions onto the protein surface as they start to migrate. Figures 4.P and 4.Q respectively illustrate Cl. 8+ and Cl. 8R cells 10 days after plating onto fibronectin and tissue culture plastic. In each instance, Cl. 8+ and Cl. 8R cells, on fibronectin, have migrated into very dense aggregates which are connected by elongated fascicles and chains of cells (which is especially evident with the Cl. 8+ cells).

Imaginal cells that adhere and flatten on a given substrate appear dark when viewed using phase contrast microscopy. Conversely, cells which have failed to spread on the substrate, or do not adhere to it at all, tend to be more refractile and have smaller profiles. Cl. 8+ cells that have been plated onto fibronectin in medium that does not contain any serum components spread extensively as shown in Figure 4.R.1, appearing as phase-dark cells dotted on

the substrate surface. Figure 4.R.2 illustrates Cl. 8+ cells which have been grown under similar conditions to those in Figure 4.R.1, but differ in that they have been co-incubated with the anti-βPS antibody, aBG-1. These particular cells are morphologically distinct from their counterparts in Figure 4.R.1, in that they are smaller and refractile. The cells in this culture have apparently failed to spread and, on agitation of the culture vessel, were found to be very loosely adherent to the culture substrate. Figure 4.R.3 shows cells that have been co-incubated with an equivalent amount of pre-immune serum, which seem to spread well and apear phase-dark in this photograph. From these assays it seems that aBG-1 is, in some way, disrupting the normal imaginal cell-substrate interaction. Figures 4.S and 4.T detail the effect of adding aBG-1 to Cl. 8+ cells grown either on tissue culture plastic or on human fibronectin respectively. There seems to be a contrast between assays with regard to the adhesion, spread and morphology of individual cells, with those cells in the assays minus the antibody appearing phase-dark and flattened and those in the assays with the antibody appearing small and refractile, often with spindle-like morphology.

Cultures were also set up to examine the possible influence of the anti-PS integrin antibodies on 'long-term' imaginal cell morphogenesis *in vitro*. Figure 4.U illustrates two different cultures of L127D6 cells which have been grown on tissue culture plastic, in MM3 medium containing 2% FBS, for 3 days. The control sample (Figure 4.U.1) which lacks any antibody, already shows pronounced signs of aggregation with cells migrating into numerous foci dotted over the substrate leaving areas denuded of cells. However, the experimental samples have a markedly different appearance to this. Those cells which have been cultured with the aBG-1 antibody (Figure 4.U.2) seem unable to aggregate properly, with little cell adhesion, spread or migration in evidence. After a further 4 days of culture the contrast in the states of aggregation in these cultures has become more marked. Cells in the samples

containing aBG-1 seem to have aggregated (Figure 4.V.2), but not to the same extent as in the control samples (Figure 4.V.1); the aggregates tending to be smaller and fewer when co-incubated with this antibody. The aggregation of Cl. 8+ cells is similarly affected by the presence of these antibodies. Figure 4.W illustrates Cl. 8+ cells 2 days after plating either in the presence or absence of aBG-1. Control cells have already adhered and begun to migrate as indicated by the darkened and elongated cells (Figure 4.W.1). Cells in the aBG-1 containing samples (Figure 4.W.2) show some adhesion and migraion. By day 7 of culture the control cells have reached an advanced stage of aggregation (Figure 4.X.1) whilst the cells in the experimental samples do not seem to have progressed as far (Figure 4.X.2), although some cell-substrate adhesion is seen in the aBG-1 containing cultures.

GRGDSP and GRGESP peptides were added to cultures of Cl. 8+ cells that had already been allowed to adhere and spread on tissue culture plastic in MM3 medium plus 2% FBS for 2 hours, to test for the possible involvement of these peptides in cell-substrate adhesion. Figures 4.Y.2 and 4.Y.3 show samples of Cl. 8+ cells after 6 hours of incubation with GRGDSP and GRGESP respectively. Neither of these cultures seem to differ morphologically from the control sample shown in Figure 4.Y.1; all sets of cells adhering strongly to the substrate. The cells in each of the experimental samples then went on to aggregate as normal, as detailed in Figure 4.Z.

Figure 4.A   A confocal micrograph detailing the distribution of PS integrins in a group of Cl. 8R cells generated using the monoclonal anti-β_{PS} antibody, aBG-1. The image on the left was taken using phase contrast optics while the image on the right is immunofluorescent, the brighter regions representing areas of intense antibody staining. In the image to the right, PS integrins seem to be expressed over the entire cell surface, and on single cells, with an apparent accumulation of antigen at cell-cell contact sites. Pseudocolour was added to Figures 4.A, 4.B and 4.C.

Scale bar represents 10 μm

Figure 4.B   A confocal micrograph of an aggregate of Cl. 8R cells stained for the antibody, aBG-1, as in Figure 4.A. PS integrin expression is again evident on the surface of these cells and especially at sites of cell-cell contact.

Scale bar represents 25 μm
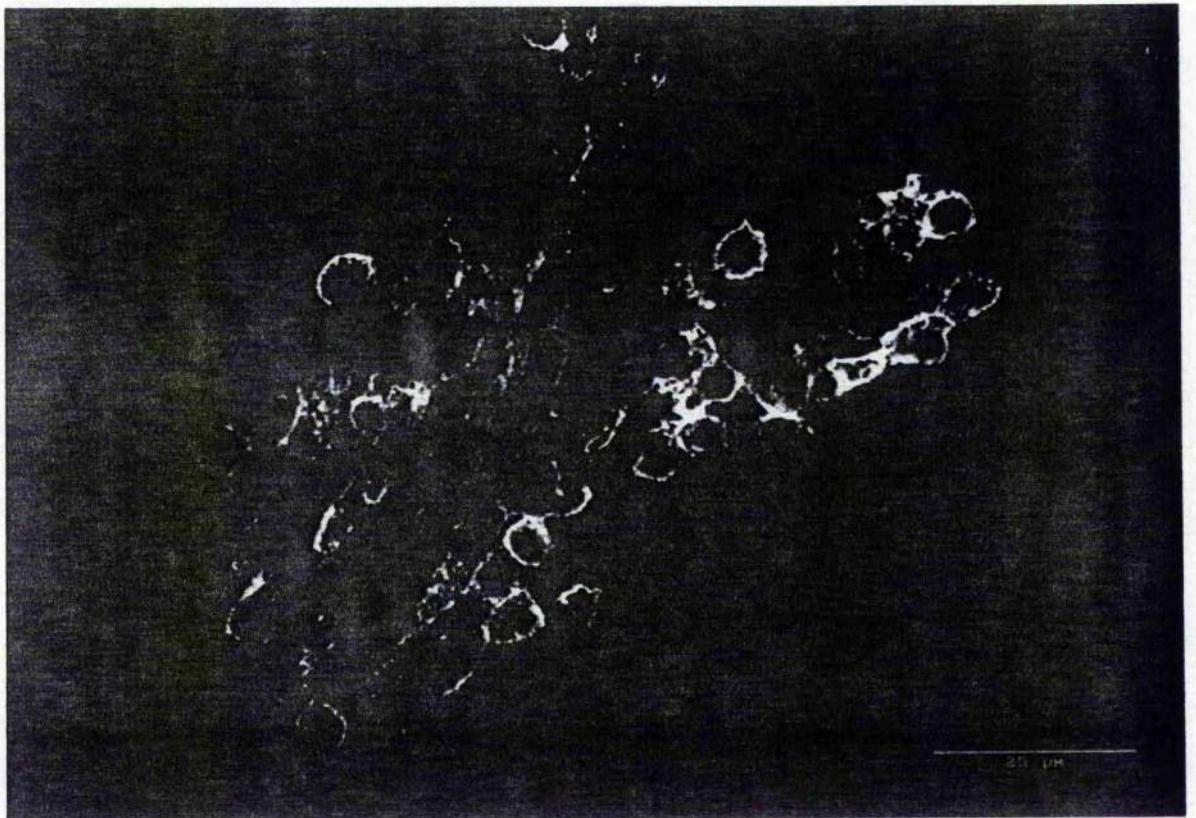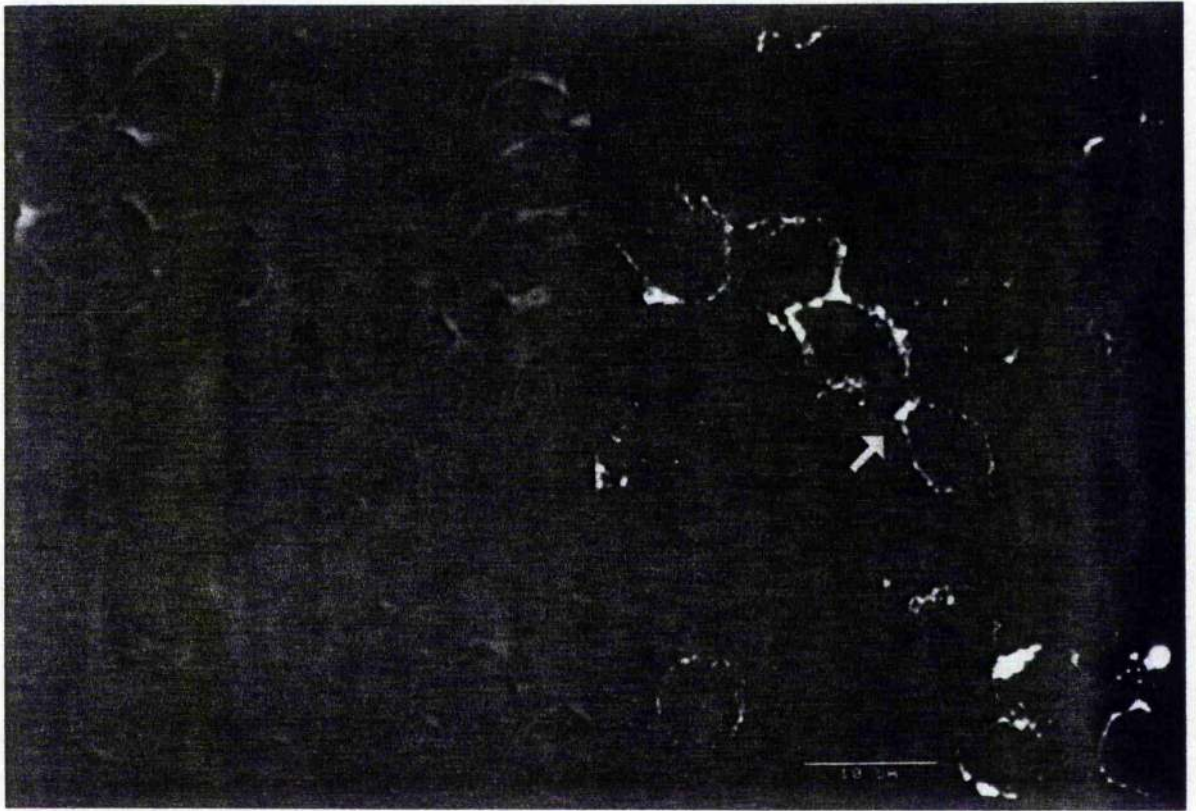
Figure 4.C These two images, again, detail the staining patterns of aggregating Cl. 8R cells for β_{PS} using aBG-1. Here, a cell in the upper aggregate appears to be extending an elongated process to the aggregate below. Again, PS integrin expression is seen on the surface of each of these cells, and on the process itself.

Scale bar represents 10 μm

Figure 4.D This series of confocal micrograph represents the expression of the PS2 integrin within an aggregate of Cl. 8+ cells fixed after 3 days of growth. This pattern of expression is distinct from the β_{PS} expression in that PS2 antigen is only seen at sites of cell to cell contact and not over the entire surface of each cell. Each image was taken at different optical levels, as part of a Z-series, at 1 μm intervals. In the fourth, lower right hand image ("number 6 of 7") the profiles of six cells within the aggregate have been numbered.
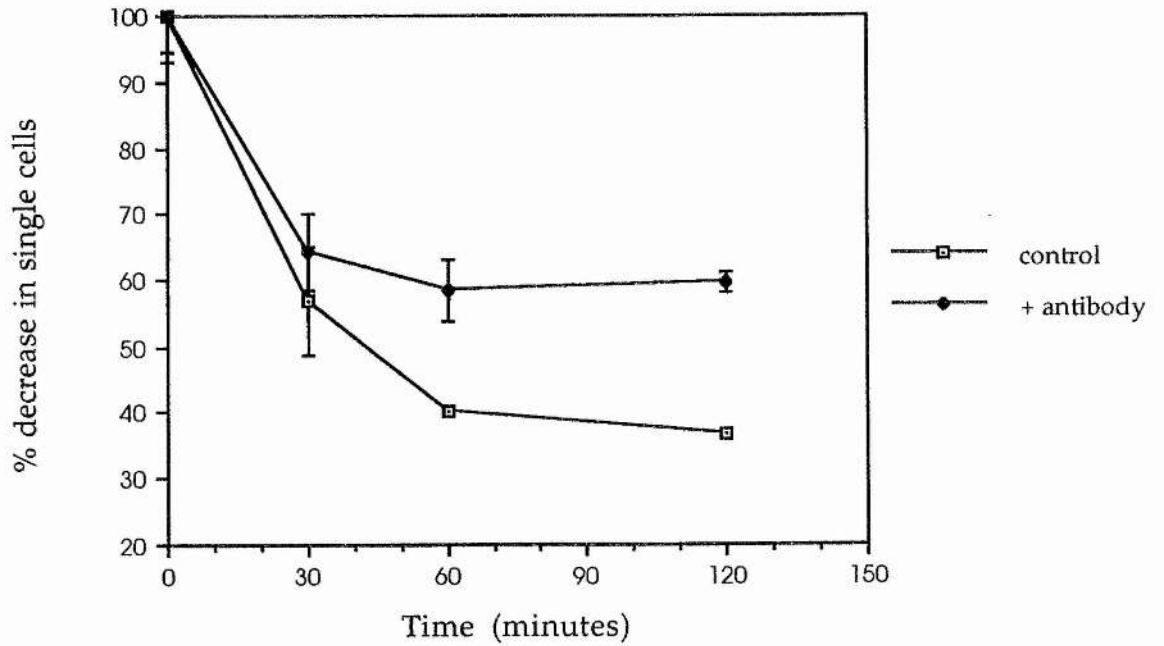
Scale bar represents 10 μm
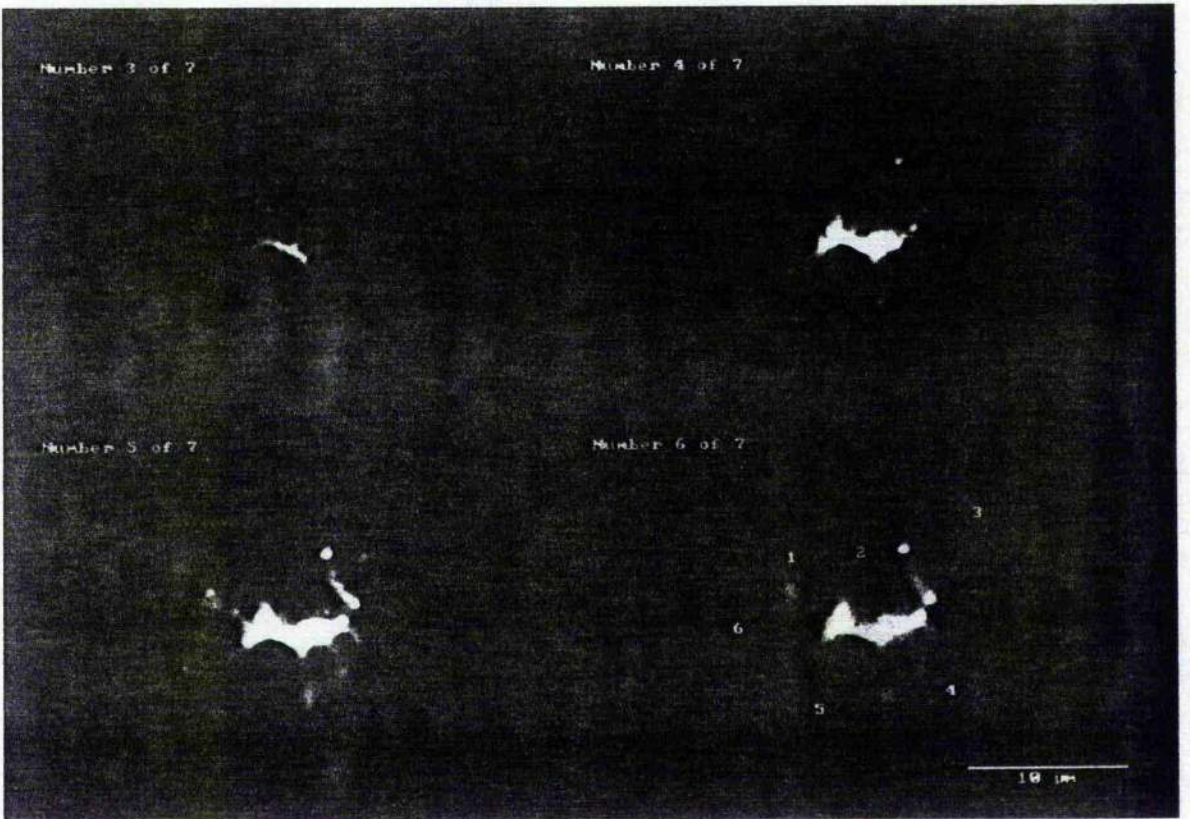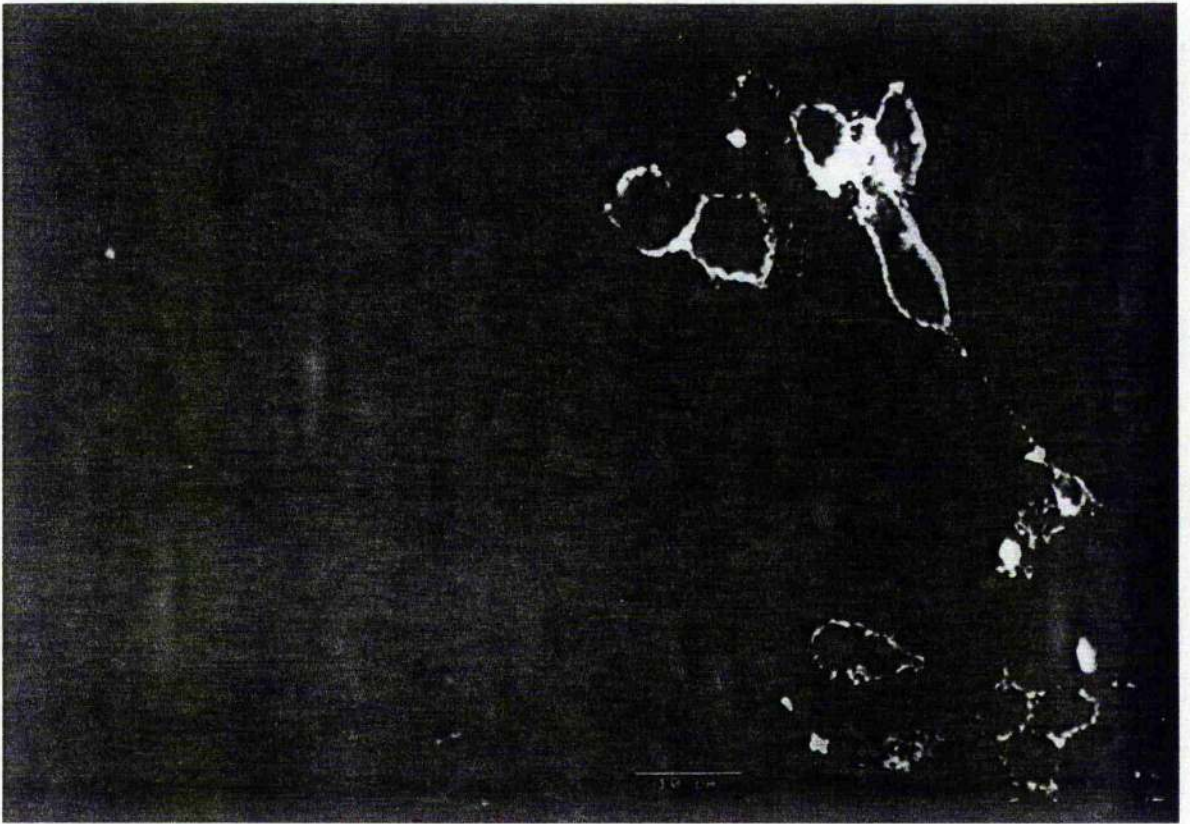
Figure 4.E The rate of reaggregation of two imaginal cell lines , Cl. 8+ (1) and L127D6 (2), when incubated either in the presence or absence of the anti-β_{PS} antibody, aBG-1. In both graphs, it can be seen that those cells which have been incubated with the antibody tend to reaggregate less than those cells which have not been exposed to the antibody, although some reaggregation does occur.

Fig. 4.E(1). Cl.8+ reaggregation: aBG-1
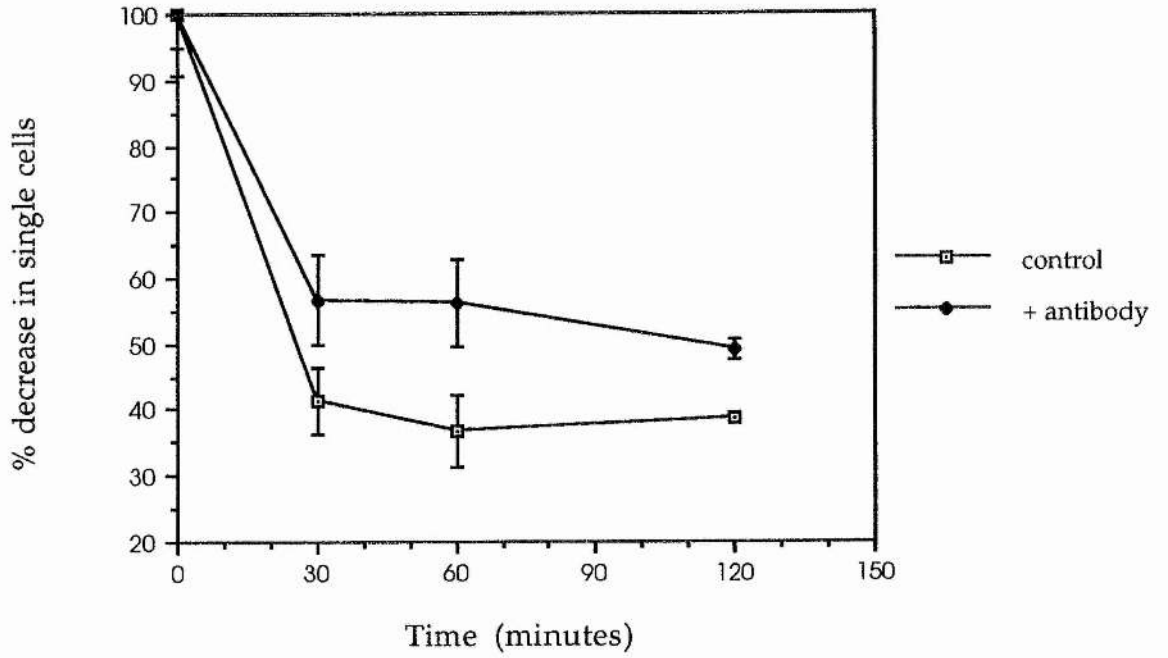
Fig. 4.E(2). LI27D6 reaggregation: aBG-1

Figure 4.F   The rate of reaggregation of two imagnal cell lines , Cl. 8+ (1) and L127D6 (2), when incubated either in the presence or absence of an antibody specific for the α_{PS} subunit of the PS integrins (CF2C7). In these graphs there seems to be less variation between the control and the experimental sample rates of reaggregation; suggesting that α_{PS} does not have an adhesive function during such reaggregation.

Fig. 4.F(1). Cl.8+ reaggregation: CF2C7
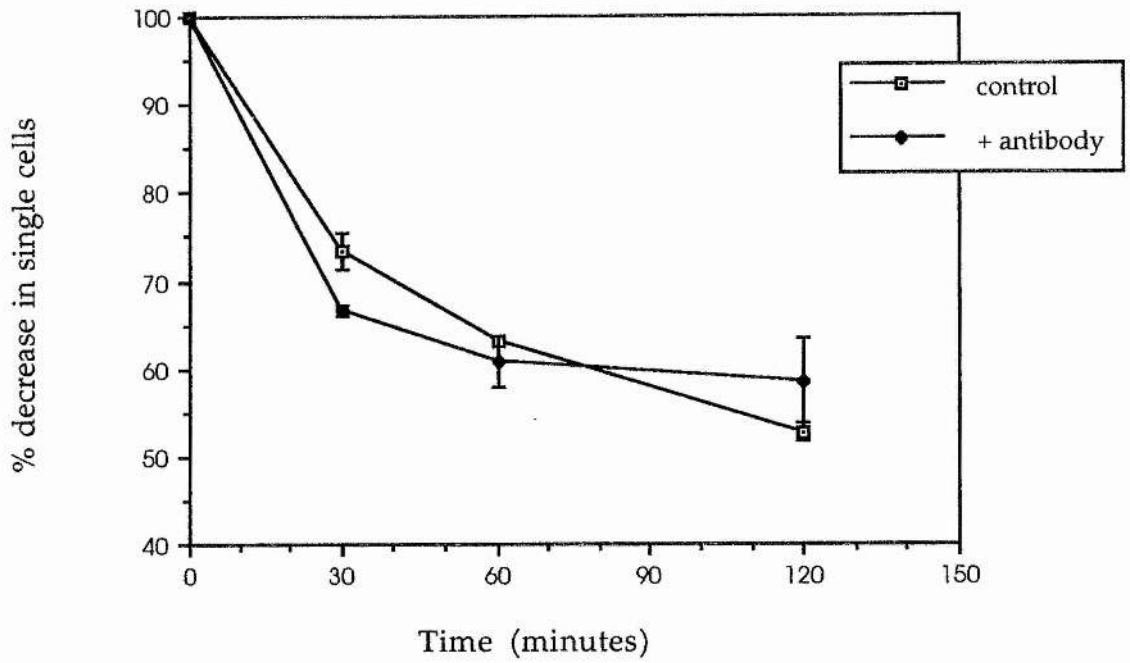
Fig. 4.F(2). LI27D6 reaggregation: CF2C7
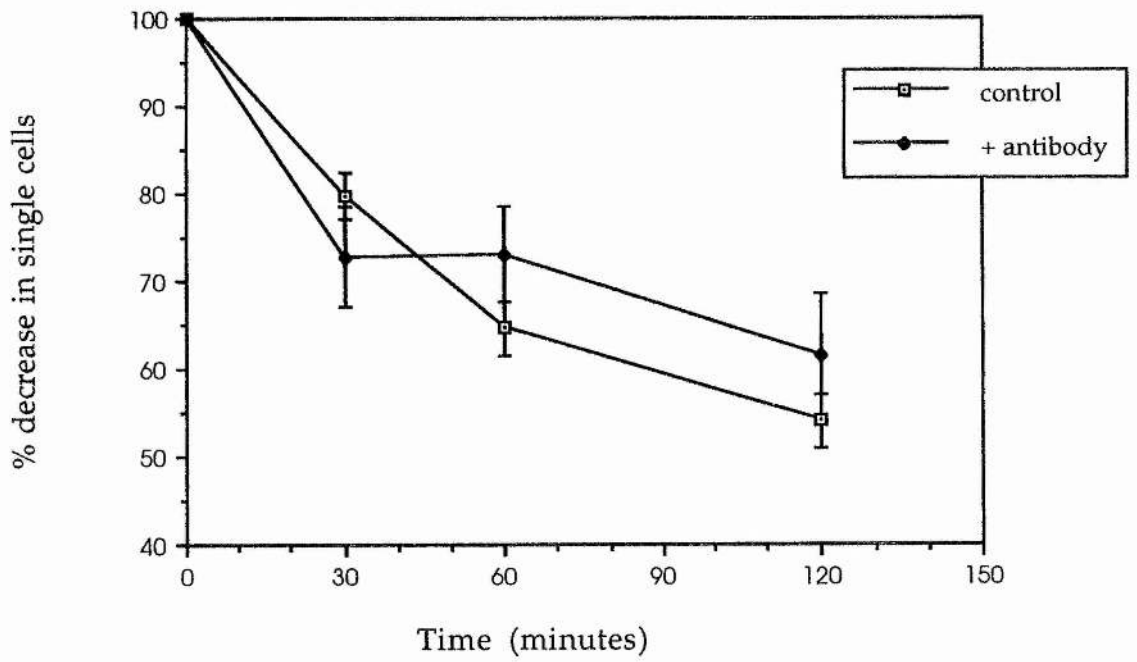
Figure 4.G   The rate of reaggregation of Cl. 8+ cells when incubated in the
presence of four different titres of either GRGDSP (1) or GRGESP (2)
peptide. Change in peptide titre does not seem to significantly alter the
rate of reaggregation in either of the two graphs. There does, however,
seem to be a slight overall difference observed in the rate of
reaggregation seen between the two different peptides, those cells in the
GRGDSP (1) sample tending to reaggregate to a greater extent than those
in GRGESP (2); suggestive that certain ECM components may play a role
in cell-cell adhesion.

Fig. 4.G(1). Cl. 8+ reaggregation: GRGDSP

Legend:
- 10 µg/ml
- 50 µg/ml
- 100 µg/ml
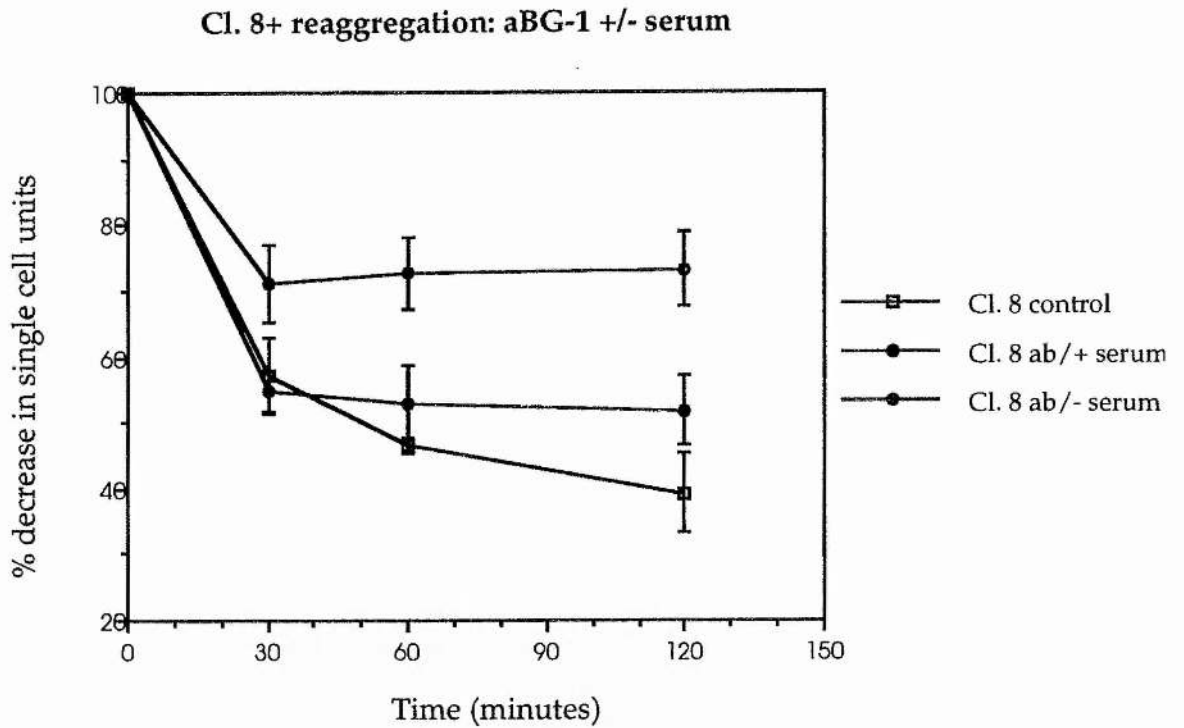- 200 µg/ml

Y-axis: % decrease in single cells units
X-axis: Time (minutes)

Fig. 4.G(2). Cl. 8+ reaggregation: GRGESP

Legend:
- 10 µg/ml
- 50 µg/ml
- 100 µg/ml
- 200 µg/ml

Y-axis: % decrease in single cell units
X-axis: Time (minutes)

Cl. 8+ reaggregation: aBG-1 +/- serum

Legend:
- Cl. 8 control
- Cl. 8 ab/+ serum
- Cl. 8 ab/- serum

Y-axis: % decrease in single cell units

X-axis: Time (minutes)
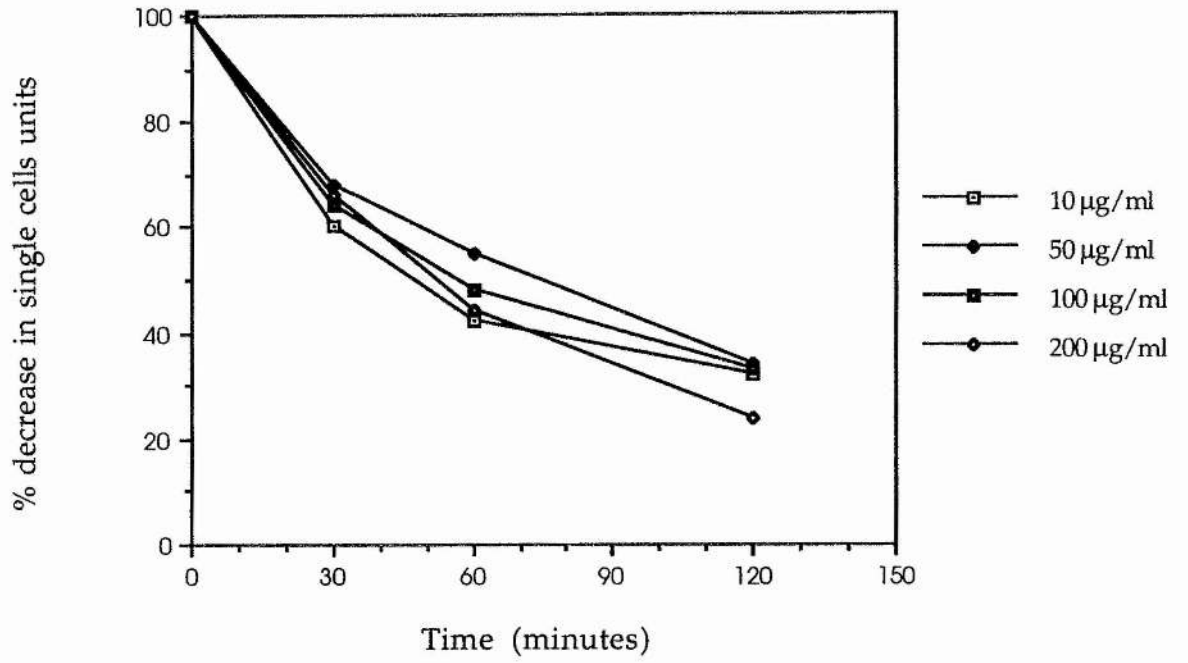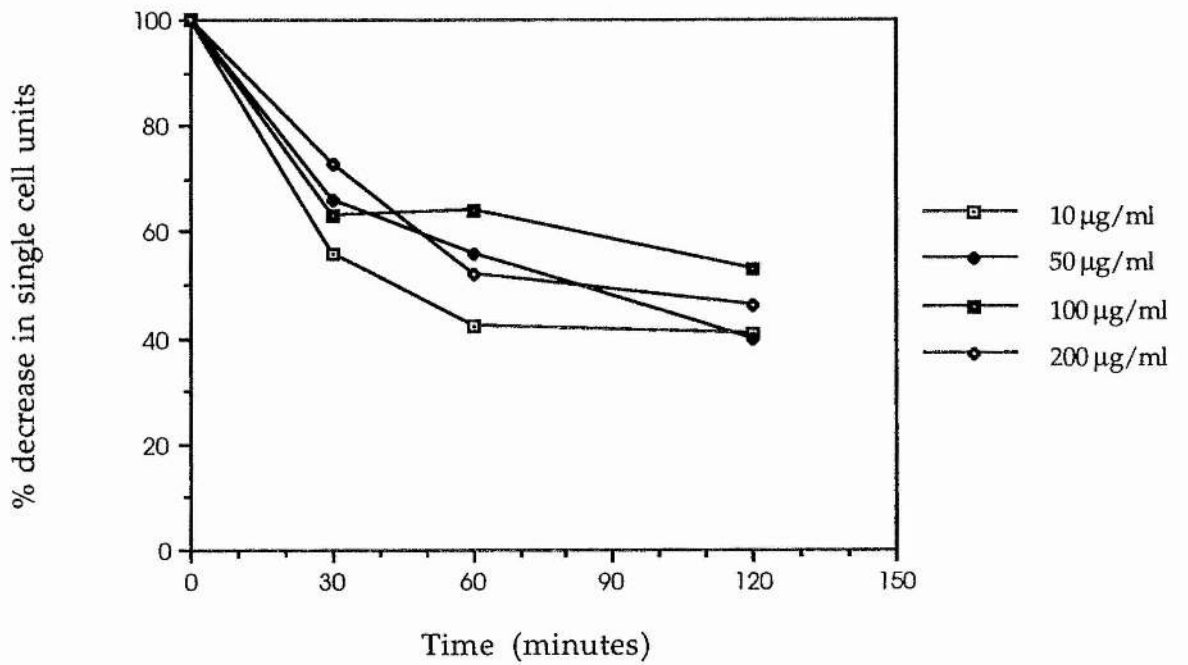
Figure 4.H The effect of the anti-β_{PS} antibody, aBG-1, on the rate of reaggregation of Cl. 8+ cells when either in the presence or absence of foetal bovine serum (FBS). Those cells which are in medium which contains serum tend to reaggregate to a greater extent than those which are in medium which is serum deficient, evidence that the ECM components of the serum may act as ligands for the PS integrins during cell-cell adhesion and are blocking the action of the antibody.

Figure 4.I   The reaggregation of W20C6 cells in either PBS (1), CSM (2) or D= (3). The cells pictured in these micrographs were photographed after 2 hours of incubation in roller culture. Cells in D=, unsurprisingly, have failed to aggregate by this stage, with pronounced aggregation being seen in the PBS and CSM cultures. There also seems to be a variation in aggregate size between the PBS and CSM cultures, the larger cells being found in the latter.

Scale bars represent 50 μm

Figure 4.1

1.
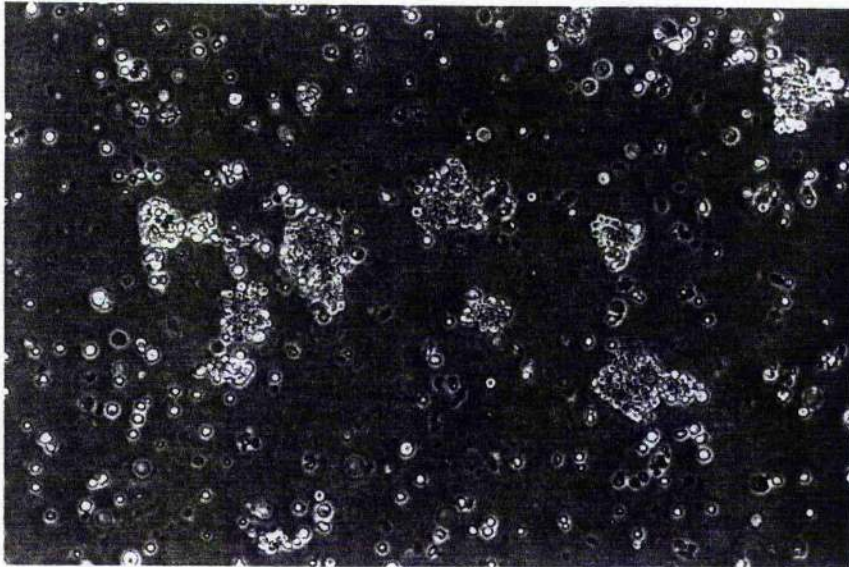
2.
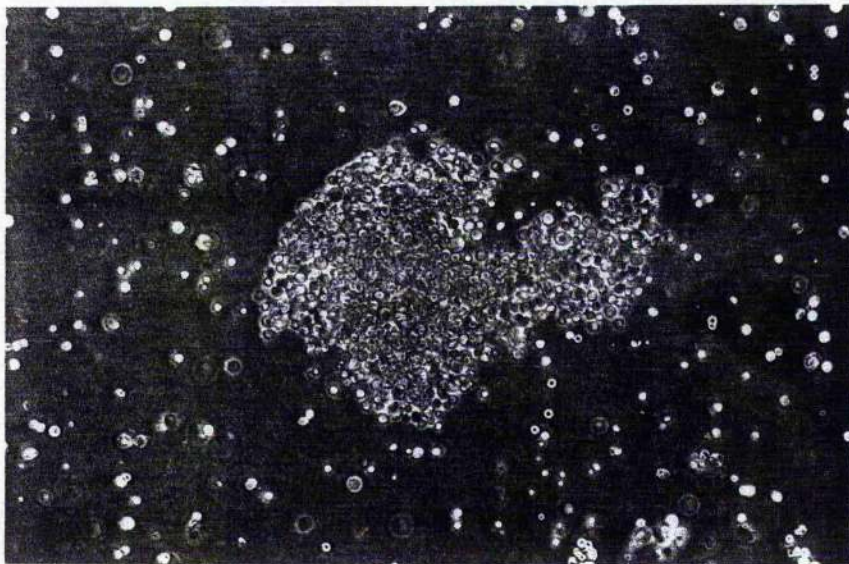
3.

Figure 4.J   The growth of Cl. 8+ cells on a 'pre-used' tissue culture

substrate. The cells in these micrographs are shown 2 days after plating

onto either tissue culture plastic (1) or tissue culture plastic which had

already supported 7 days of imaginal cell growth (2). The cells on the

pre-used Petri dishes (2) have already begun to move into aggregates

leaving denuded areas of plastic whereas the cells plated onto

unused plastic exist as a monolayer which covers the entire substrate.

Scale bars represent 25 μm
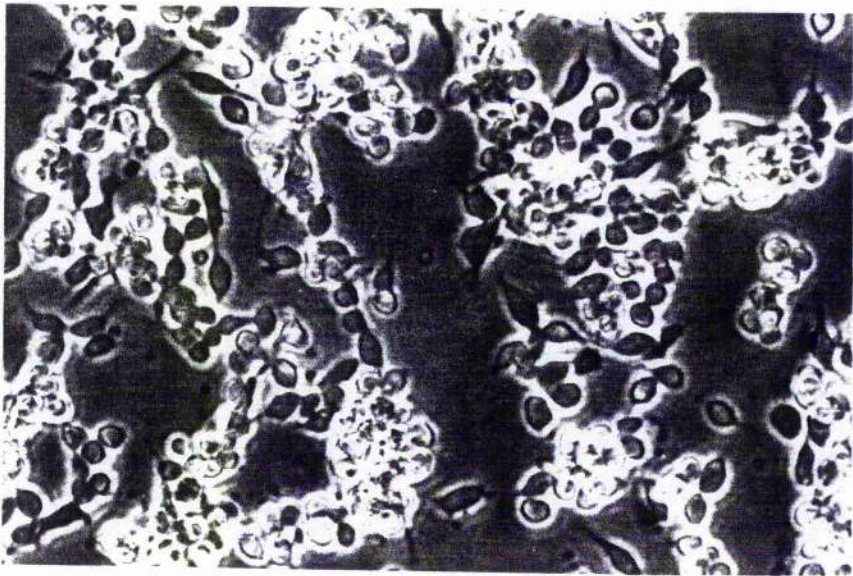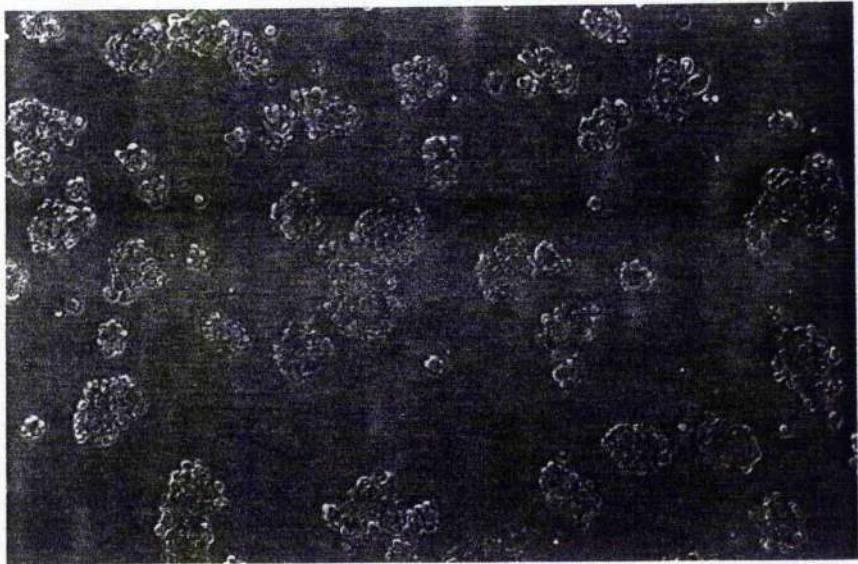
Figure 4.J

1.
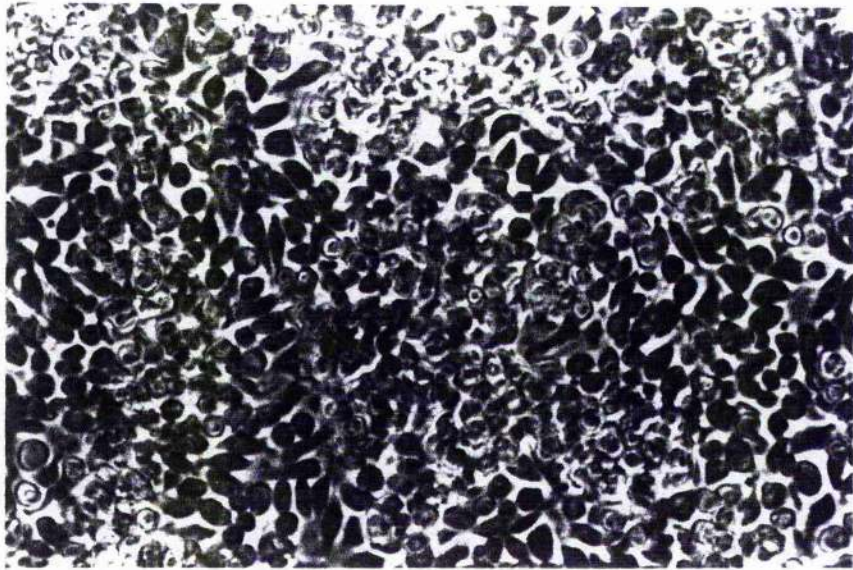
2.

Figure 4.K The response of Cl. C9 cells 2 days after plating onto a substrate of collagen III and photographed using phase contrast optics. Cells in the control sample (1) have adhered and have already begun to aggregate. The cells plated onto collagen (2) (3), however, have failed to adhere to the substrate and have instead formed numerous free-floating aggregates (A).

Scale bars represent 50 μm in (1) and (2), and 15 μm in (3)
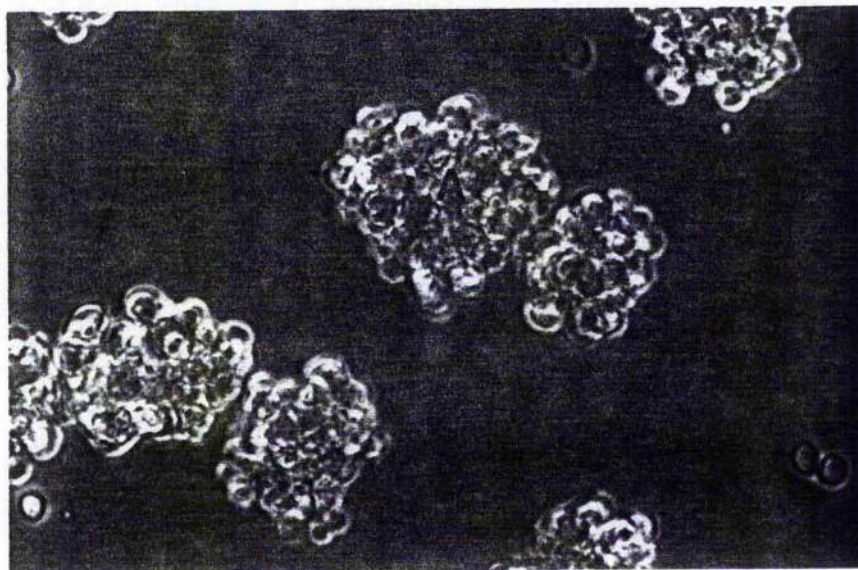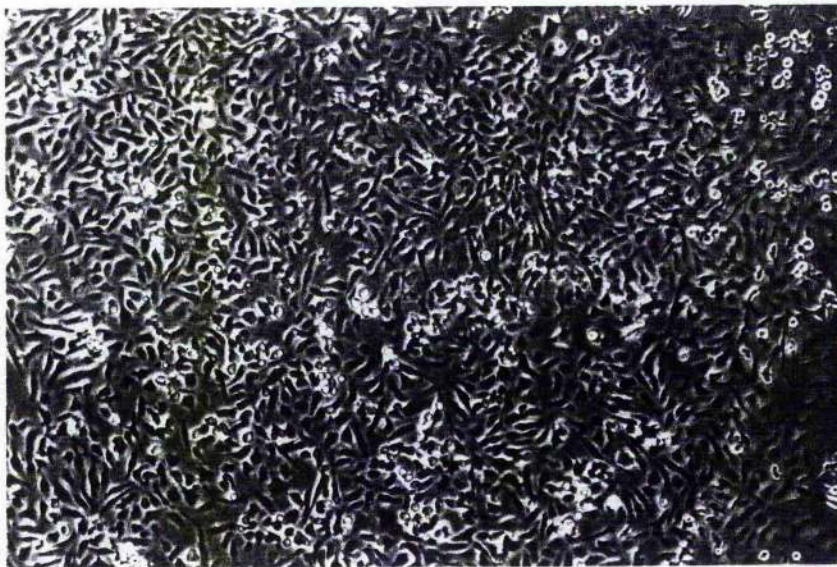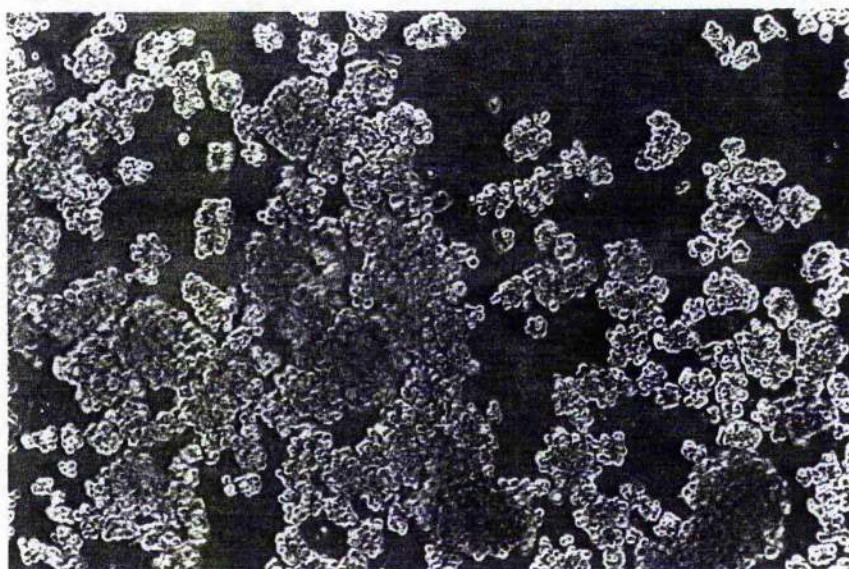
Figure 4.K

1.

2.

3.

Figure 4.L   This set of micrographs details the response of Cl. 8+ cells when grown on a substrate of collagen III. As before, cells in the control sample (1) have adhered well to the substrate whilst cells plated onto collagen (2) (3) have failed to adhere to the substrate and have formed large free-floating aggregates (A). There also seems to be some adhesion between separate clumps, forming groups and chains of aggregates.

Scale bars represent 50 μm in (1) and (2), and 15 μm in (3)
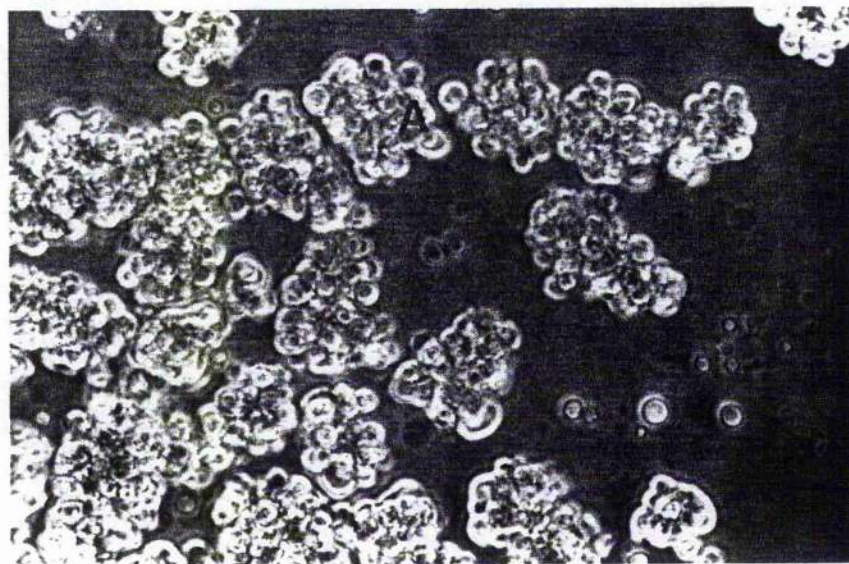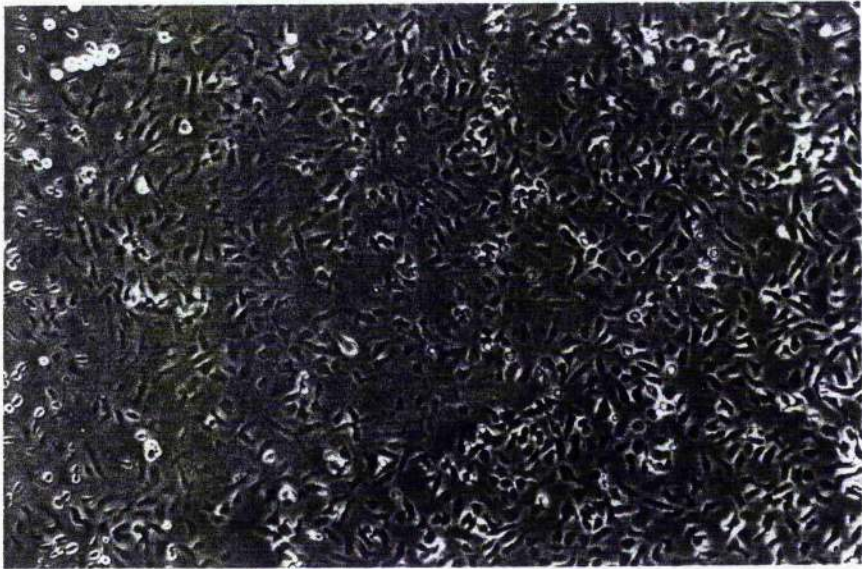
Figure 4.L

Figure 4.M This set of micrographs illustrates the appearance of Cl. 8+ cells 2 days after being plated onto a substrate of collagen IV. As with collagen III, cells on collagen IV fail to adhere (2) (3) whereas cells in the control sample have adhered well. Again, the cells which fail to adhere to the substrate instead form free-floating aggregates.

Scale bars represent 50 µm in (1) and (2), and 15 µm in (3)

Figure 4.M

1.

2.
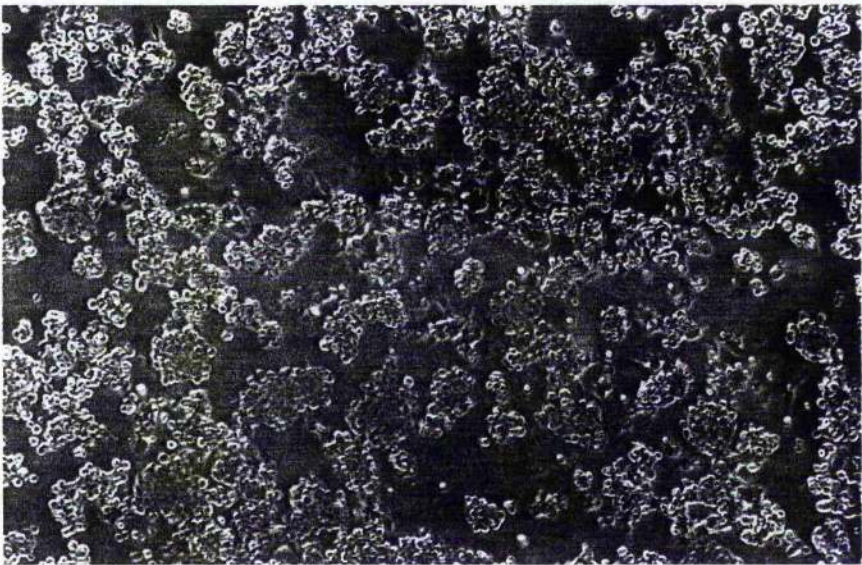
3.
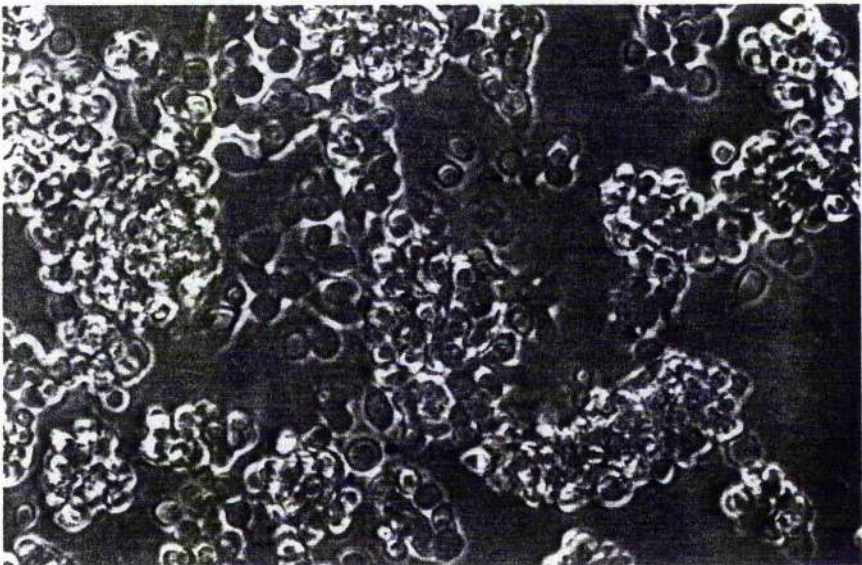
Figure 4.N   The response of Cl. 8+ cells to growth on a laminin substrate. The growth of imaginal disc cells on a substrate of laminin is much the same as is seen with cells which have been plated onto collagen, with cells in the control cultures (1) adhering well to the culture surface and cells in the experimental cultures (2) failing to adhere and forming loose agglomerations of aggregates instead.

Scale bars represent 50 μm

Figure 4.N

1.
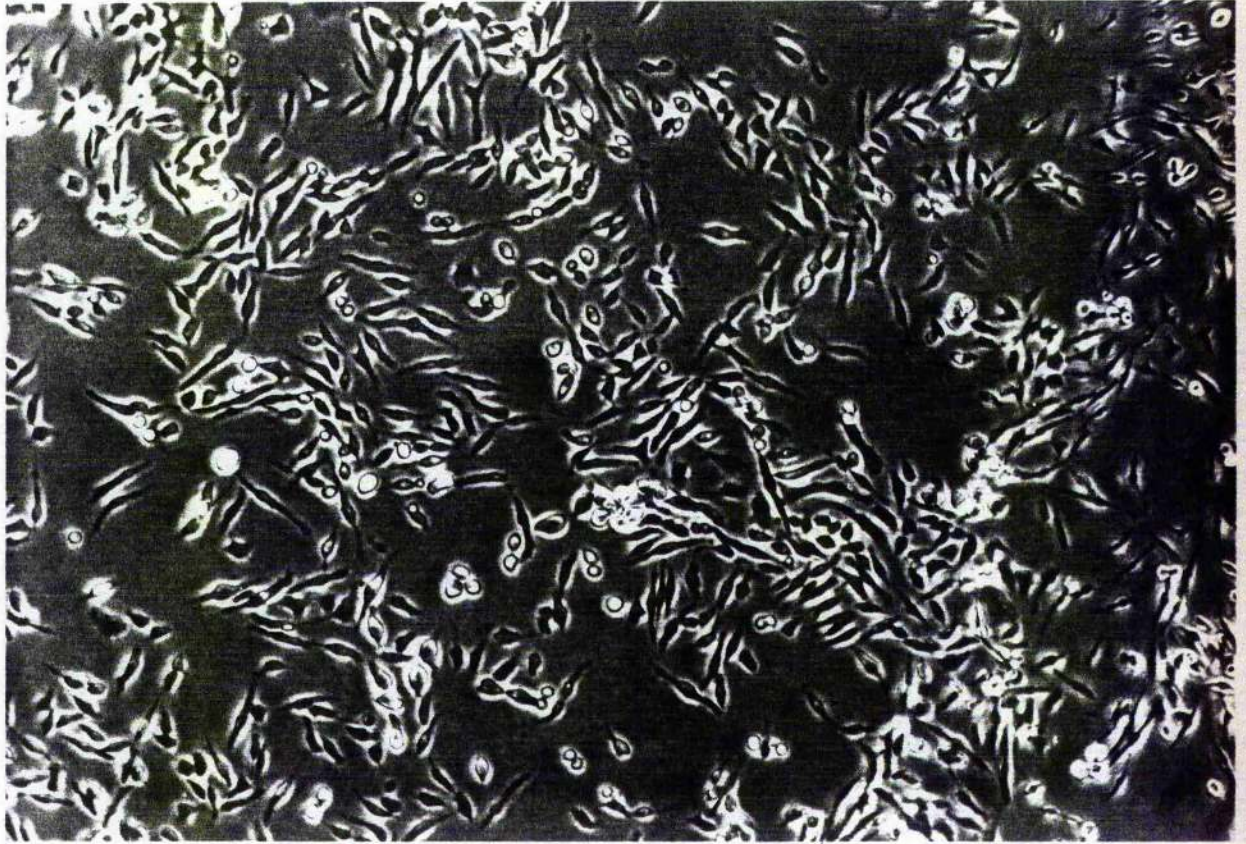
2.
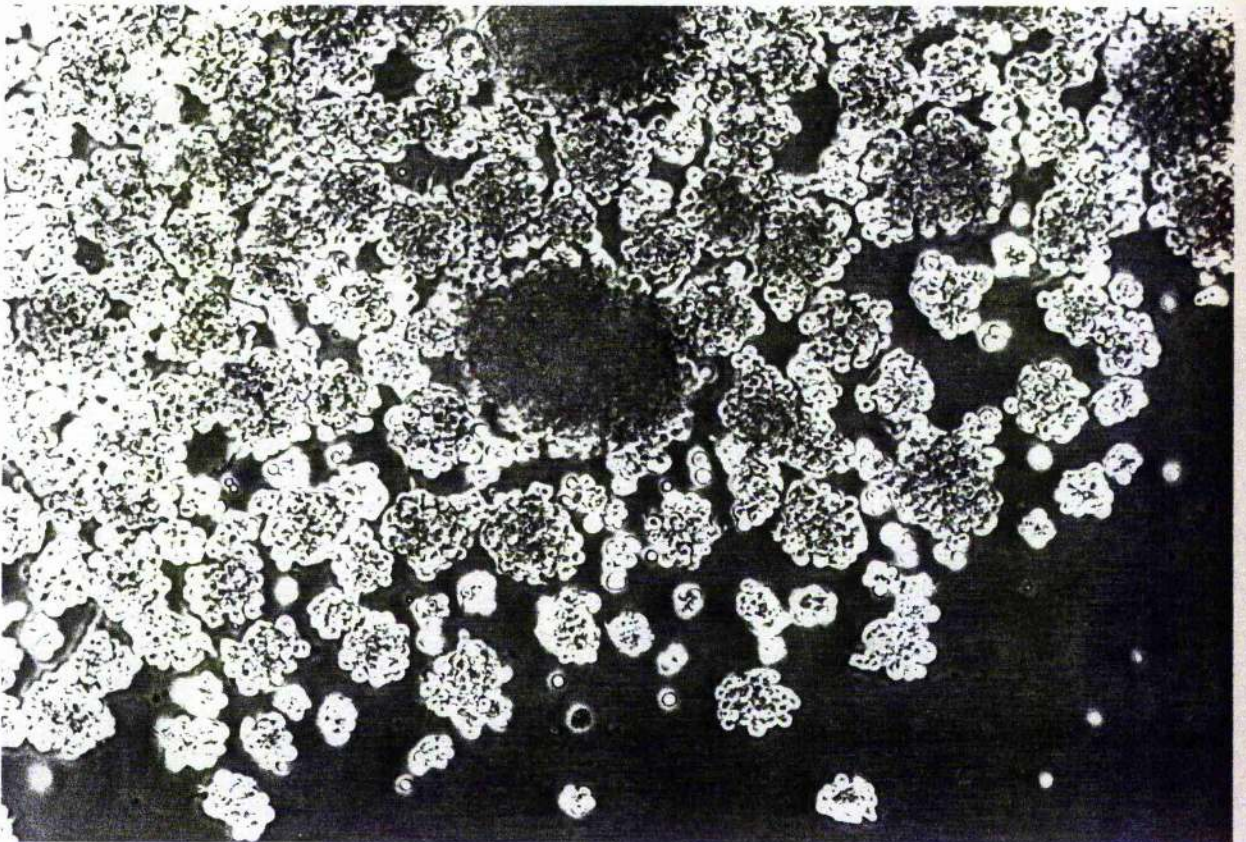
Figure 4.O The growth of imaginal cells when plated on a substrate of fibronectin. The cells in these micrographs belong to the Cl. 8+ line and were photographed 1 day after plating onto the substrate. Here cells in both the control (1) and experimental (2) (3) cultures have adhered to and spread on the substrate. Some of the cells in (3) appear phase dark and fibroblast-like in appearance and can be seen to extend numerous extensions and processes as they migrate and begin to aggregate. Other cells appear more rounded and epithelioid and are more refractile perhaps prior to division.

Scale bars represent 50 μm in (1) and (2), and 15 μm in (3)

Figure 4.O

1.
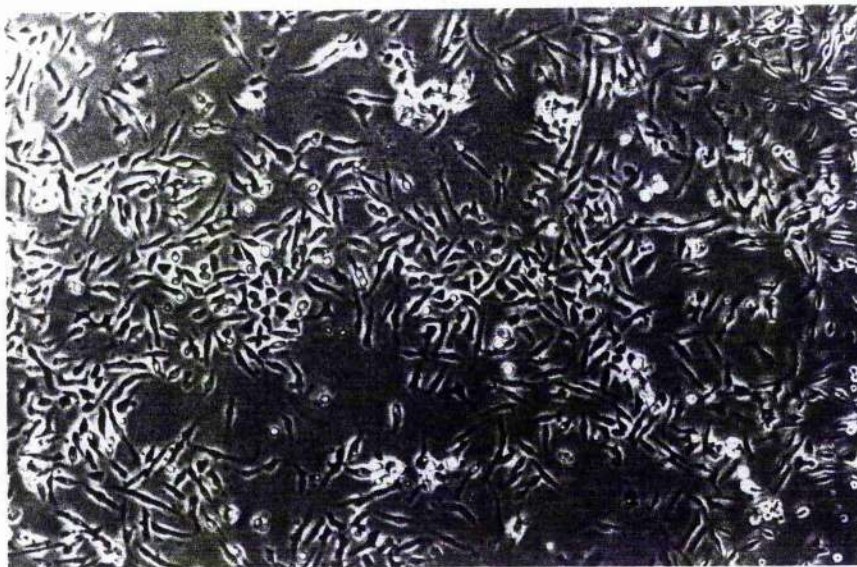
2.
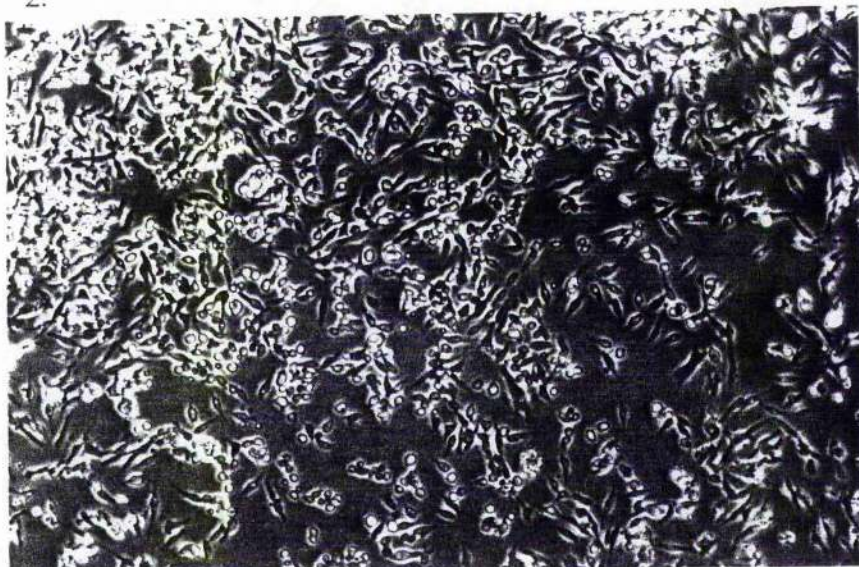
3.
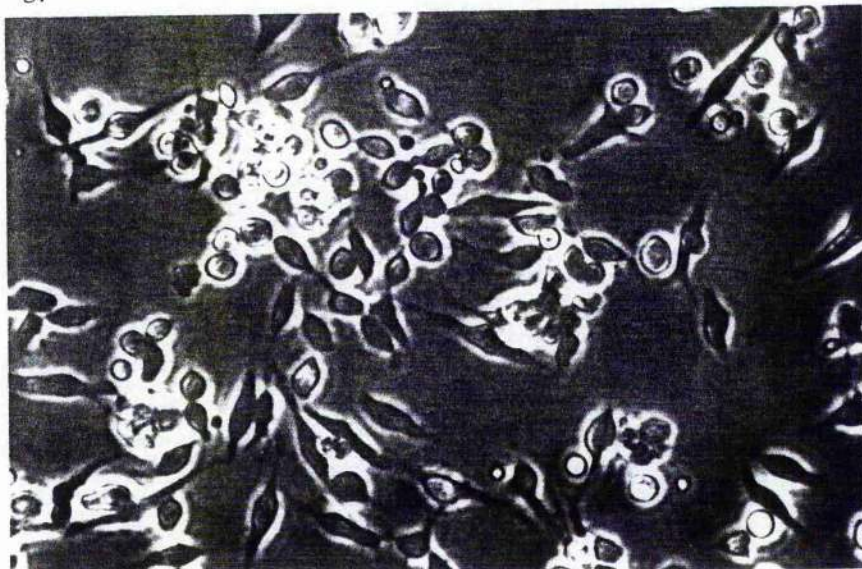
Figure 4.P   Cl. 8+ cells 10 days after plating to a substrate of human
fibronectin. The cells plated onto fibronectin (2) are seen to migrate into
dense aggregates, much the same as is seen in the control cultures (1)
although the aggregates in (2) seem much darker and compact leaving
the surrounding substrate completely denuded of cells.

Scale bars represent 50 µm

Figure 4.Q (overleaf)   Here Cl. 8R cells are shown aggregating on human
fibronectin, 10 days after plating, and again dense aggregates are
seen in each. As in Figure 4.P, cells plated onto fibronectin (2) have
formed very dense aggregates dotted on the substrate. Some stellate like
fascicles of cells (arrows) can be seen radiating between aggregates
which are more distinct than those in the control culture (1).

Scale bars represent 50 µm

Figure 4.P

1.

2.
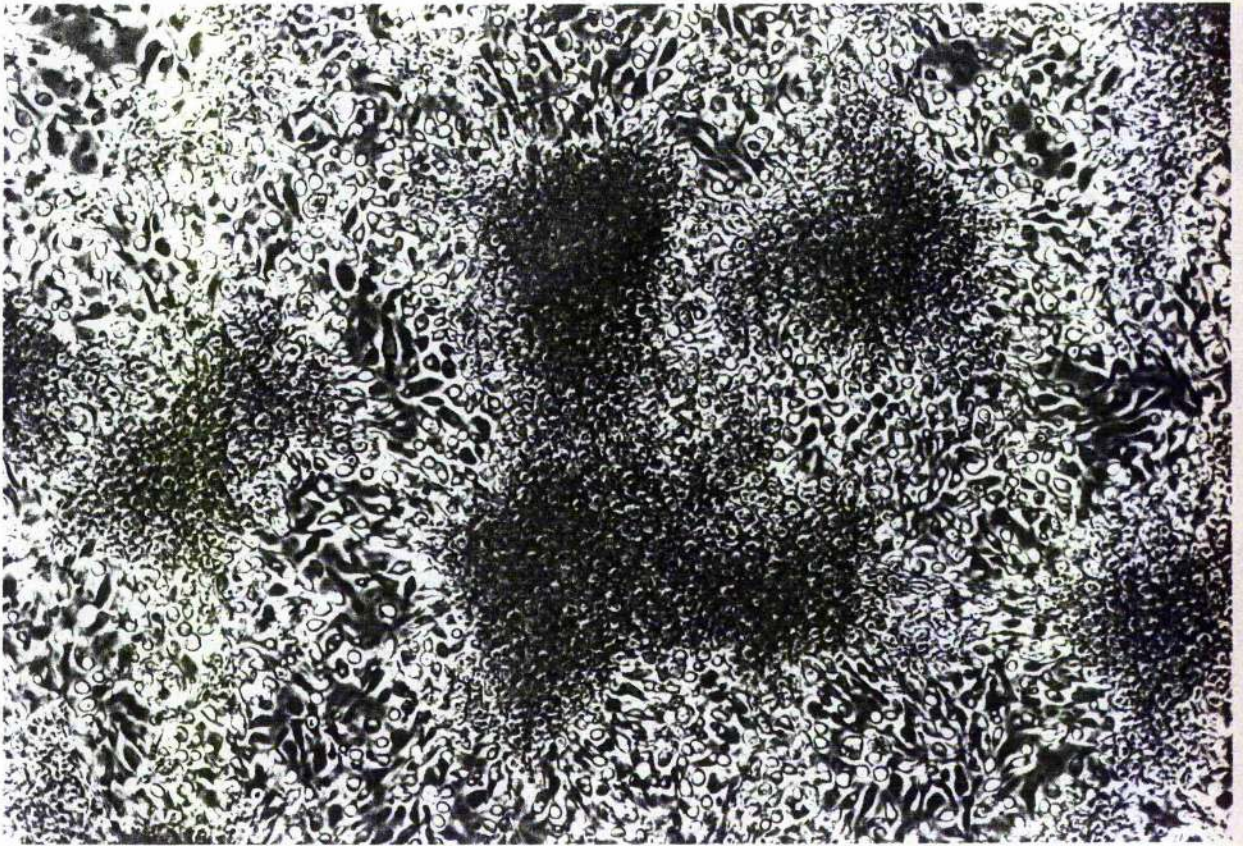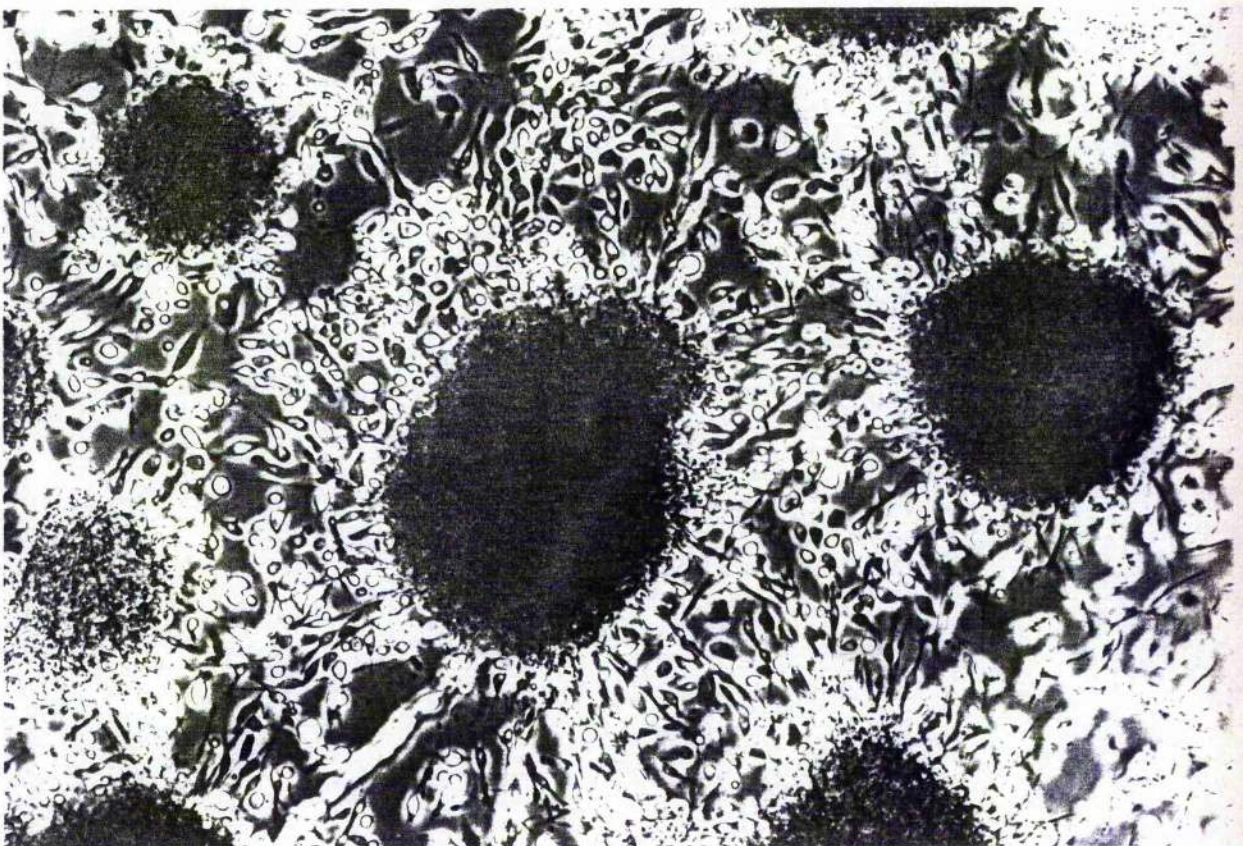
Figure 4.Q

1.
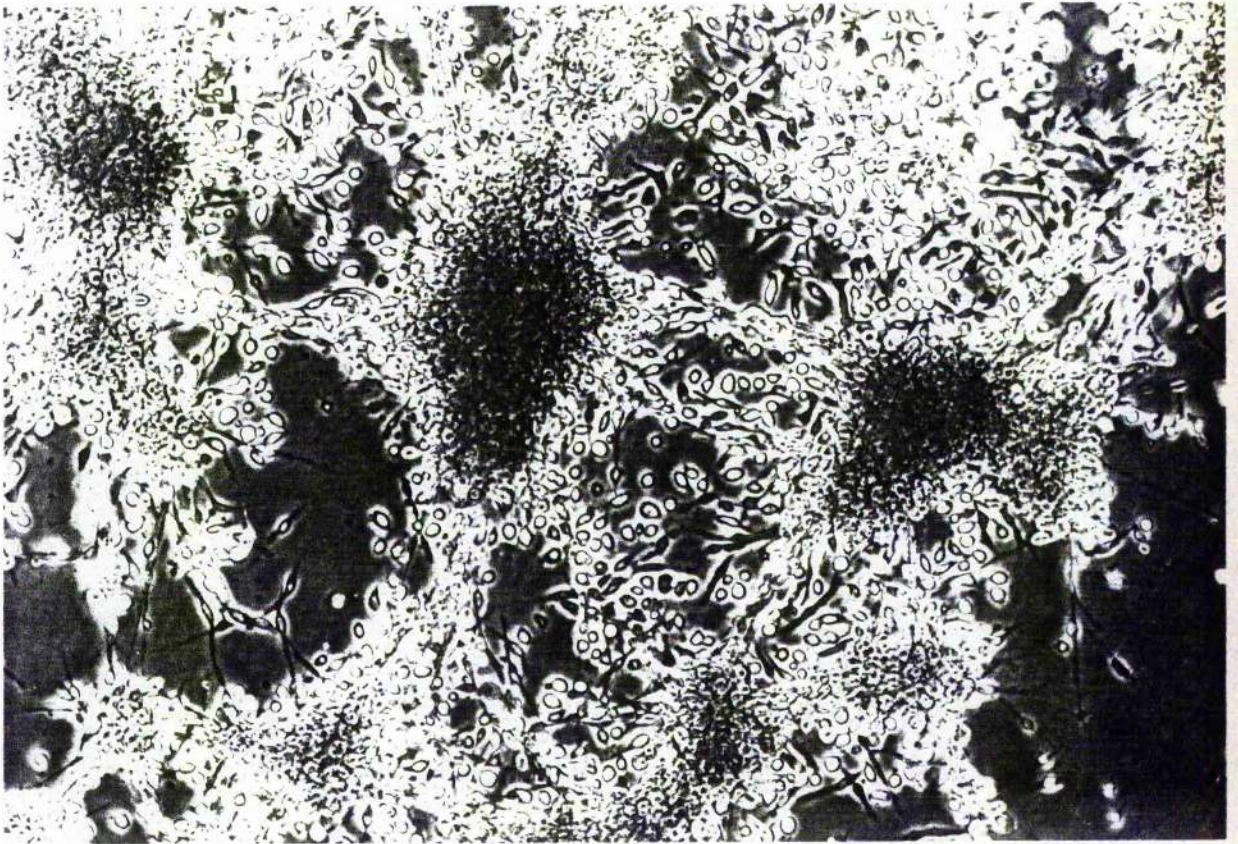
2.
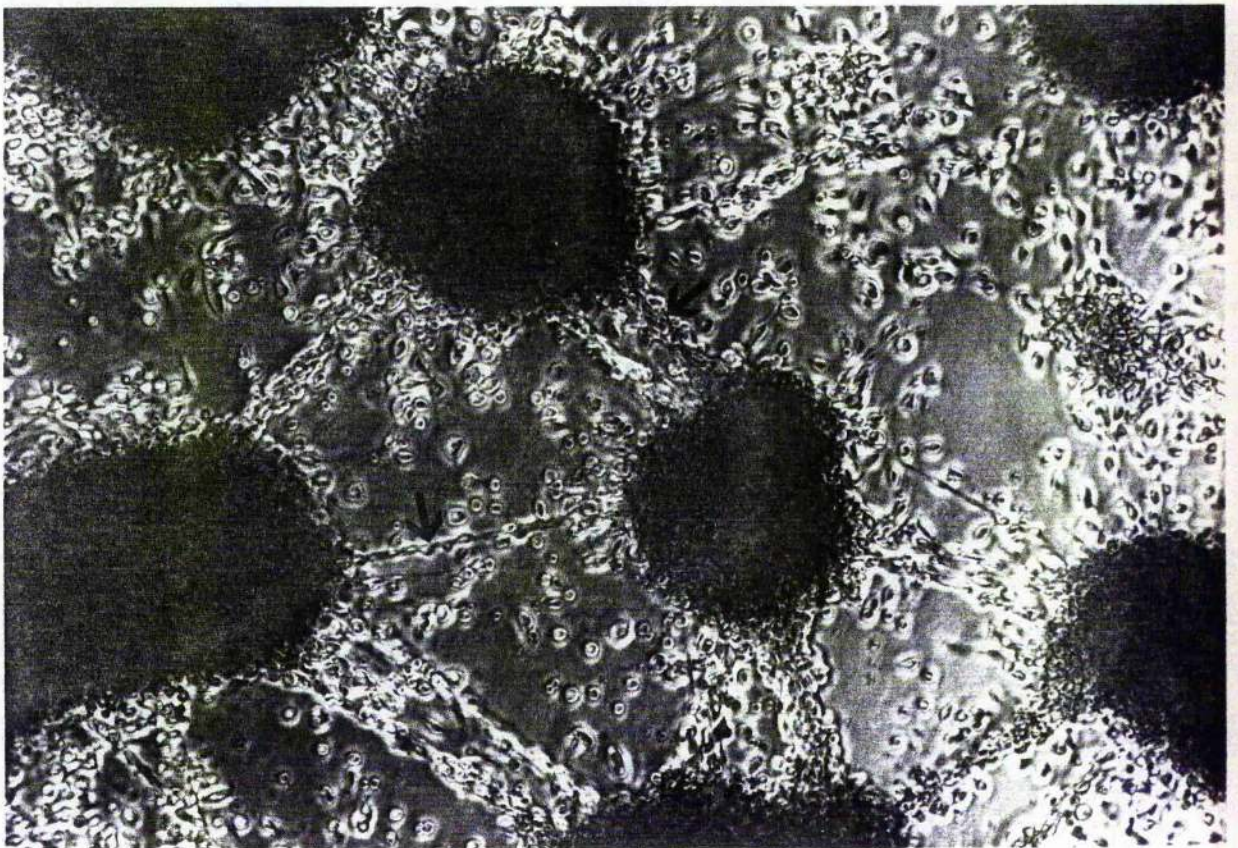
Figure 4.R   Micrographs of the response of Cl. 8+ cells to aBG-1 when spread on fibronectin in the absence of serum. Cells that are either cultured in MM3 with no serum (1) or with pre-immune serum (3) remain flattened to the substrate surface and appear phase-dark whereas cells grown under similar culture conditions but allowing for the presence of the anti-β_{PS} antibody, aBG-1 (2), are free-floating and refractile in appearance.

Scale bars represent 25 μm

Figure 4.R

1.
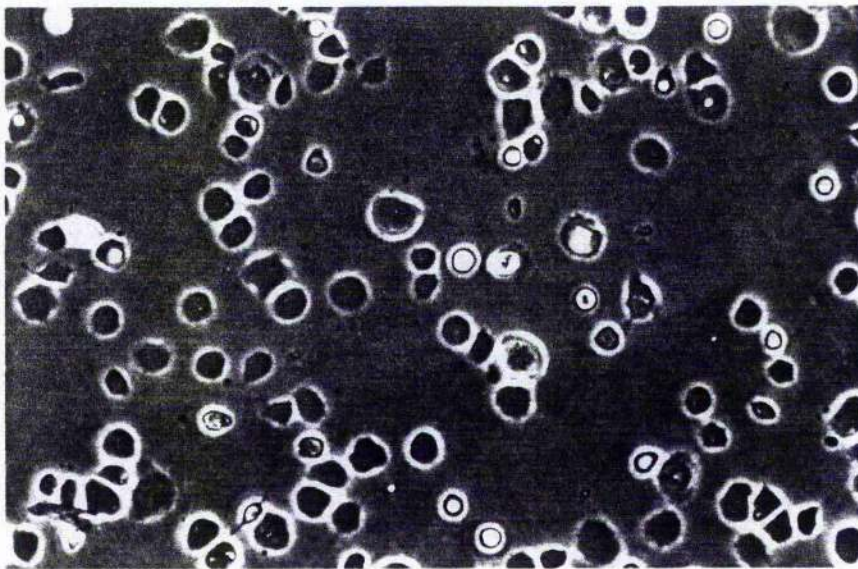
2.

3.
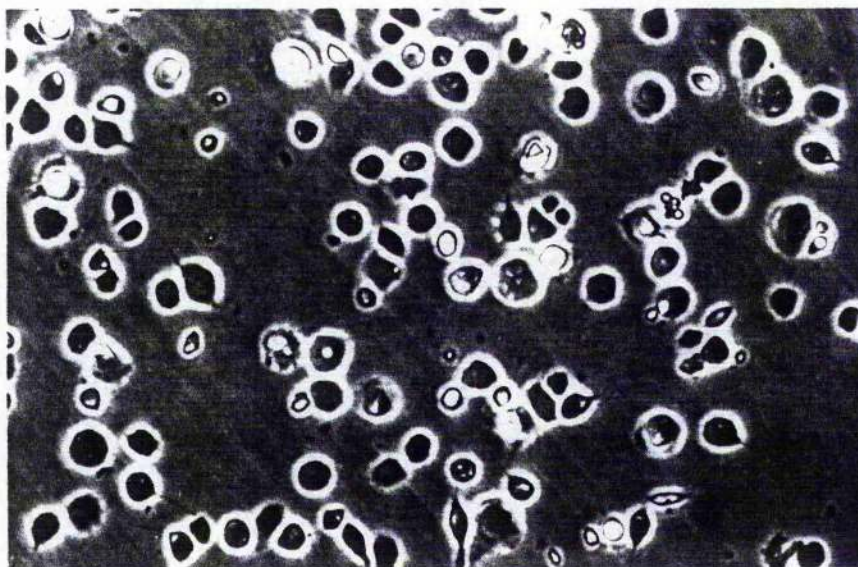
Figure 4.S   Cl. 8+ cells grown on tissue culture plastic in serum-free

medium either in the presence of pre-immune serum (1) or presence of

the anti-PS antibody, aBG-1 (2). In both this and the subsequent set of

figures (Figure 4.T) those cells which have been plated onto a substrate

in the absence of the antibody appear flattened and phase dark (4.S.1),

(4.T.1), whereas those where the antibody is present appear refractile

and free-floating (4.S.2), (4.T.2).

Scale bars represent 25 µm

Figure 4.T (overleaf)   In these micrographs, Cl. 8+ cells have been

incubated, as in Figure 4.S, in serum-free medium either in the presence

of pre-immune serum (1) or aBG-1 (2). In this set of assays, however, the

substrate consisted of human fibronectin.

Scale bars represent 25 µm

Figure 4.S

1.

2.
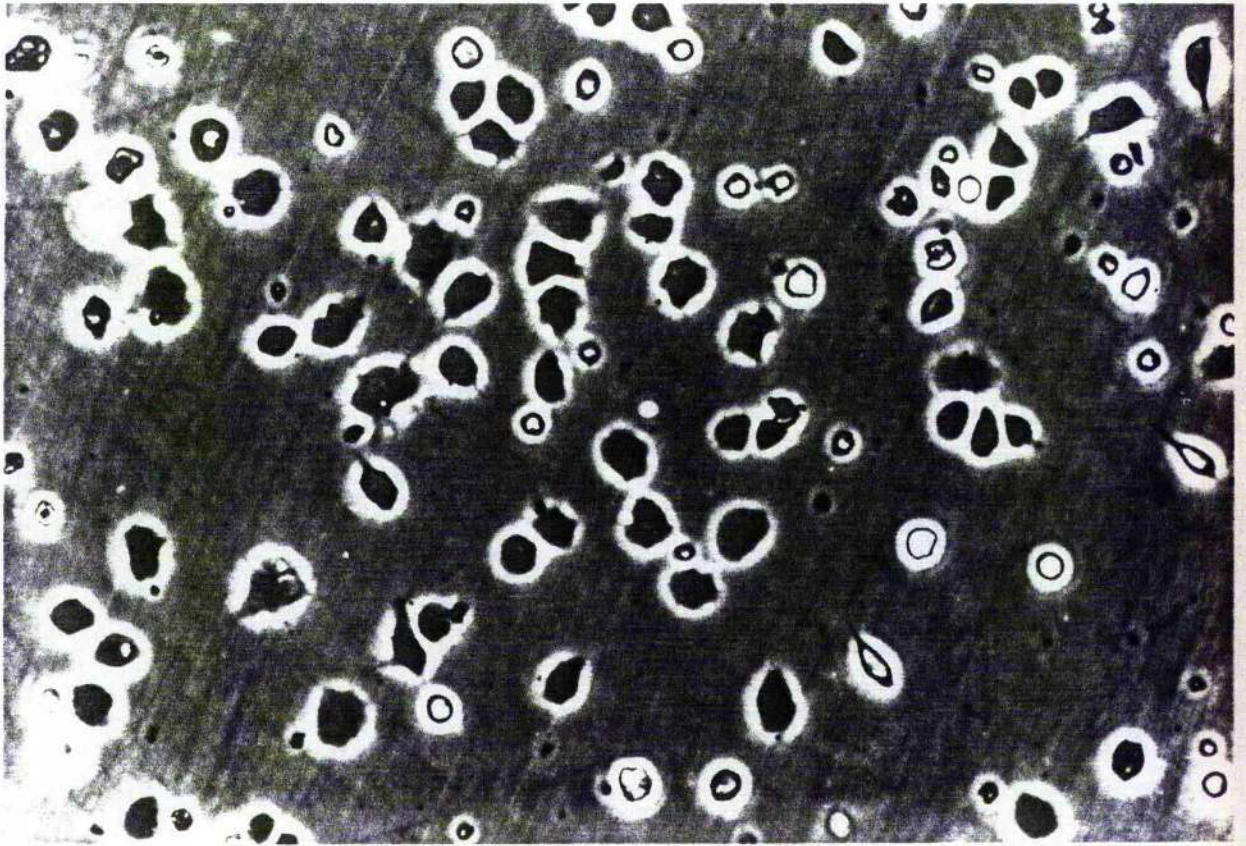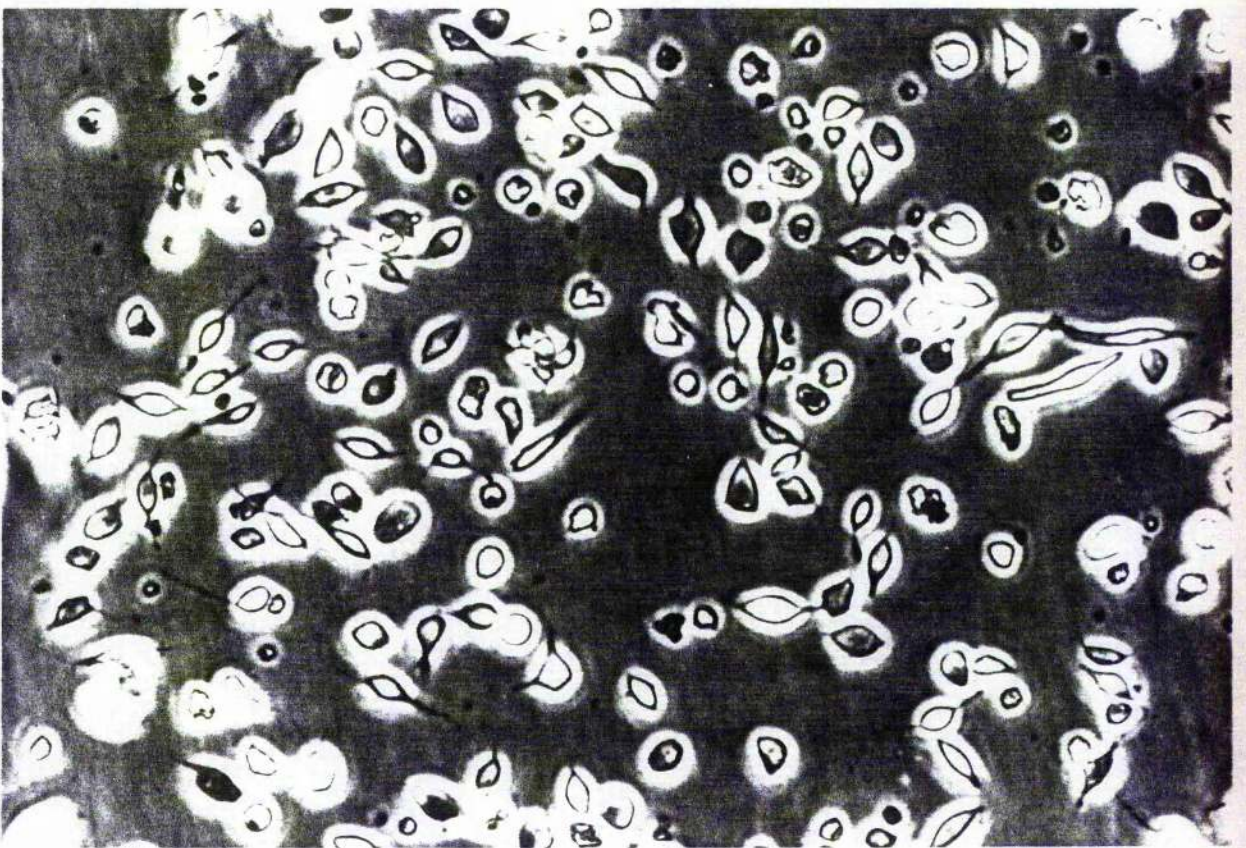
Figure 4.T

1.
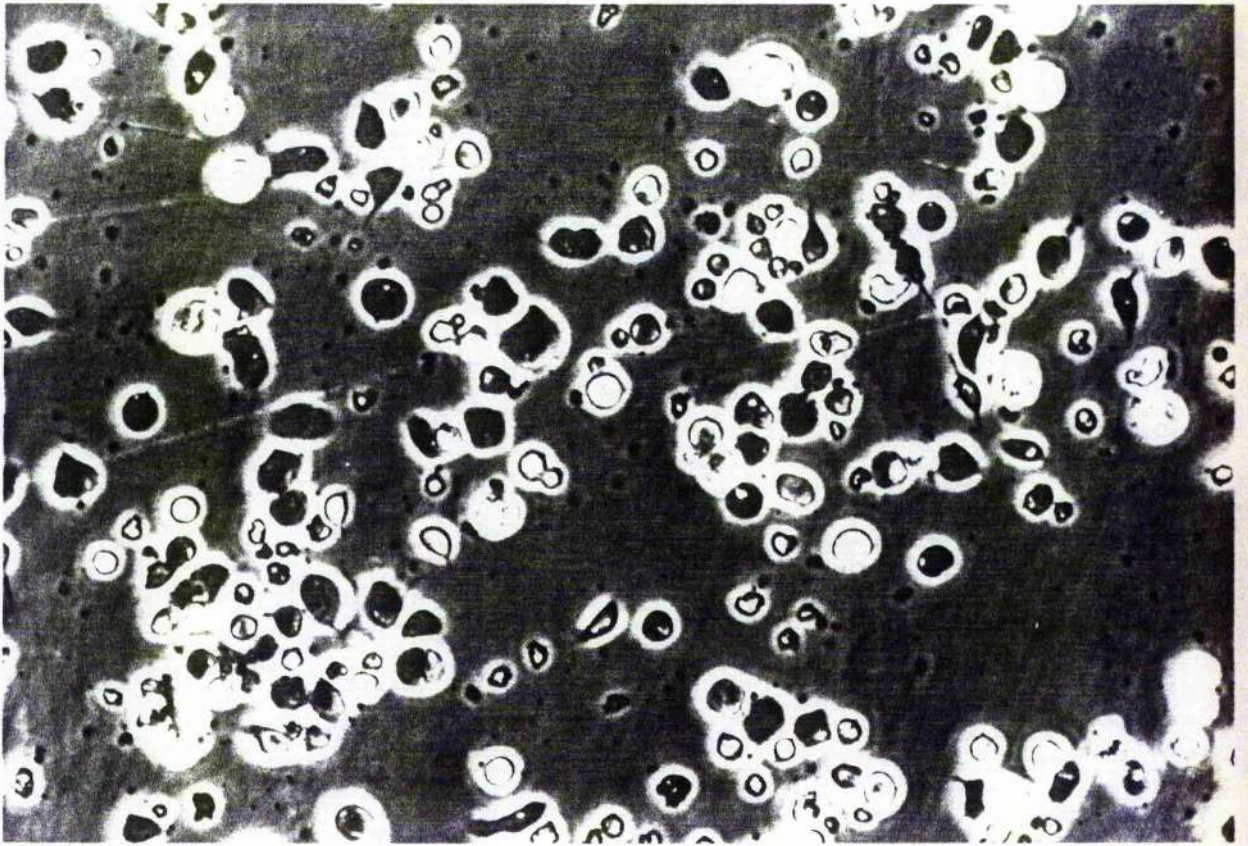
2.
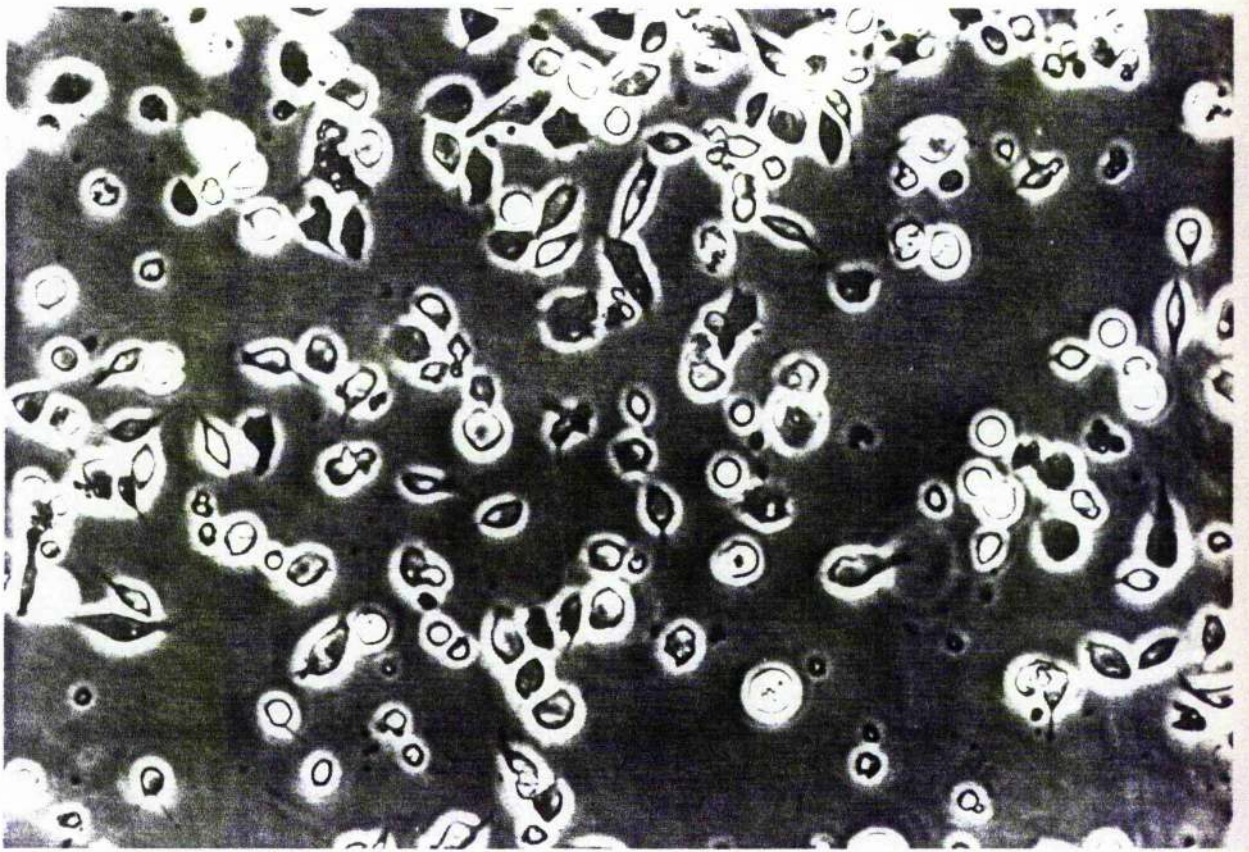
Figure 4.U Morphological effects of aBG-1 antibody on the long-term
aggregation of L127D6 cells. After 2 days growth, those cells which have
been grown in culture without aBG-1 (1) are already showing
pronounced aggregation. Cells in the experimental culture (2),
however, seem to have partially adhered to the substrate but have not
begun to aggregate.

Scale bars represent 50 μm

Figure 4.V (overleaf) Effects of aBG-1 on imaginal disc cell growth , 6
days after plating. The difference between the cultures by this stage is
more marked than in Figure 4.U, as cells in the control culture (1) form
dense aggregates and cells in the experimental sample (2) remain in
more or less a disaggregated state. However, it is notable that
some small aggregates have formed in the presence of the antibody but
little cell-substrate adhesion and proliferation seems to have taken place.

Scale bars represent 25 μm

Figure 4.U

1.

2.
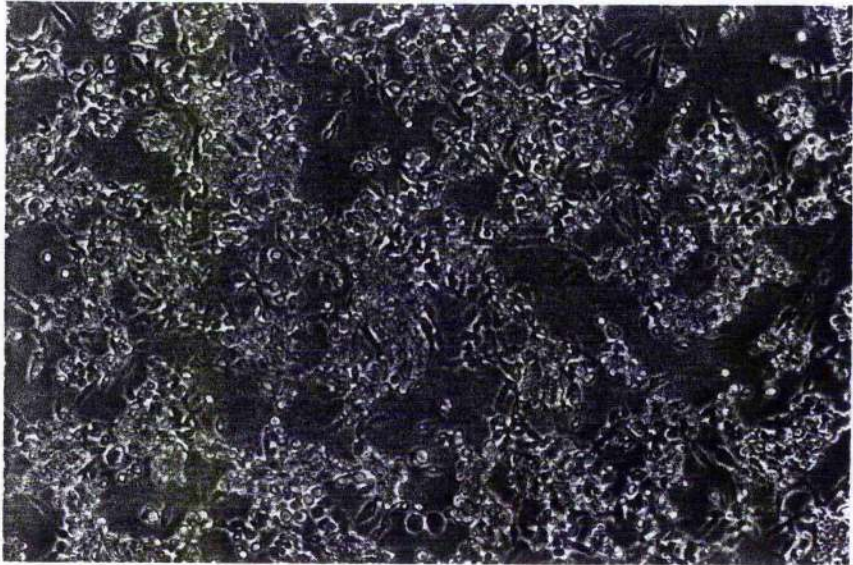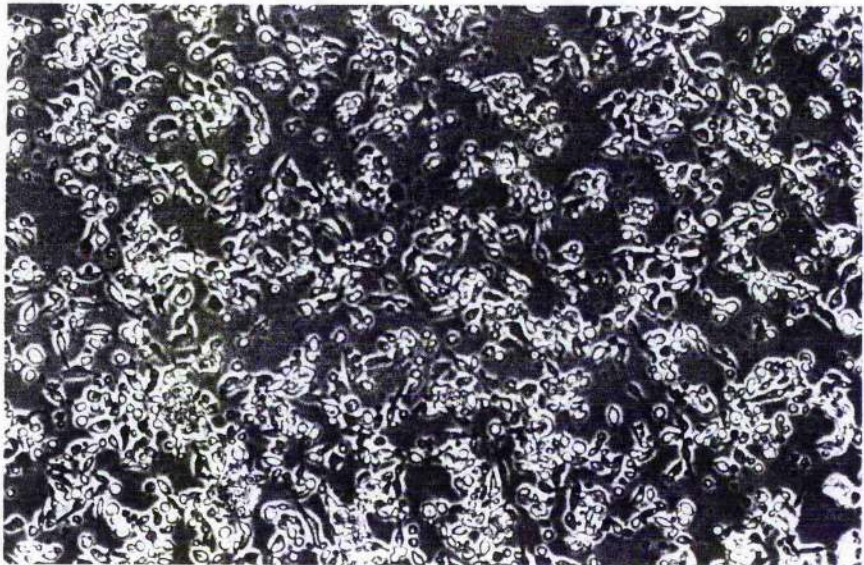
Figure 4.V

1.
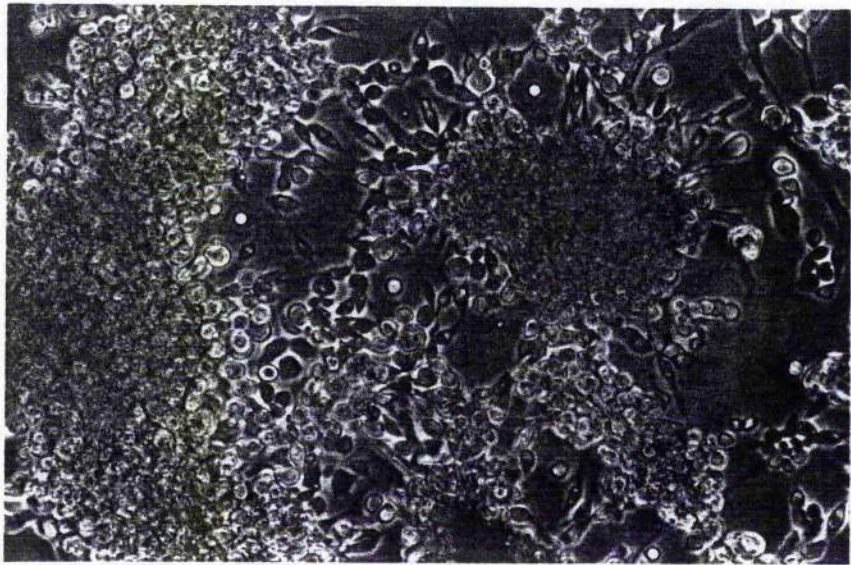
2.
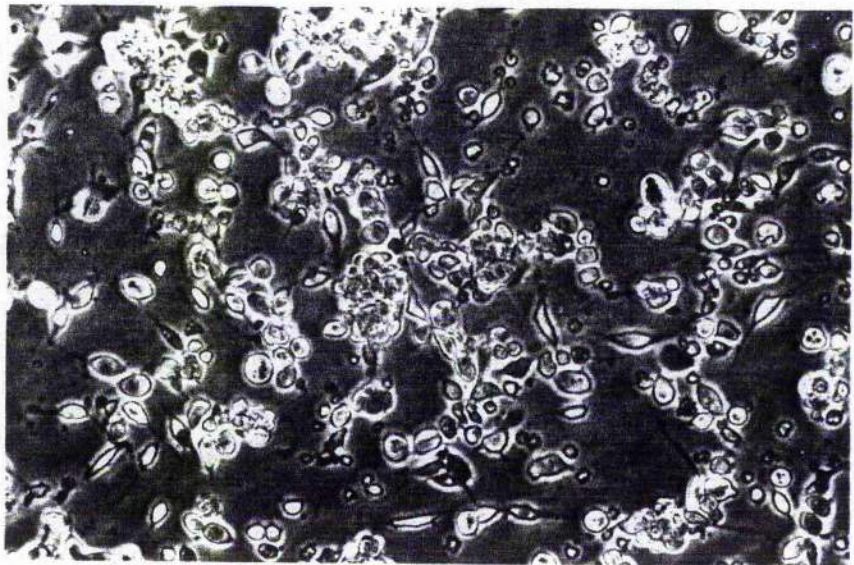
Figure 4.W   Effects of aBG-1 on Cl. 8+ cell growth, 2 days after plating. Cells in the control culture (1) have adhered and spread well on the substrate, appearing as darkened and elongated in appearance whereas cells in the experimental culture (2) similarly how some adhesion and migration, but the presence of debris suggests that some cell death may be occurring.

Scale bars represent 50 μm

Figure 4.X (overleaf)   effects of aBG-1 on Cl. 8+ cell growth, 7 days after plating. As in Figure 4.S, the difference between samples by this stage is more marked, with cells in the control sample (1) proliferating and migrating into aggregates and cells in the experimental sample (2) failing to progress; either to proliferate or migrate into aggregates.

Scale bars represent 25 μm

Figure 4.W

1.

2.
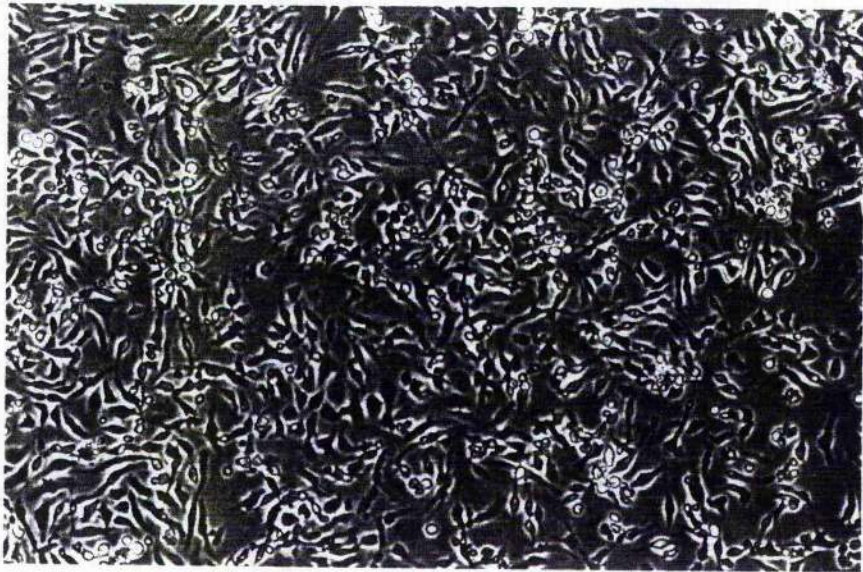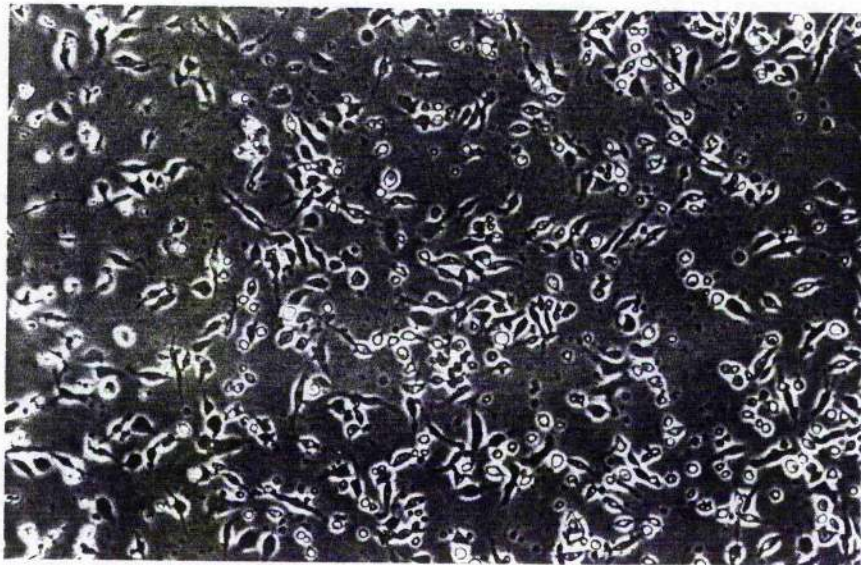
Figure 4.X

1.
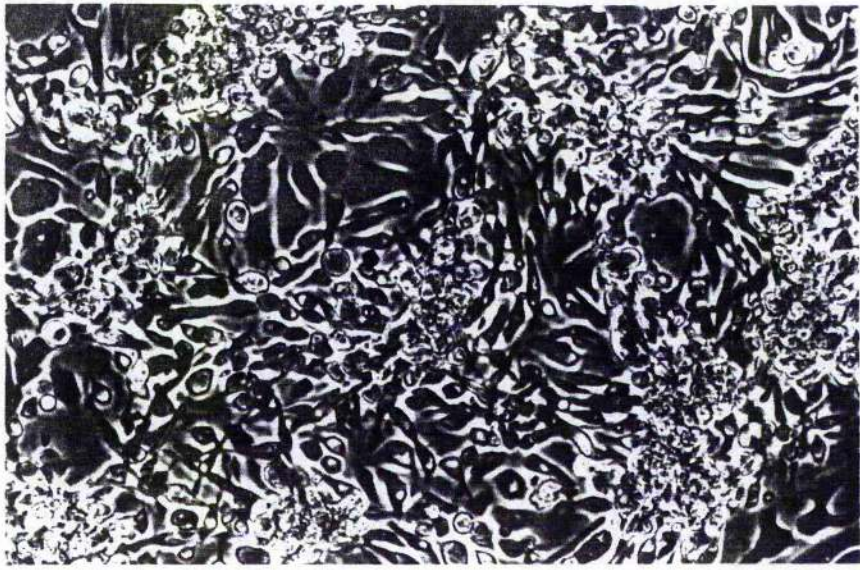
2.
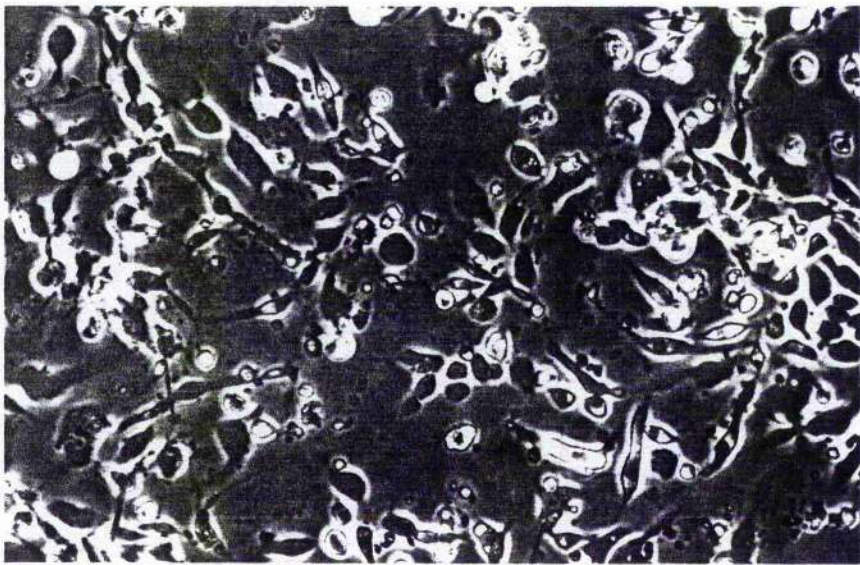
Figure 4.Y   The effect of adding the pentapeptides GRGDSP and GRGESP

to aggregating imaginal disc cells *in vitro*. Here, both the RGD (2) and

RGE (3) containing peptides were added to pre-substrate-adhered Cl.

8+ cells and were photographed after a 2 hour incubation period. The

control sample, containing an equivalent amount of BSA is pictured in

(1). Neither the RGD or RGE containing peptides seems to have had any

effect in altering the state of cell-substrate adhesion Of the Cl. 8= cells

during this period.

Scale bars represent 25 μm

Figure 4.Z (overleaf)   The effect of adding RGD and RGE containing

peptides to cultures of Cl. 8+ cells. Here the cultures pictured in Figure

4.W are shown 3 days after passage. Again, neither the GRGDSP (2) or

GRGESP (3) containing cultures seem morphologically distinct from

the control culture (1). Indeed, a very distinct aggregate has formed in

the GRGDSP containing culture; the peptide seemingly failing to block

imaginal cell morphogenesis.

Scale bars represent 50 μm in (1) and (2) and 25 μm in (3)

Figure 4.Y

1.

2.

3.

Figure 4.Z
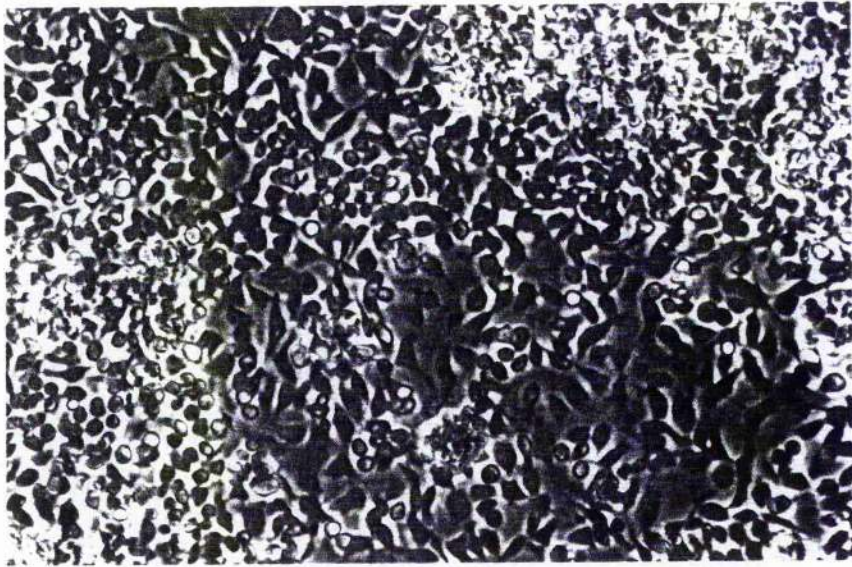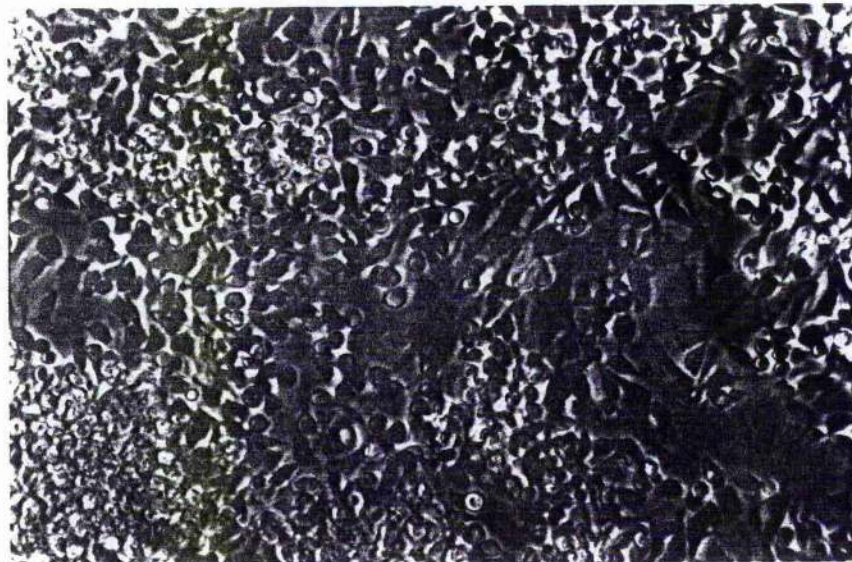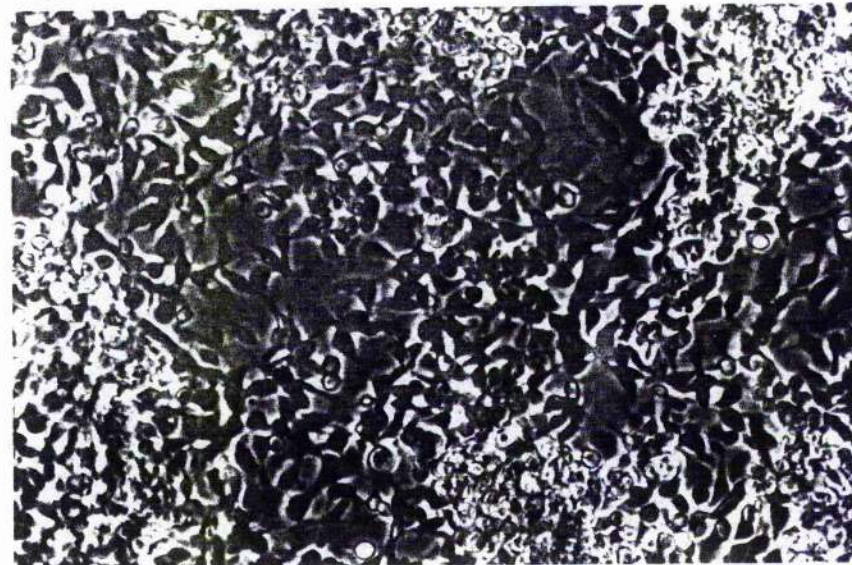
1.

2.
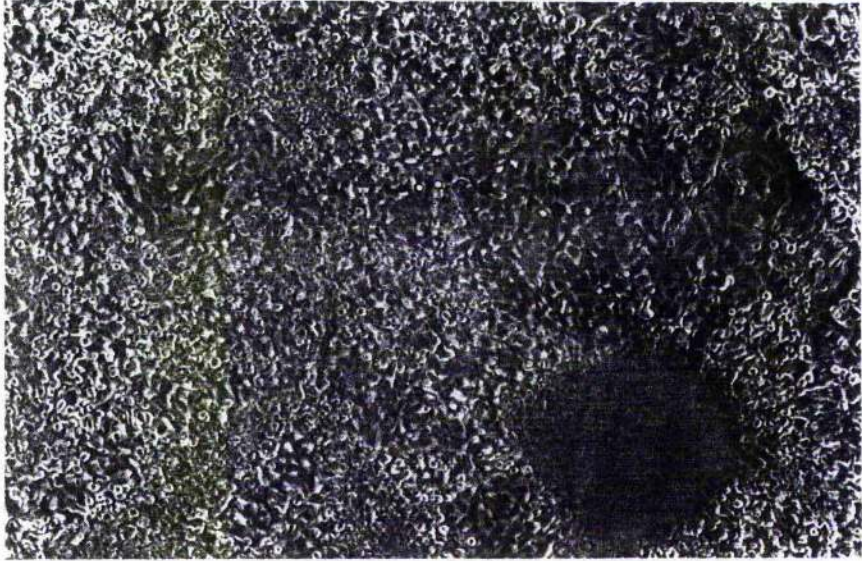
3.
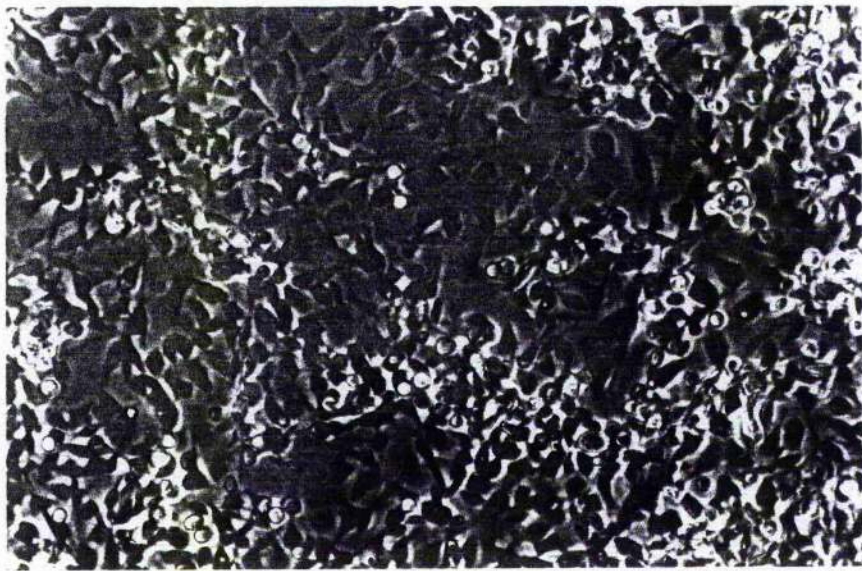
4.4   Discussion

4.4.1   Immunocytochemistry

From the evidence provided by immunocytochemical staining, analysed by laser scanning confocal microscopy, it seems that PS integrins are widely expressed in *Drosophila* imaginal cells throughout morphogenesis. This staining was achieved primarily through the use of the monoclonal anti-β_{PS} probe, aBG-1. These results suggest that PS integrins are being expressed on the surface of imaginal cells and especially at sites of cell-cell contact.

One might expect to find a restricted pattern of expression of PS integrins in cloned wing cell lines, depending on whether the clones were derived from a dorsal or ventral wing cell. However, in their study of PS1 and PS2 staining in cloned wing cell lines, Peel and Milner reported that they could find no homogeneity of PS integrin expression; each line tested was found to express both PS1 ($\alpha_{PS1}\beta_{PS}$) and PS2 ($\alpha_{PS2}\beta_{PS}$) in a heterogeneous manner (Peel and Milner, 1992a). In the same study, Peel and Milner reported that both PS1 and PS2 are preferentially expressed in aggregates of imaginal cells and at sites of cell-cell contact only. The accumulation of β_{PS} at cell-cell contact sites, as interpreted from aBG-1 staining, is consistent with these findings and reflects the expression of PS integrins at myoepidermal attachment sites in the embryo and at dorsal-ventral epidermal attachment sites in the pupal wing (Bogaert *et al.*, 1987; Wilcox *et al.*, 1989). The specific identity of the heterodimers at these sites is unclear as the β_{PS} subunit is shared by both the PS1 and PS2 integrins in imaginal discs.

However, there seems to be a slight inconsistency between the staining patterns generated using aBG-1 reported here and the PS integrin probes used in the Peel and Milner study. Peel and Milner reported that all the PS integrin antibodies, against α_{PS1} α_{PS2} and β_{PS}, tested on imaginal cells strongly

stained sites of cell-cell contact with no, or very diffuse, expression elsewhere, whilst aBG-1 tends to stain entire cells; single or aggregated. Because βPS cannot exist as a monomer at the cell surface (Hynes, 1992), it does not seem likely that aBG-1 is recognising β subunits that are not bound to α subunits. The fixation and staining protocols used in both studies were very similar, therefore it is unlikely that this was the cause of different staining patterns.

One explanation for this apparent contrast of staining of the two anti-βPS probes could be that the probe used by Peel and Milner tends to only recognise an epitope on integrins that are engaged in adhesion, and those that are present as unbound heterodimers on the cell surface do not get recognised by that particular βPS antibody. However, this also sounds unlikely as the same antibody against PS2 (CF2C7) was used in separate studies and was found on surfaces of epidermal cells other than those that were adherent to other cells (Brower et al., 1984; Fristrom et al., 1993). A more likely explanation is that the cells stained in the present study were at an early stage of morphogenesis (3 days in culture) and the original plating density of cells was low in comparison to the Peel and Milner study (2×10^5 compared to 7.5×10^5). Therefore, it is likely that the cells in the present study were at a stage in morphogenesis of cell-substrate rather than cell-cell adhesion, which may explain the peripheral expression of PS integrins. The cells stained in the Peel and Milner study were more likely to have been involved in cell-cell adhesion, due to the increased cell number and advanced stage of morphogenesis, which seems to favour cell-cell rather than cell-substrate adhesion (Peel et al., 1990).

From the staining patterns of βPS one can attribute possible functions for the PS integrins in imaginal cell morphogenesis in vitro (see Figure C). The tissue with which this particular system may be compared is the developing wing during Drosophila metamorphosis. During the apposition stages in wing morphogenesis, the basal surfaces of the dorsal and ventral

epithelia are known to extend so that they come into close proximity and then subsequently adhere (Waddington, 1941; Milner and Muir, 1984; Fristrom *et al.*, 1993). Dianne Fristrom and co-workers (pers. comm.) have discovered that this phenomenon involves the extension of basal cell processes across the ECM-filled wing cavity prior to making contact with the cells of the opposing epithelium and it seems likely that the PS integrins play an important role in this process (for review, see: Hynes and Lander, 1992).

The PS integrin β subunit is known to be expressed on the basal parts of these cell extensions during apposition (Fristrom *et al.*, 1993) and it is possible that these molecules may form transient cell-substrate connections that are important in this process of cell extension. The phenomenon of *Drosophila* imaginal cell migration *in vitro* may be homologous to what is seen *in vivo*. As *in vivo*, βPS is expressed on the extended parts of cells *in vitro* which extend towards, and are coming into contact with, other cells or groups of cells prior to aggregation. Like the epidermal cells that are found in the metamorphosing pupal wing, imaginal cells *in vitro* extend microtubule-rich processes to other cells and it has been suggested that they may play an active role in driving aggregation (Peel *et al.*, 1990; Peel, 1991). Vertebrate integrins have been implicated in the process of cell migration (Hynes and Lander, 1992) and there is evidence to suggest that the PS integrins can mediate cell-substrate adhesion (Volk *et al.*, 1990; Hirano *et al.*, 1991; Bunch and Brower, 1992; Gullberg *et al.*, 1994; Fogerty *et al.*, 1994).

Therefore, instead of a basally oriented growth across a wing cavity, imaginal cells *in vitro* presumably send out cell processes distally on either their own, secreted, ECM or on the ECM found on the surface of other cells. Imaginal cells have been observed to grow in thick fascicles between aggregates, and can thus presumably grow on, and migrate over, other cells (Peel *et al.*, 1990).

The second proposed function for PS integrins in imaginal cell morphogenesis *in vitro* is the mediation of cell-cell adhesion, which again evokes what is seen in *Drosophila* pupal wing development. During the stages of apposition and adhesion in the pupal wing, PS integrins are expressed on the lateral surfaces of cells, as well as on the basal sides which extend toward the opposite epithelium, and are believed to be important in maintaining contact between adjacent cells during these stages (Fristrom *et al.*, 1993). In a similar fashion, after a cell *in vitro* establishes contact with a neighbouring cell they then aggregate into large clumps containing hundreds of tightly packed cells (Peel *et al.*, 1990). The expression of PS integrins at these sites of cell-cell contact indicates that they may be important in helping maintain the structural integrity of such aggregates, (Figure C).

One further potential role for PS integrins in imaginal disc cell morphogenesis would be in the formation of tension transmitting junctions between cells. This exists, again, in the pupal wing of *Drosophila* and is believed to be important in adhering the basal contact zones of the dorsal and ventral epithelia during wing expansion. Integrins are present at these basal sites prior to the formation of junctions, which consist of a complex of hemidesmosomes that connect an array of microtubules and microfilaments that originate and extend from the apical end of each cell. This cytoskeletal structure is referred to as either the transalar cytoskeletal array (Tucker *et al.*, 1986; Mogensen and Tucker, 1987; Mogensen and Tucker, 1988;) or the transalar apparatus (Fristrom *et al.*, 1993).(Figure B). These arrays (Figure D) are believed to structurally support the wing, maintaining wing blade thickness, during the separation stages when the wing is distended; during which time the epithelia are furthest apart and the basal connections are hugely elongated (Tucker *et al.*, 1986).

PS integrins are known to be of importance in establishing this contact between basal projections because any defects in integrin expression, such as

in flys mutant for *inflated* (which encodes α_{PS2}) and *myospheroid* (which encodes β_{PS}) can result in a wing blister phenotype due to the incorrect apposition of the dorsal and ventral surfaces (Zusman *et al.*, 1990; Brower and Jaffe, 1989; Wilcox *et al.*, 1989; Brabant and Brower, 1993).

What may be occurring with imaginal disc cells *in vitro* is that the PS integrins that are distally expressed on extended cell processes, as mentioned above, come into contact with neighbouring cells and adhere to them. This adhesion may then allow for the formation of a tension transmitting junction that may facilitate aggregation. These processes resemble the epidermal 'feet' that are found at the basal ends of insect epidermal cells, which are known to be developmentally important (Locke and Huie, 1981; Locke, 1985). These processes are believed to actively drive aggregation by adhering to other cells and then contracting, and so bringing the cells together (Peel *et al.*, 1990; Chapter 6, this thesis). PS integrins are present on these processes throughout aggregation and it is possible that they help establish and maintain the required tension between cells.

It was the purpose of the various adhesion and blocking assays carried out to determine in greater detail the nature of imaginal cell-substrate and imaginal cell-cell adhesion *in vitro* and whether these activities involved the function of PS integrins.

4.4.2. Short-term cell-cell adhesion assays

The monoclonal antibody aBG-1 was found to reduce the extent of reaggregation of both *Drosophila* wing and leg cell lines in roller culture. PS integrins also appear to be important in the clumping together of aggregates of MLDmBG-1 cells; as determined by blocking studies using aBG-1. This was reflected in the staining pattern of the antibody, which was found at cell-cell contact sites (Hirano *et al.*, 1991). Talila Volk and co-workers have discovered

that PS2 integrin accumulates at sites of cell-cell contact in *Drosophila* embryonic muscle cells which have been cultured on laminin prior to myotube formation (Volk *et al.*, 1990). Integrins are also believed to function in cell-cell adhesion in vertebrate cells. Carter and co-workers discovered that both $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins were being expressed at cell-cell contact sites of human foreskin keratinocytes which could be selectively blocked by anti-integrin antibodies (Carter *et al.*, 1990) while Larjava *et al* reported that β_1 subunits were being expressed at contact sites of human epidermal keratinocytes, both *in vivo* and *in vitro*, again determined by staining patterns and antibody-blocking assays (Larjava *et al.*, 1990).

However, the presence of aBG-1 in roller cultures, whilst reducing the rate of aggregate formation, did not totally block cell-cell adhesion; some small clumps of cells being formed. An interpretation of this result is that PS integrins may indeed function in mediating imaginal cell-cell adhesion but there may be other cell adhesion molecules (CAMs) that work in concert with them: such as the cadherins (Takeichi, 1988) or members of the Ig superfamily of adhesion molecules, (such as fasciclin III, the adhesive properties of which are reported in Snow *et al.*, 1989) the roles of which are addressed in more detail elsewhere in this thesis.

Similarly, there are reports that aBG-1 prevented the adhesion of aggregate to aggregate of MLDmBG-1 cells, but not the formation of the small aggregates themselves, again indicating that PS integrins may play a role in cell-cell adhesion as part of a hierarchy of CAM function (Hirano *et al.*, 1991). Here the suggestion was that PS integrins effected a 'higher' order of adhesion, adhering aggregate to aggregate whilst the other CAMs, such as the cadherins and immunoglobulins which are known to function homotypically, perform the 'lower' order of adhering single cell to single cell. The concept of PS integrins performing this sort of task is reflected in what is seen *in vivo*; in embryonic and adult tissues which lack integrin expression, cell adhesion

between different tissues (such as the epidermal-epidermal junction in the pupal wing) may be lost while adhesion within the plane of an epithelium remains (Brown *et al.*, 1993). This, however, does not entirely seem to be the case with imaginal cells which have been incubated with aBG-1. Here, cells that have been incubated with this antibody can exist as single cells by the end of the assay, therefore this suggests that PS integrins may, to some extent, be involved in the 'lower' order of cell-cell adhesion. These assays do not give any indication of the 'higher' order cell-cell adhesion (aggregate to aggregate clumping), as only single cell numbers were counted, therefore one cannot interpret from these if the PS integrins function in that sort of adhesion. One way to test this would be to photograph and count the number of cells in each aggregate at the end of each assay. If many small aggregates are seen in the experimental samples, as compared to a few large aggregates in the control sample, then one could assume that PS integrins play a hierarchical role in short-term cell-cell adhesion.

PS integrins and the extracellular matrix (ECM)

What is unclear from the immunocytochemical evidence is whether the PS integrin mediated adhesion between cells is either homophillic or dependent on a ligand, which may be provided in the ECM, (either from the culture medium or produced endogenously by the cells themselves).

The rate of cell-cell adhesion of imaginal cells in roller culture was slightly reduced by the presence of GRGDSP peptide in relation to GRGESP peptide. If the PS integrins are involved in mediating short-term cell-cell adhesion, then this adhesion may rely on the presence of the RGD sequence which is known to be recognised by many integrins (for a review, see Ruoslahti and Pierschbacher, 1987) including $\alpha_{PS2}\beta_{PS}$ (PS2) which is believed to recognise the RGD sequence on the recently discovered *Drosophila* matrix

protein, tiggrin (Fogerty *et al.*, 1994). It is also known that the presence of serum during reaggregation can lessen the normally deleterious effect of aBG-1, suggesting that the presence of ECM components may be out-competing the antibody at the PS integrin matrix binding sites and that the ECM does have an important role in short-term cell-cell adhesion. This is also reinforced by the finding that aggregate size seems to be increased when the incubation medium contains serum.

It has been suggested that imaginal cells *in vitro* could function via the homotypic binding of integrin to integrin (Peel and Milner, 1992), as is seen, for instance, in the interaction of the $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins in keratinocyte intercellular adhesion (Symington *et al.*, 1993). However, this seems unlikely for various reasons: the first being that the PS integrins have a strong sequence similarity to certain vertebrate integrins which have ligands in the extracellular matrix. The PS integrins were known to have the outward biochemical characteristics of the vertebrate ECM receptors, namely those which bind to fibronectin, vitronectin, laminin and collagen, of which laminin and collagen are known to exist in *Drosophila* (Leptin *et al.*, 1987; Fessler and Fessler, 1989). Indeed, the β_{PS} subunit was originally identified as such because of its close identity with human and chicken β subunits, which are both known to function as fibronectin receptors (MacKrell *et al.*, 1988; Leptin *et al.*, 1989).

It is also known that ECM deposition in embryos mutant for the β_{PS} subunit is delayed (Newman and Wright, 1981). Another reason to believe that PS integrins do not adhere homophilically is that *inflated* (*if*) mutants (lacking α_{PS2}) are somewhat less severe than *myoshperoid* (*mys*) mutants (lacking β_{PS}); if the integrins functioned solely by the direct interaction of PS1 to PS2, one would expect these two mutants to have the same phenotype (Brown, 1994). Indeed, from clonal analysis studies, *if* + function is only required in cells on the ventral surface of the developing wing, dorsal cells

being unaffected (Brabant and Brower, 1993). It perhaps makes good developmental sense that two closely apposed tissues should be prevented from mixing by the presence of a relatively inert 'buffer', in this case the ECM.

Cells that have reaggregated in the presence of anti-PS2 antibody (CF2C7) are not blocked as much as cells that are reaggregated with anti-β_{PS} (aBG-1). Assuming that both antibodies are equally capable of blocking integrin function, and that a PS1 and PS2 combination is needed to effect adhesion, this also provides evidence against the possibility that PS integrins are homotypic in function. If integrins adhered homotypically, blocking PS2 would have the same effect as blocking all integrin function, and therefore favours the existence of integrin-ECM interaction in short-term cell-cell adhesion.

The cell-substrate assays were devised with the intention of finding ECM molecules on which imaginal cells could adhere, spread and migrate which could then be tested for PS integrin involvement.

4.4.3   Cell-substrate assays

All imaginal cells tested failed to adhere to either collagen type III, collagen type IV or laminin; the cells in each of these assays tending to clump together rather than adhere to the substrate. These cells did, however, adhere, spread and migrate on human and bovine fibronectin, which is reversed in the presence of aBG-1. MLDmBG-1 cells were observed to spread well on human vitronectin and to a lesser extent on bovine fibronectin but not at all to bovine collagens I and IV, reflecting to a degree what is seen with the imaginal cells (Hirano *et al.*, 1991). However, they differed from imaginal cells in that they attached to, but did not spread on mouse laminin.

It therefore seems possible, from these results, that the observed cell-substrate adhesion of imaginal cells to human fibronectin is being mediated

by PS integrin function. Hirano and associates observed that cell spreading to vitronectin and fibronectin was partially inhibited by aBG-1, but not by RGD peptides (Hirano *et al.*, 1991). Imaginal cells do not even adhere to fibronectin in the presence of aBG-1, but are similarly unaffected by RGD containing peptides. From this analysis it seems that both MLDmBG-1 and imaginal cells contain PS integrins that can use fibronectin as a ligand. This is the first indication that *Drosophila* imaginal cells *in vitro* can use a vertebrate ECM molecule for morphogenesis and that this morphogenesis is mediated by PS integrins.

In a similar study, Bunch and Brower transformed normally non-adhesive Schnieder's line 2 (S2) cells with an inducible PS2 gene construct (Bunch and Brower, 1992). From these studies it seemed that PS2 integrin was being expressed on the surface of these cells and was mediating adhesion on vertebrate vitronectin and fibronectin. The transformed cells were also known to adhere to substrates coated with RGD peptides, which was reversible on the addition of soluble RGD peptides (Bunch and Brower, 1992; Gullberg *et al.*, 1994). More recently, tiggrin has been identified as a *Drosophila* ECM protein which contains the RGD recognition that PS2 seems to use as a ligand. Tiggrin expression is found at muscle apodemes and at Z-bands, colocalised with PS integrin expression, and may yet prove to be a PS integrin ligand employed in the pupal wing (Fogerty *et al.*, 1994).

The fact that cells which express PS2 adhere to RGD-containing vertebrate ECM proteins, such as fibronectin and vitronectin may be either fortuitous or may indicate the existence *in vivo* of other, as yet, unknown *Drosophila* ligands that resemble these molecules. A tentative report that an extracellular molecule similar to fibronectin had been identified in the haemolymph of *Drosophila* (Gratecos *et al.*, 1988) has, to date, remained unsubstantiated.

Because RGD peptides fail to influence the adhesion of imaginal and MLDmBG-1 cells to tissue culture plastic one could venture that the PS1 receptor does not use the RGD sequence as a ligand. Indeed, S2 cells that have been transformed to express PS1 do not spread on RGD coated surfaces, but they have recently been reported to adhere and spread on laminin (Gotwals *et al.*, 1994). The role of laminin as a ligand for PS integrins originally seemed unlikely. Volk *et al.*, (1990) reported that embryo myocyte adhesion and formation of myotubes on laminin occurs in the absence of βPS function and Fristrom *et al.*, (1993) reported that laminin and PS integrins do not have mutual sites of expression in the late pupal wing; although neither of these findings precludes the possibility of PS1 functioning as a laminin receptor. *Drosophila* laminin, however, *is* found at sites of integrin expression in the early pupal wing, although this complementary expression is lost later in development (Fristrom *et al.*, 1993). Adult flies that are mutant for laminin A tend to display many non-cell autonomous cuticular defects; such as bent and malformed legs and blistered wings, again indicating a requirement for laminin late in development (Henchcliffe *et al.*, 1994).

Interestingly, imaginal cells do not adhere to laminin, and these cell lines are reported to express PS1 (Peel and Milner, 1992). MLDmBG-1 cells express PS1, but the attachment of these cells to laminin does not depend on PS integrin function, as indicated by blocking studies (Hirano *et al.*, 1991). Whether this lack of laminin adhesion in imaginal and MLDmBG-1 cells is due to some sequence variation between vertebrate and *Drosophila* laminin, or whether PS1 is only expressed later in morphogenesis, and not in the initial cell-substrate interaction in imaginal cells is not presently known.

What is clear from the cell-substrate blocking studies is that the anti-βPS antibody can block the adhesion and spread of imaginal cells on fibronectin; and one can assume from this that the PS integrins may use fibronectin as a ligand. One can perhaps predict that this adhesion and spread

on fibronectin would be reversed if RGD peptides were introduced, and that only PS2 is effecting this adhesion.

The results of the preliminary study, that cells grown on 'pre-used' Petri dishes tended to aggregate more quickly than cells grown on bare tissue culture plastic was suggestive that components of the extracellular matrix function in directing imaginal cell movement *in vitro*. It seems likely that cell-substrate interactions are as important in aggregation as interactions between cells. It was the proposition of Chapter 3 that cell-substrate interactions predominate early in imaginal cell passage which were then superceded by a period of cell-cell adhesion. Often, a substrate of fibronectin was also found to enhance the rate of imaginal cell aggregation (Figure 4.P), forming discrete, dense aggregates while cells in the control still exist essentially as a monolayer.

These findings are consistent with the model of traction-induced aggregation, a mechanism originally posited by Harris *et al.*, in 1984 (and discussed in: Bard, 1990) to explain the uniform distribution of mesenchymal cells *in vitro* and, more controversially, *in vivo*. Fibroblasts plated onto a collagen gel were observed to break up the cell-matrix mixture into regularly spaced aggregates consisting of cells and compressed matrix. It seemed that the force generated for such movement was generated by traction; the cells exerting a tensile force on their environment leading to substrate deformation. Harris *et al.*, (1980) had originally suggested that fibroblasts could generate traction by observing that they could cause a substrate of silicone rubber to wrinkle, generating a "center effect." The cells appeared to be pulling on their collagenous substrate, as evidenced by the alignment of collagen fires between aggregates, thus bringing other cells into their proximity (Harris *et al.*, 1984). This movement was absent when these cells were plated onto plastic. Such a mode of traction induced aggregation could thus exist during

the early phase of *Drosophila* imaginal cells growth, *in vitro*. Indeed, in cultures of imaginal cells grown on fibronectin, chains and fascicles of cells, very similar in structure to those seen between aggregates of mesenchymal cells in culture (Bard, 1990), suggesting that imaginal cells may be actively deforming and aligning the fibres of this substrate. Occasionally cells were seen to align themselves lenghtways early on in culture, presumably along strands of fibronectin.

The presence of cell adhesion molecules on the surface of imaginal cells throughout aggregation might suggest that cell-cell rather than cell-substrate interactions are of a paramount importance in aggregation, as suggested by Peel *et al* (1990), but, as stated in Chapter 3, it is difficult to distinguish between cell-cell and cell-substrate interactions during this process. It now seems distinctly possible that some ECM components are present between aggregating cells, which serves to blur the traditionally sharp operational terms of cell-cell rather than cell-substrate adhesion. It is possible that force could be generated between cells, via tension transmitting junctions (see also Chapter 6), but it seems unlikely that aggregation is driven exclusively by either cell-cell or cell-substrate interactions.

Long-term cell-cell adhesion assays

RGD peptides fail to prevent aggregation of imaginal cells on tissue culture plastic in medium containing 2% FBS. It therefore seems likely that an RGD independent adhesion molecule is functioning here; either PS1 to laminin or perhaps even an unidentified PS integrin to an ECM ligand, such as a molecule which is believed to function on the dorsal surface of the pupal wing epithelium, and is in the process of being characterised (Brower *et al.*, 1995; Brown, pers. comm.).

Normal aggregation is blocked in cultures of imaginal cells grown in 2% FBS on tissue culture plastic which contains anti-β_{PS} antibody; there being a greatly reduced level of cell migration and only a few small aggregates being formed. This limited cell-substrate and cell-cell adhesion reflects what is seen in the reaggregation study where other CAMs, such as the cadherins and Ig molecules, are believed to function in the absence of the integrins and these molecules seem capable of providing a limited degree of cell adhesion, spread and migration. These particular studies indicate that PS integrins appear to be important but not exclusively so for the growth and aggregation of imaginal cell lines *in* vitro. However, it is impossible to tell if the lack of aggregation in these particular assays was due to the blocking of either cell-cell or cell-substrate interactions, instead merely indicating the importance of PS integrins in cell movement.

PS integrins and tension transmitting junctions

As mentioned earlier in this chapter, the PS integrins may function in adhering a cell process to a neighbouring cell, forming a tension transmitting junction at contact zones prior to contraction and aggregation. In the pupal wing during metamorphosis, this contact zone between dorsal and ventral cell processes is characterised by a convoluted interdigitation joined by a complex of desmosomes and packed with microfilaments and microtubules (see Figure D) (Mogensen and Tucker, 1987; Mogensen and Tucker, 1988; Tucker *et al.*, 1986). There has been little ultrastructural evidence to suggest that any cytoskeletal structures as elaborate as the contact zone found between basal cell processes in the pupal wing are found between aggregating imaginal cells *in vitro* (Peel *et al.*, 1990).

However, f-actin has been seen to accumulate at sites of imaginal cell-cell contact *in vitro*, along with extended bundles of microtubules (see

Chapter 6), indicating that PS integrins at these sites may still be able to transduce tension across the plasma membrane to the cell's cytoskeleton and generate the force required to drive aggregation. The circumstance of cell-cell adhesion *in vitro*, as compared to *in vivo*, is also quite different and may account for the lack of junctional differentiation. Instead of cell process meeting cell process, here a cell process adheres to the main body of the cell.

Furthermore, the need for a powerful tension transmitting junction may not be so great *in vitro*, if one assumes that these junctions are formed to withstand pressure, *in vivo*. The transalar arrays *in vivo* presumably have to withstand a powerful rise in pressure between the dorsal and ventral surfaces as haemolymph is pumped in, inflating the wing (Waddington, 1941; Fristrom *et al.*, 1993). However, in wing discs that have metamorphosed *in vitro*, and where the rise in disc pressure is presumably absent, many transalar arrays and basal junctions are formed (Tucker *et al.*, 1986). An increase in disc lumen pressure does not seem to be a necessary prerequisite for basal junction formation, therefore this would suggest that such junctions could exist *in vitro*.

One possible reason why basal junctions are not seemingly as complex and differentiated *in vitro* is the apparent lack of restriction of PS integrin expression. In wing discs there is a well characterised apposition of integrin expression, with PS1 integrin expression *only* being found on the dorsal wing epithelium and PS2 *only* being found on the ventral epithelium (Brower *et al.*, 1985). This restriction of expression seems to be missing in imaginal disc cell lines screened to date (Peel and Milner, 1992), each cell expressing both PS1 and PS2. An intriguing possibility which could be addressed is whether a complementary distribution of PS1 and PS2 is actually *required* for the formation and function of a tension transmitting junction similar to those seen in myo-epidermal and epidermal-epidermal contact sites. It might be the case, as with present findings, that PS integrins accumulate at sites of cell-cell

contact, functioning along with the other CAMs, but for a 'real' junction to form, complementary α_{PS1} and α_{PS2} subunits are needed.

It is possible that a clonally derived cell line could be found that is homogenous in expression of a particular α–chain, by screening each cell line with existing antibodies. If two such lines, expressing PS1 and PS2 respectively, could be co-cultured and each cell line could be distinguished after cell-cell adhesion to ensure suitable integrin apposition, (such as by 'vital-dye' staining with DiI and DiO as used by Elkins et al. (1989) in their cell-sorting assays), then one could go on to examine, ultrastructurally, if junctions (or even cytoskeletal arrays) are formed between cells.

Another possible function of the PS integrins other than acting as mechanical linchpins in tension transmitting junctions during development is the integrin acting as a signal transducer; a subject of much current interest and research. In vertebrate models *in vivo* and *in vitro*, there are found arrangements of cytoskeletal proteins at sites of focal adhesion which bind to the integrins, connecting the integrins to actin filaments. The sites of expression of f-actin in imaginal disc cell lines are addressed later in this thesis. From those expression patterns PS integrins and f-actin seem to be co-localised and it certainly seems possible that the two could be functionally related in some way, with the effect of an integrin binding an ECM component being transduced to the cell's cytoskeleton.

These arrays also form signalling complexes which include the focal adhesion kinase (FAK), which in turn interact with signalling molecules (for review see: Clark and Brugge, 1995). It is possible that PS integrins may have similar signal-transducing properties to the vertebrate integrins; but these remain, as yet, unidentified. *Drosophila* imaginal disc cell lines may prove to be an amenable model for studies testing for the signal transducing properties of the PS integrins.

5.1   Introduction

Established epithelial cell lines in culture are morphologically distinct from their predecessors, namely cells from the imaginal disc epidermis and peripodial membrane. Primary cultures of imaginal disc fragments led to the outgrowth of epithelial cells growing as sheets, epithelial-like and fibroblast-like cells adhering to the substrate, or as vesicles (Currie *et al.*, 1988; Peel *et al*. 1990; Peel, 1991). In established and cloned cell lines the cells also displayed a variety of morphologies; having the outward charateristics of epthelial cells, fibroblasts and lamellocytes (Peel and Milner, 1990). It is thus apparent that the major morphological difference between established imaginal cell lines and the epithelial cells in the *in vivo* state is the absence of apical-basal polarity.

The classic apical-basal phenotype of an epithelium can be characterised by four features, firstly by the restricted distribution of plasma membrane proteins and lipids to three distinct surface domains (apical, lateral and basal); secondly by circumferential occluding junctions that separate and form a barrier between apical and lateral surface domains, thirdly by cohesive, cell-cell interactions mediated by cell adhesion molecules (CAMs) and a highly differentiated junctional complex; and fourthly by the polarised configuration of cytoplasmic organelles and the cytoplasmic and cortical cytoskeleton (Rodriguez-Boulan and Nelson, 1989).

Imaginal disc epithelia, in common with all vertebrate and invertebrate epithelia, exhibit apical-basal polarity (Poodry and Schneiderman, 1970). The apical surface is covered in microvilli and is specialised for the secretion of the pupal and adult cuticles and will become the external surface of the fly. The basal, serosal, surface faces the basement membrane and haemolymph and is, by comparison, relatively uniform in appearance. Processes known as 'epidermal feet' have been observed to extend from the basal surfaces of

epithelial cells in insects such as *Calpodes* and *Rhodnius,* and are believed to be important in morphogenesis (Locke and Huie, 1981; Locke, 1985; Fristrom and Fristrom, 1993). In *Drosophila* such cell processes are present at the onset of eye-antennal imaginal disc fusion *in vitro* and are thus implicated to be of importance in proper imaginal disc morphogenesis *in vivo* (Milner *et al.,* 1984).

Cells of this convoluted, pseudostratified, epithelium tend to vary in shape depending on their position within the epithelium, ranging from tall (30 μm) and columnar cells on convex folds to short and cuboidal cells found within concave folds. Between each of these cells are the specialised junctions which are shared by all invertebrate epithelial cells alike. Most apically, the zonula adherens is found, corresponding to the tight junctions found in vertebrate cells, followed basally by the septate and gap junctions respectively. Cytoplasmic bridges are seen, on occasion, between sister cells for a short time after division (Poodry and Schneidermann, 1970; Poodry, 1980). Many cytoplasmic organelles are distributed along the apical-basal axis. Nuclei tend to be found towards the basal ends of cells whereas the Golgi apparatus and centrioles of the microtubule organising centres (MTOC) are more usually found towards the apex of the cell (Fristrom, 1988) The golgi body is itself polarised and is believed to direct vesicular transport from one membrane domain to another (Simons and Fuller, 1985).

It was, thus, the purpose of this study to determine if apical-basal polarity could be re-established in these cell lines *in vitro.* If successful, this would thereby provide a model which may be useful for isolating and identifying the envronmental cues and support which are needed for such development *in vivo.*

Various factors are known to be important for the induction and maintenance of apical-basal polarity *in vitro.* One such factor seems to be the polarity of nutrient uptake, which *in vivo* is unidirectional via the basement membrane. When cells are plated onto tissue culture plastic or glass the cells

are forced to feed from their apical surface, thus potentially affecting the control of polarity (Simons and Fuller, 1985). Another consideration is the presence of cell-cell and cell-substratum contact to neighbouring cells and an extracellular matrix at the lateral and basal sides of the cells respectively. Some epithelial cells have undergone improved biochemical and cytological differentiation when seeded onto collagen, in collagen gels or on more complete extracellular matrices (Gospodarowicz *et al.*, 1984; Rodriguez-Boulan and Nelson, 1989).

The ultrastructure of a high-passage number, uncloned leg cell line (L127D6) was thus studied under differing environmental conditions with a view to re-establishing apical-basal polarity centering around the use of a surrogate basement membrane in the form of a human-fibronectin coated microporous membrane. Human fibronectin was selected as a suitable extracellular matrix molecule for this study on the strength of adhesion assay studies carried out in Chapter 4.

As an additonal test to determine the state and control of polarity in imaginal cells *in vitro*, immunocytochemical techniques were used to assay for the expression of the *crumbs* gene (Jürgens *et al.*, 1978; Tepaß *et al.*, 1990). The product of the *crumbs* gene is an integral membrane protein with 30 EGF-like repeats which has a localised pattern of expression in embryonic and imaginal epithelia being found exclusively at the apical membranes and borders of cells. There is evidence to suggest that this protein may be a diffusible factor which could act as a signal, passing between cells, important for the initiation and subsequent maintenance of epithelial cell polarity (Tepaß and Knust, 1990).

5.2   Materials and Methods

5.2.1   Immunocytochemistry

Cells were plated out at a density of 1×10^5 cells per ml, in 1 ml of CSM, into individual chambers of a LabTek slide (Nunc) and left to grow for 3 days. Cells, which were now adherent to the slides, were washed for 5 minutes in PBS and fixed for 10 minutes in 4% paraformaldehyde or, alternatively, in methanol at -20°C for 5 minutes. The slides were subsequently given two 5 minute washes in PBS with 1% goat serum and were permeabilised in PBT buffer (0.01% BSA and 0.01% Triton-X in PBS) for 2 hours at 25°C. The slides were then blocked for 1 hour at 25°C in PBS with 10% goat serum. Next, the slides were drained and incubated overnight at 4°C, in a solution of the primary antibody. Initially, the antibodies used, P4B4 and P2.8, were mouse polyclonal antisera directed against different parts of the extracellular domain of the CRUMBS protein, diluted 1:1000 in PBS with 1% goat serum. After a limited success with these two antibodies in immunofluorescent staining studies, a monoclonal antibody, Cg4, directed against CRUMBS, was latterly use, and which was added, undiluted, to the cells on the slides, (all probes used were gifts from Elizabeth Knust and Andreas Wodarz). The slides were then washed for 1 hour in PBS with 1% goat serum and blocked, as before, for one hour in PBS with 10% goat serum. The slides were drained and incubated in a solution of the secondary antibody: biotinylated anti-mouse IgG (Vector) diluted 1:200 in PBS with 1% goat serum. The slides were then washed and blocked prior to incubation with the tertiary probe: avidin-FITC (Vector) diluted 1:250 in PBS with 1% goat serum. Control slides used in this study were prepared by substituting pre-immune antisera at the same working dilution for the primary antibody. The slides were then given a final, 30 minute, wash in PBS with 1% goat

serum before being mounted in UV-free mounting medium, consisting of 90% glycerol and 2.5% propyl-gallate, pH. 7.9, and sealed with a coverslip. The cells were then viewed using a Biorad laser scanning confocal microscope.

5.2.2   Preparation of cells for transmission electron microscopy (TEM)

The cell lines being studied were plated onto human fibronectin coated cell culture inserts (Biocoat® cell culture inserts; Becton Dickinson) for use with 24-well plates at a density of 1×10^6 cells per insert. Cells in the inserts were either grown in CSM or CSM minus FE2 and FBS (i.e. MM3 medium, having no serum additives). Feeder layers, when used, were seeded 1 day prior to insert seeding at a density of 2×10^6 cells per well. The cells were then grown in the insert for a fixed period of either one, two or three days. Prior to fixation, the particular culture medium being tested was removed and the cells rinsed once in D= and then twice in rinsing buffer: 5 mM KH_2PO_4, 50 mM Na_2HPO_4 and 50 mM Sucrose, pH 7.6. The cells, still adherent to the membrane, were then fixed in rinsing buffer with 2.5% glutaraldehyde for 20 minutes at 25°C followed by three 10 minute washes in rinsing buffer. The cells and membranes were post-fixed in rinsing buffer with 1% osmium tetroxide for 20 minutes, followed by three washes as before. The cells were then dehydrated by passing the membranes through a series of ethanol dilutions (graded in the order 50-100%), and changed twice in 100% ethanol. The membranes were cut from the cell inserts using a scalpel and permeabilised in propylene oxide for 1 hour with three changes. The cells were then infiltrated with a 2:1 ratio mixture of propylene oxide to embedding resin for 1 hour and were then infiltrated with a 1:2 ratio of the same for another hour prior to embedding, for at least 48 hours at 60°C, in 100% resin. The embedding resin used contained the following; 19 ml MY753, 21 ml DDSA, 0.6 ml dibutyl phthalate and 1.2 ml BDMA.

Ultra-thin sections were taken from the hardened resin using an LKB ultramicrotome, stained on grids with uranyl acetate and lead citrate (Reynolds, 1963), and examined using a Phillips 301 electron microscope.

5.3   Results

5.3.1   Immunocytochemistry

Initially, experiments carried out using the polylonal probes, P4B4 and P2.8 resulted in staining patterns that suggested CRUMBS was being localised at sites within the cell, especially at sites proximal to the nucleus. These staining patterns appeared to be non-specific, as one would expect any antigen to be localised to the cell surface.In later experiments, using the monoclonal probe Cg4, it appeared that CRUMBS was being expressed in a more diffuse, non-localised, manner on the surface of aggregating cells in various cell lines that were tested (Figure 5.A). However, the pattern of expression in both instances usually gave little indication that CRUMBS was being localised to sites of cell-cell adhesion, as reported for epithelial cells *in vivo* (Tepaß *et al.*, 1990). However, in a few instances, *crumbs* expression seemed to be localised at sites of cell-cell contact. Figure 5.B details the point of contact between two Cl. 8+ cells, which has a strong, restricted expression pattern for *crumbs*. These expression patterns were absent from the experimental controls that were analysed.

5.3.2   Ultrastructutral analysis

Established cell-lines, grown on tissue-culture plastic, do not display any apical-basal polarity and, thus, do not have the appearance of a typical epithelium. L127D6 cells, as with other established imaginal disc cell lines, begin growth as an monolayer of cells adherent to the tissue culture plastic substrate which then migrate into large multicellular aggregates. L127D6 cells are known to undergo a similar mode of monolayer formation followed by

pronounced aggregation when grown on human fibronectin, as reported in Chapter 4.

Cells which had been grown on fibronectin coated membranes allowing for unidirectional nutrient uptake and in the presence of a feeder layer display some of the characteristics typical of epithelial cells *in vivo*.

Some of those cells which were grown on a fibronectin membrane under these conditions, tended to have a coumnar morphology. Cells grown on fibronectin without unidirectional nutrient uptake and without a feeder layer do not have this appearance, and tend to pile-up on one another as masses of rounded cells.

Figure 5.C details a site of L127D6 cell-cell contact grown under these conditions. A bundle of microtubules can be seen running longitudinally down the left hand cell in an apical-basal orientation. Similarly oriented microtubules are known to occur in the columnar disc cells *in vivo*, as a likely means of support for the cell's elongated shape. There was also some evidence to suggest that there were some microtubules running circumferentially around the cell just below the plasma membrane. Microtubules such as those found *in vivo* are known to be associated with the zonulae adherens junctions (Fristrom and Fristrom, 1993).

In Figure 5.C there is also some evidence for microvilli-like processes at the apical end of both cells, above sites of apparently strong adhesion, reflecting the polarised *in vivo* state. This particular characteristic was not found in cells grown without unidirectional nutrient uptake and a feeder layer but was less pronounced than that which would be seen *in vivo*.

Figures 5.D and 5.E illustrate the sites of adhesion of L127D6 cells grown for 3 days on fibronectin, allowing for either bi-directional nutrient uptake (Figure 5.D) or unidirectional nutrient uptake (Figure 5.E). The cells in Figure 5.E differ from Figure 5.D in that there seems to be some level of

junction differentiation; displaying what appears to be a septate desmosome at the site of contact (Poodry and Schneiderman, 1970).

Figure 5.F again illustrates the site of adhesion between two L1276 cells, which were fixed after 1 day of growth on fibronectin. Numerous small attachment sites have formed between the cells and , towards the apical side. Some microvilli-like structures are also seen at this site of cell-cell adhesion.

Figures 5.G and 5.H show the site of adhesion between L127D6 cells and the substrate. The basal part of the cell in Figure 5.G seems to be tightly adherent to the fibronectin substrate, although no hemidesmosome are seen. In Figure 5.H some cytoplasmic extensions which are reminiscent of epidermal 'feet' and filopodia, which are believed to be of developmental importance in insects such as *Drosophila* and *Calpodes*, are seen between the cell and the membrane (Milner *et al.*, 1984; Locke 1985; Locke 1987). One large cell process is is seen bulging into, and possibly entering, a pore in the membrane, through which the cell is deriving nutrient.

Figure 5.A   The expression of the putative signalling and adhesion molecule CRUMBS in four different imaginal disc cell lines (Cl. 13, Cl. C9, L127D6 and W20C6), stained for immunofluoresence using the Cg4 monolconal antibody and recorded using confocal laser scanning microscopy. With every example, the phase contrast image is on the left and the immunofluorescent image is on the right. Consistently, there appears to be little localisation of the antigen to any specfic site on the surface of the aggregating cells.

Scale bar represents 25 μm

Figure 5.B   The expression of CRUMBS at a site of contact between two aggregating Cl. 8+ cells *in vitro*. A cell process at upper right is extending down and coming into contact (arrow) with the opal shaped cell at the centre. Occasionally, as with this example, CRUMBS did appear to have a localised pattern of expression in aggregating imaginal cells, consistent with a proposed role in cell adhesion and signalling.

Scale bar represents 10 μm
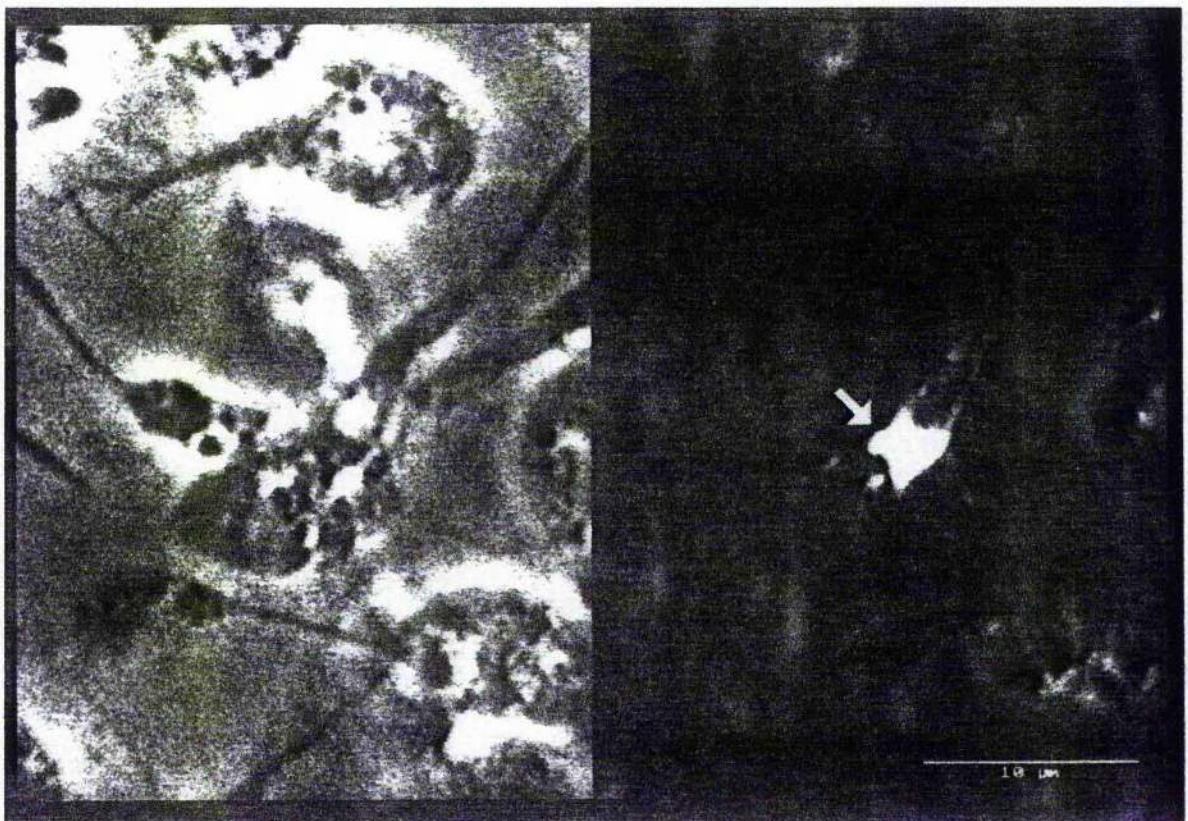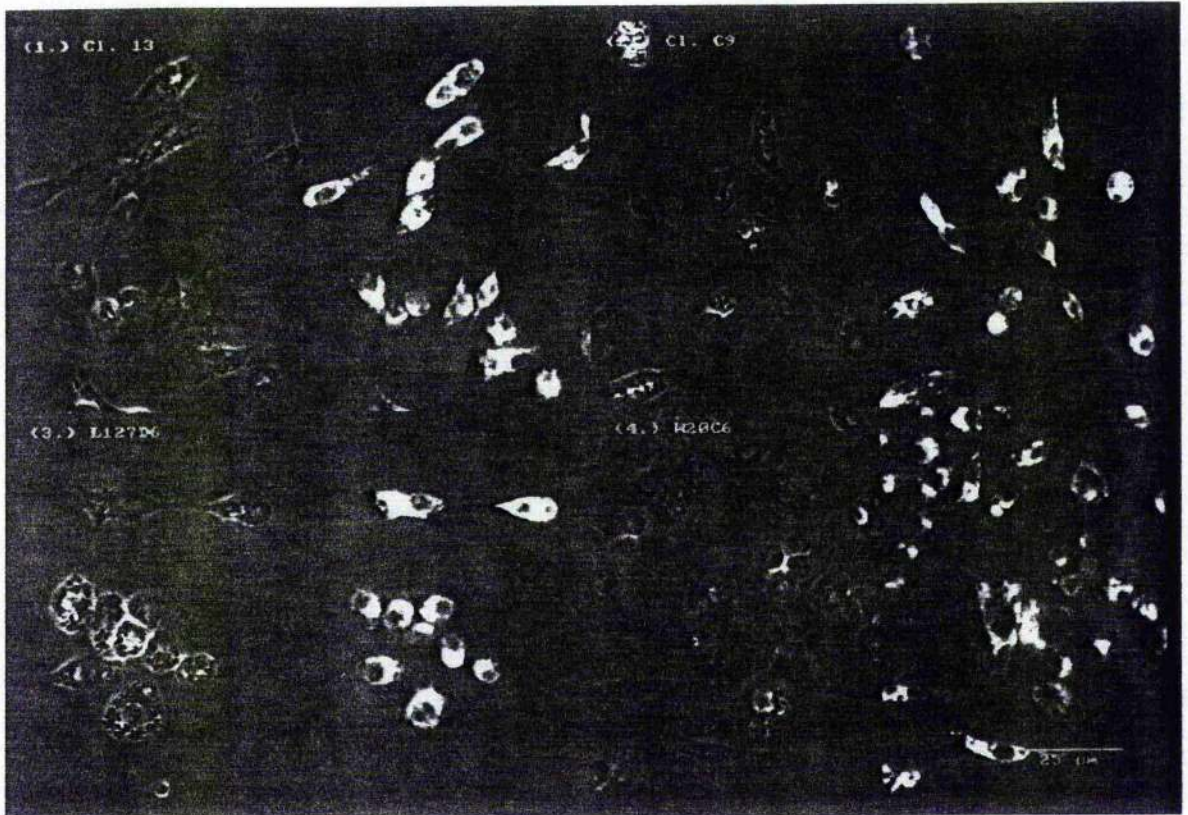
Figure 5.C An electron micrograph of two closely apposed L127D6 cells

after plating onto a fibronectin coated membrane in the presence of

a basal feeder-layer. Towards the apical end of the cells (top) are

numerous microvilli (mv), towards which some microtubules (mt)

can be seen to extend. Moving basally, the cells are seen to be

adhering very strongly to each other but no differentiated junctional

complexes can be seen.

Scale bar represents 0.5 μm

mv
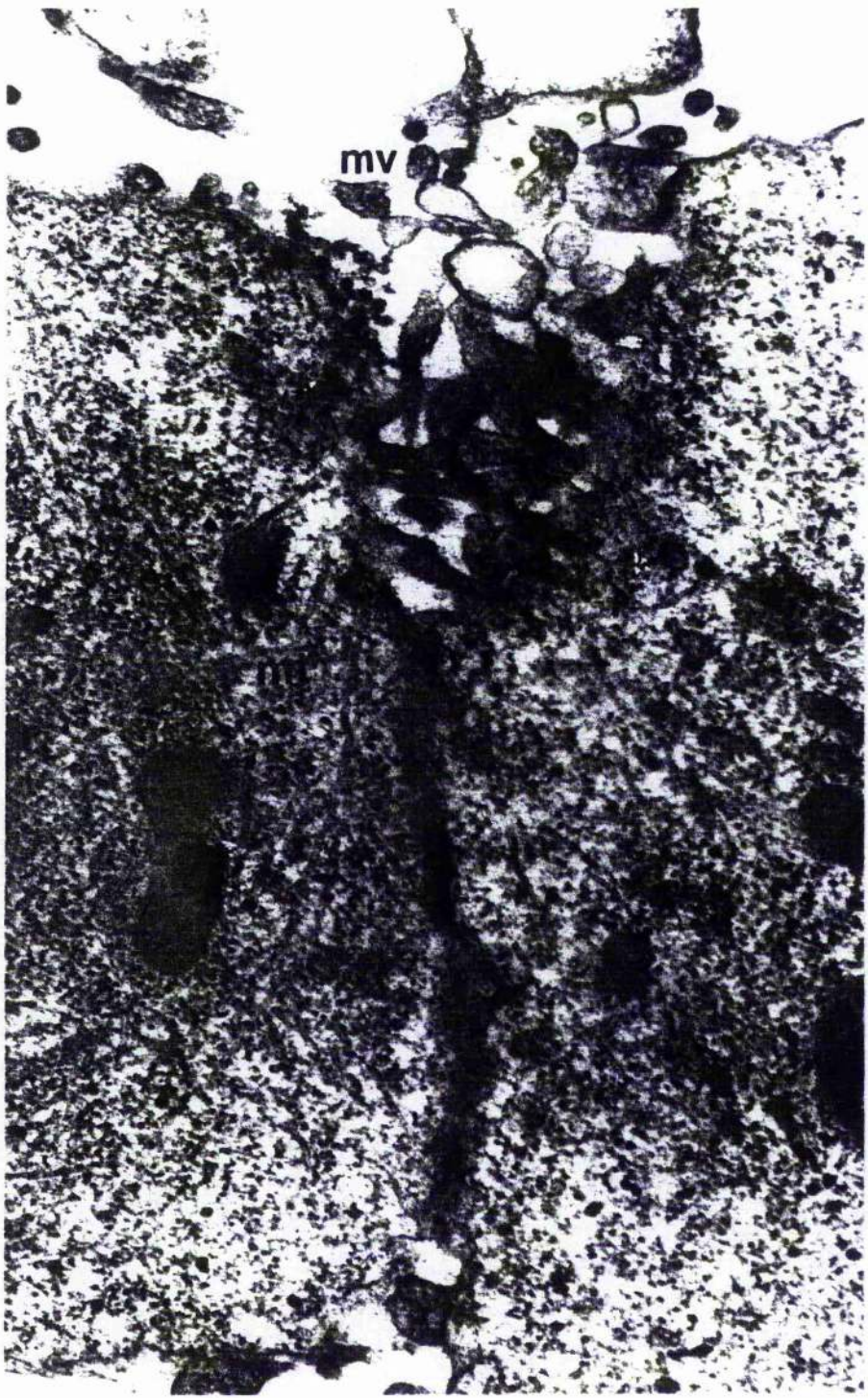
c

Figure 5.D   An electron micrograph detailing the site of adhesion (ad) of two L127D6 cells plated on a fibronectin coated membrane allowing for the bi-directional uptake of nutrient from the apical (upper left) and basal (lower right) sides. The cells seem very strongly adherent but there is no evidence of any junctional differentiation.

Scale bar represents 0.3 µm
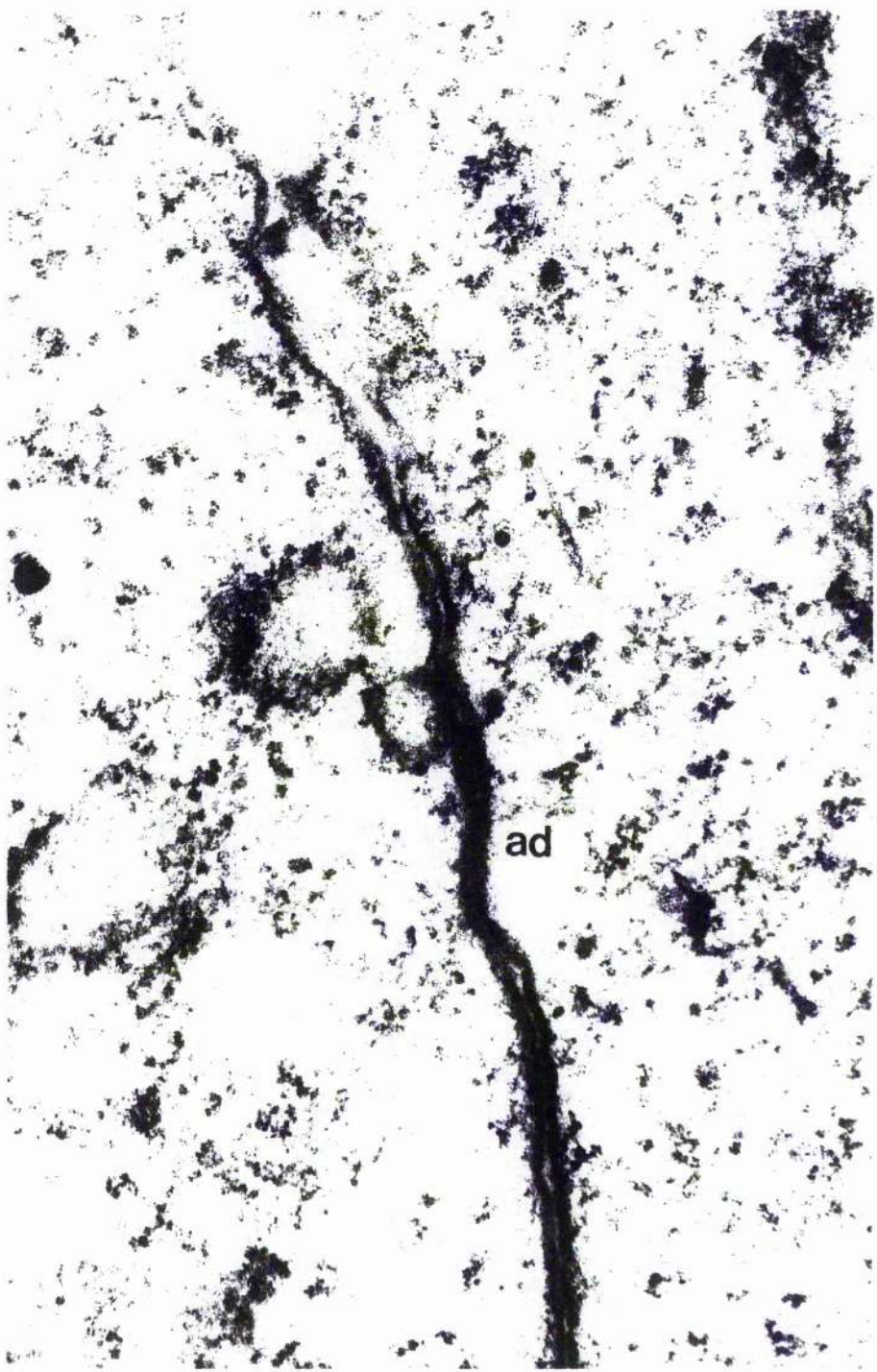
D

Figure 5.E An electron micrograph detailing the site of adhesion of two L127D6 cells grown on a fibronectin coated membrane allowing for the unidirectional uptake of medium from the basal (lower) side. The arrow points to a possible site of junctional differentiation evocative of a septate desmosome both in structure and positioning.

Scale bar represents 0.5 μm
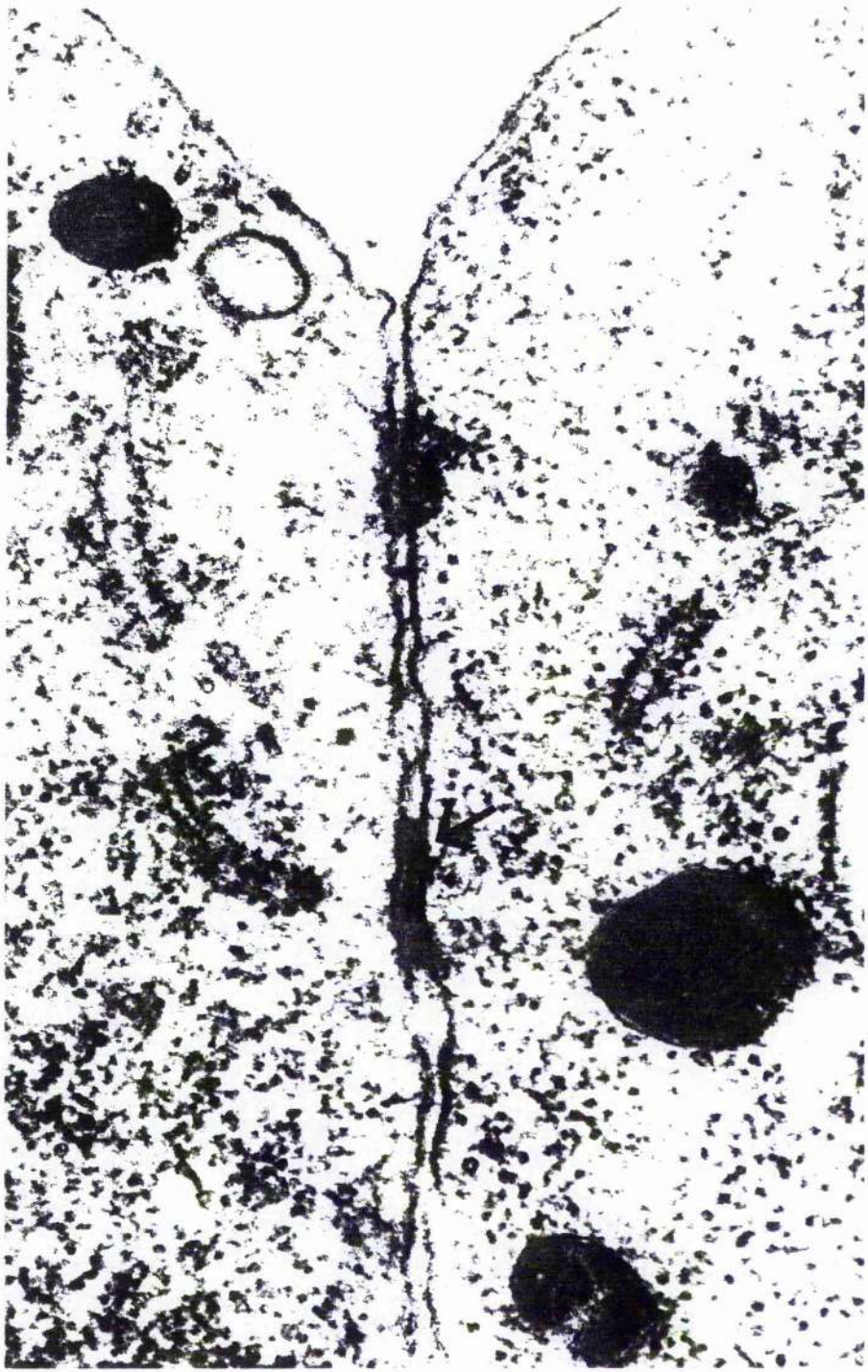
E

Figure 5.F   An electron micrograph of two L127D6 cells grown on
fibronectin, allowing for unidirectional nutrient uptake. The apical
side of each cell is to the upper right, the basal side being to the
lower left. Numerous small attachment sites (arrows) are present at
points of contact between the cells, as are microvilli (mv) at the
apical side. No differentiated junctions can be seen.

Scale bar represents 0.5 μm
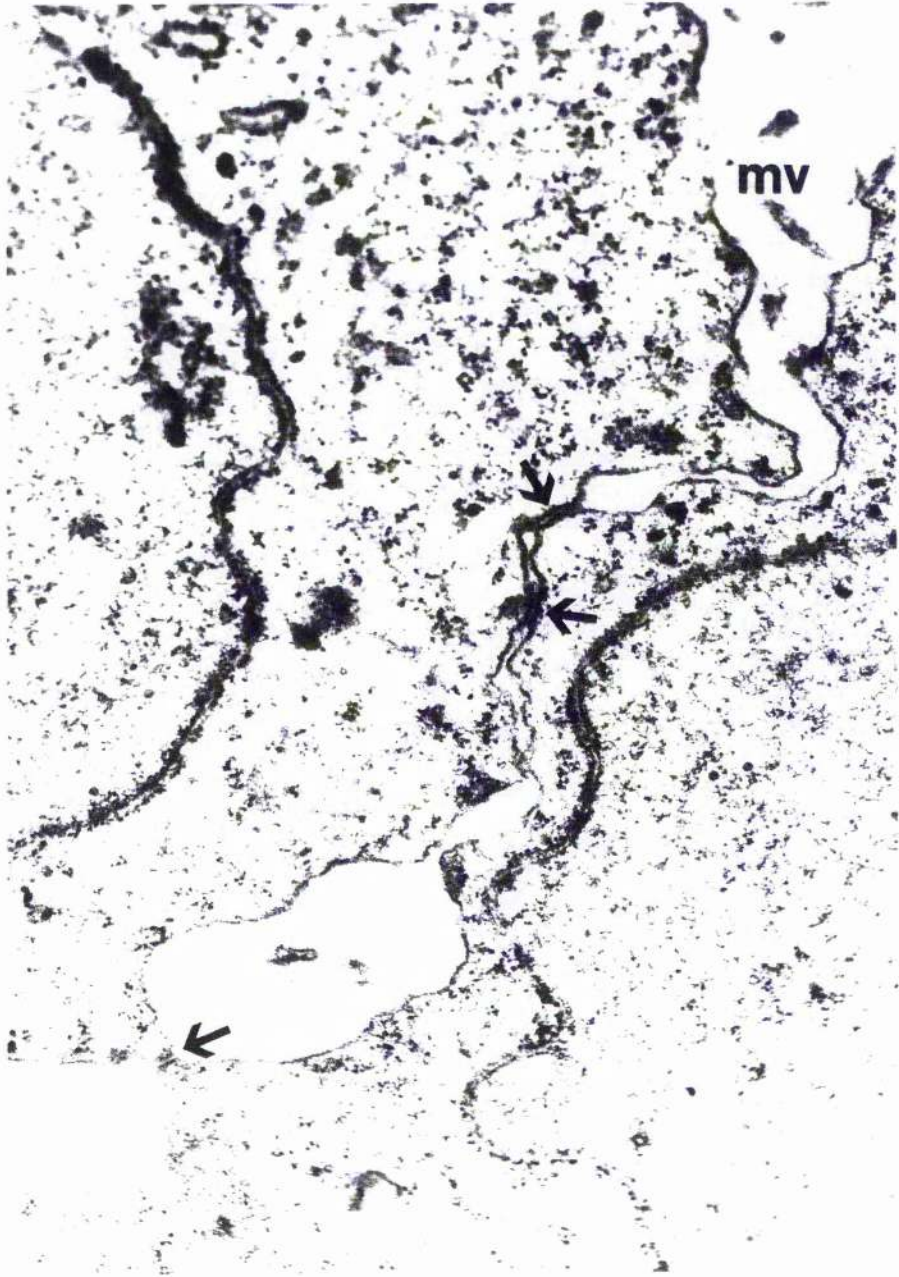
F

Figure 5.G   This electron micrograph illustrates the basal part of an L127D6 cell which is in contact with, and is adhering to, a fibronectin matrix (fn), through which it is deriving nutrient. Endoplasmic reticulum (er) is seen close to the substrate, as are some microtubules. There is no evidence of any hemidesmosomes at the cell-substrate interface.

Scale bar represents 0.5 μm

er

fn
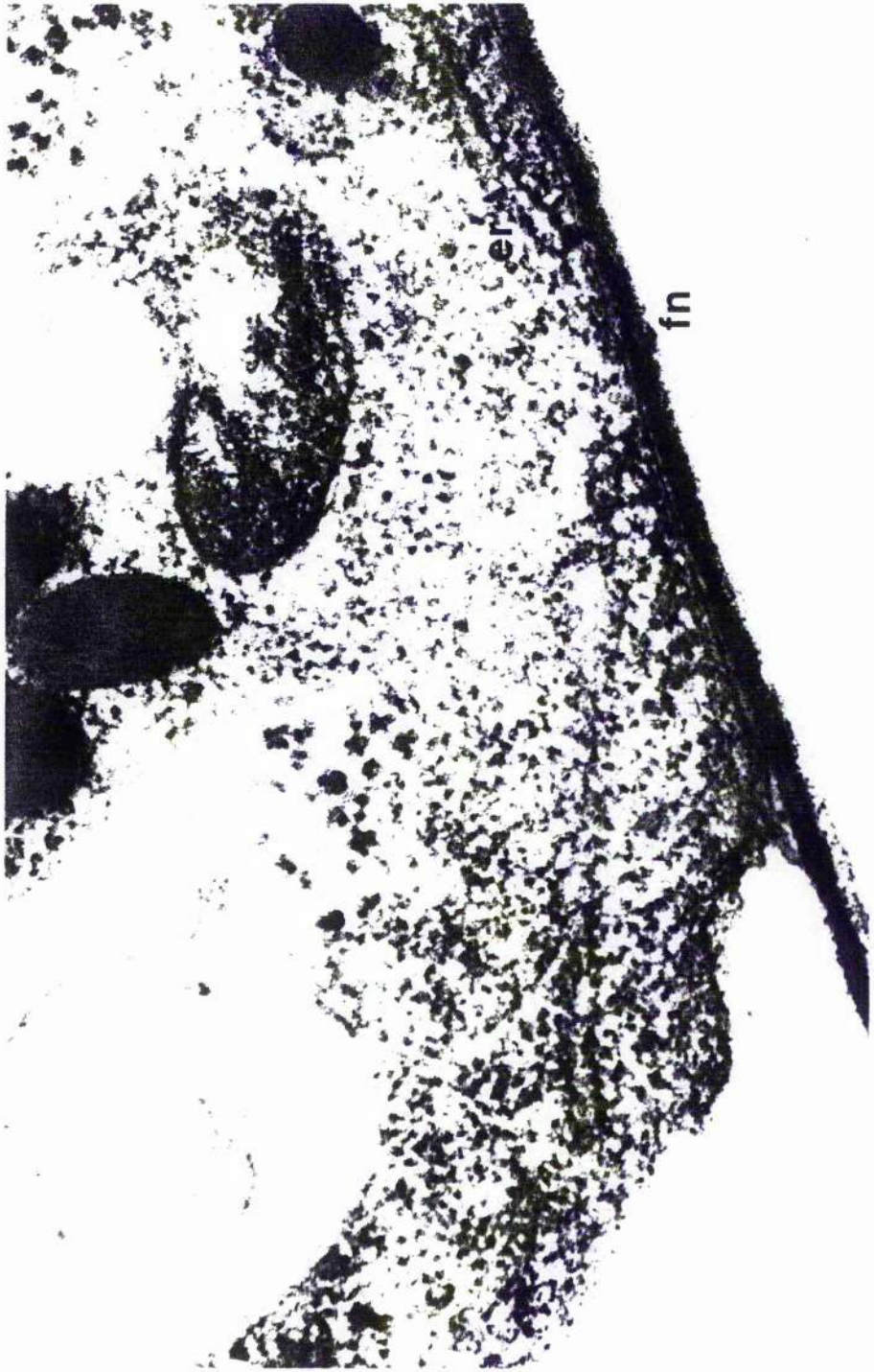
G

Figure 5.H   An electron micrograph illustrating the basal side of an L127D6 cell which is adhering to a fibronectin coated membrane (fn). Numerous cytoplasmic extensions (arrows), or 'feet', can be seen between the cell and the membrane, one of which is directly above a pore (p) through which the cell is deriving nutrient.

Scale bar represents 0.5 μm

5.4   Discussion

The imaginal epidermis of *Drosophila* is known to be derived from the ectoderm and is believed to originate from groups of cells in the blastoderm of the embryo, although the exact stage at which they become determined is, as yet, indefinite (Cohen, 1993). The imaginal discs themselves are then formed as invaginations of the embryonic epidermis at sites of anterior-posterior compartment boundaries (Fristrom and Fristrom, 1993).

Morphologically, epithelia can be distinguished from mesenchyme in that they possess apical-basal polarity and they are connected via rings of specialised intercellular junctions that integrate them into a laterally cohesive layer.

The maintenance of this epithelial organisation is expected to be active and dynamic, because epithelia are known to undergo certain morphological changes throughout development. In imaginal discs, these morphogenetic changes are much like those seen in other epithelia (for review, see: Fristrom, 1988), such as the elongation of leg discs by cell rearrangement (Fristrom, 1976) and the contraction of the peripodial membrane, which changes from a squamous to a columnar epithelium and, hence, drives disc evagination (Milner *et al.*, 1984a). The imaginal disc epithelium remains as a single cell layer throughout this morphogenesis.

These changes in epithelial morphology are complex and may necessitate the breakage and formation of intercellular junctions. For this to occur, cells must first be able to recognise their neighbour and their own orientation, and secondly be able to adhere correctly to that cell, employing the suitable cell adhesion molecules (CAMs).

Research into the development of epithelia in the past has relied mostly on experimentation using mammalian embryos or *in vitro* tissue and cell systems. These studies have revealed much about the morphogenesis of

the epithelium, but little is still known about the molecular and genetic control of this development (for review see: Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989).

Through the use of *Drosophila* as an experimental subject, some researchers have begun to address the question of how these tissues develop epithelial characteristics, such as the formation of a monolayer and the maintenance of apical-basal polarity.

Through the isolation and study of mutants that are known to be defective in ectodermal and cuticular development, these researchers have begun to isolate certain genes whose functions are known to be important in establishing and maintaining the characteristic epithelial phenotype. One such area of research has culminated in the discovery of the *crumbs* (*crb*) gene, which is believed to function in the control of epithelial polarity.

The protein encoded by *crumbs* (*crb*) is known to be expressed in all wild-type imaginal epithelial cells, except for the adepithelial cells; being found at the apical end of cells, facing the peripodial cavity (Tepaß *et al.*, 1990). A strong *crb* mutant phenotype is characterised by the disorganisation of many epithelial tissues throughout development and subsequently by a severe loss of cuticle (Jürgens *et al.*, 1984). This lack of integrity of epithelial tissues seems to be caused by the loss of apical-basal polarity, which is apparently stabilised by the *crb* product (Tepaß and Knust, 1990).

The *crb* gene encodes a predicted 234-kDa transmembrane protein with 30 EGF-like repeats, which are found in four clusters in the extracellular domain, with a short 28 residue cytoplasmic domain. This sort of structure is suggestive that the *crb* product may be involved in protein-protein interactions via the EGF-repeats, as either a cell adhesion molecule or as a signalling molecule, the latter of which seems more likely. This molecule has no known vertebrate counterpart (Tepaß *et al.*, 1990).

In embryos where this protein is diffuse in expression, or is completely absent, the ectoderm forms normally but fails to construct a tightly organised epithelium and the cells subsequently die (Tepaß and Knust, 1990). The expression of *crb* in imaginal disc cells under normal growth conditions on tissue culture plastic is similarly diffuse and fails to localise at sites of cell-cell adhesion, as it does *in vivo*. Occasionally, some antigen was seen to acumulate at cell-cell contact sites (see Figure 5.B), perhaps functioning in adhesion or signalling. However, this seemed to be a rare event. Because the CRUMBS protein *does* seem to be expressed in these cells and that these cells are not polarised may indicate that this protein is involved in the maintenance rather than the establishment of polarity, as expression alone does not seem enough to bring about a change in phenotype.

Beyond being simply a suitable marker for the absence of apical-basal polarity, *crb* expression may function in conferring viability in these cells, as mutants which lack this molecule have a marked rate of epidermal cell death (Tepaß and Knust, 1990).

One might expect, with regard to the ultrastructural evidence, that *crb* expression may be more restricted and concentrated in imaginal disc cells grown on fibronectin-coated membranes under the culture conditions described here which seem to enhance the apical-basal polarised phenotype.

Established cell lines *in vitro* do not seem to display apical-basal polarity, this is believed to be due in part to the lack of a unidirectional uptake of nutrient from the medium via an authentic extracellular matrix (ECM). Other factors known to be important in the generation of polarity are cell-cell and cell-substrate interactions (for reviews see Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). The significance of these factors in the generation of epithelial polarity is illustrated by the observation that apical-basal polarity can exist in vesicles of *Drosophila* epithelial cells *in vitro*

but not in the derived cell lines which grow directly on the tissue culture surface (Peel, 1991).

Cells within these vesicles, which grow as outgrowths from fragments of *Drosophila* imaginal tissue during primary cell culture (Currie *et al.*, 1988; Peel., 1991) exhibit a phenotype which is similar to what is found in imaginal discs. Here, the apical side, complete with microvilli, faces in towards the lumen and the basal side faces out towards the culture medium. However, the belief that unidirectional uptake was the sole factor for the formation of a polarised tissue here is questionable as it has not been demonstrated that these vesicles constitute one cell type. Mesodermal cells, such as adepithelial cells and haemocytes, may also have been present and could have provided an inductive role at the basal side of the epithelial cells.

It is possible that the epithelial cells in these vesicles are being influenced by factors other than direction of nutrient uptake; such as by the presence of ECM molecules that are missing from non-polarised imaginal cells growing *in vitro*. Studies using explanted vertebrate epithelia indicate that the basement membrane, or basal lamina, is important in maintaining the characteristic epithelial phenotype (Surgue and Hay, 1981; Surgue and Hay, 1986).

Imaginal cells have reportedly been grown on nitrocellulose membranes with little evidence for the development of apical-basal polarity (R. Wheater, personal communication in: Peel, 1991). The main difference between that particular study and this is that the vertebrate ECM molecule fibronectin was present for cell attachment and growth.

Vertebrate fibronectin is a ubiquitous extracellular glycoprotein, which can exist as either a soluble form in body fluids or as an insoluble component of the ECM. Fibronectin is known to function in many important physiological processes, such as embryogenesis, woundhealing and

thrombosis (Potts and Campbell, 1994) . The role of fibronectin in adhering epithelial cells to the ECM is well known. Many different cells are known to attach and spread rapidly on fibronectin, either on its own or when bound to collagen matrices (Ruoslahti and Pierschbacher, 1982). This interaction between vertebrate cell and matrix molecule is often mediated by specific interactions between integrins at the cell surface and tripeptide arginine-glycine-aspartic acid (RGD) sites on fibronectin (Ruoslahti and Pierschbacher, 1987), presenting a versatile recognition system which can act as a cue for polarity. The findings, detailed more fully in Chapter 4, that imaginal cells could adhere to, and migrate on, human fibronectin coupled with the fact that there is at present a comparative lack of availability, or indeed characterisation, of analogous invertebrate molecules determined the use of fibronectin in these assays.

In 1988, Danielle Gratecos and co-workers claimed to have discovered and isolated a protein in *Drosophila* which was thought to be similar to mammalian fibronectin (Gratecos *et al.*, 1988) and was referred to as *Drosophila* fibronectin. However, there has been little evidence since then that such a molecule exists.

Drosophila cells have been observed to adhere and spread on human fibronectin, including imaginal disc cells (see Chapter 4), MLDmBG-1 cells (Hirano *et al.*, 1991) and S2 cells which have been transformed to express the PS2 integrin, $\alpha_{PS2}\beta_{PS}$ (Bunch and Brower, 1992). These findings also implicated the importance of the RGD sequence in mediating *Drosophila* cell-ECM adhesion. Indeed, a recently identified *Drosophila* ECM protein, tiggrin, which contains the RGD sequence, has been identified (Fogerty *et al.*, 1994) and is known to function as a substrate for embryonic cell adhesion and as a ligand for PS2, although it is possible that a fibronectin analogue may yet be found in the *Drosophila* ECM.

It therefore seems likely that imaginal disc cells can adhere to human fibronectin in much the same way as their vetebrate counterparts do. namely, via the integrins. Indeed, the *Drosophila* PS integrins are known to have a significant sequence homology to some vertebrate integrins, including receptors for fibronectin and vitronectin, and thus may be functionally related (Leptin *et al.*, 1987; Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Leptin *et al.*, 1989).

The PS integrins are known to localise to the basal surface of the imaginal disc epidermis, presumably acting as receptors to components of the ECM, but their exact function in develpment is not yet known (Brown, 1993; Fristrom *et al*, 1993; Gotwals *et al.*, 1994). Thus, it is reasonable to suggest that the PS integrins could have a degree of functional similarity with the vertebrate molecules; specifically in the binding to and being influenced by human fibronectin in much the same way as vertebrate cells and thereby coordinating the formation of apical-basal polarity. If PS integrins were known to accumulate at sites of cell-ECM contact in these assays, then this would further suggest that fibronectin can function as a ligand for these receptors.

An apical-basal phenotype is found, to some degree, in imaginal disc cells grown on fibronectin-coated inserts, as indicated by a columnar cell shape and ultrastructurally by the presence of apical microvilli, differentiated intracellular cell-cell junctions and basal processes. However, the findings of this study suggest that growth on fibronectin alone is not sufficient for inducing apical-basal polarity in these cells. The induction of polartiy seems to depend also on the direction of nutrient uptake, as has been suggested earlier in this Chapter.

It seems that the closest phenotype to the *in vivo* epithelial state is produced when the cels are in the presence of *both* a basally presented fibronectin matrix and growth medium. Those cells grown in cultures such

that the only source of serum components, namely fly-extract (FE2) and foetal bovine serum (FBS), was from the basal side of the cells exhibited a phenotype which was reminiscent of that seen *in vivo*. The presence of a basally situated feeder layer of L127D6 cells also seemed to augment this state of polarity.

However, the serum components which are present in the growth medium can also be interpreted as a soluble form of ECM. The findings of Surgue and Hay (1981; 1986) indicate that soluble ECM components *in vitro*, such as fibronectin and laminin, can interact with the basal surface of explanted vertebrate cells growing on membranes and revert them from a blebbing phenotype to a smooth, *in vivo*, state. The same can thus be said of imaginal cells growing on membranes *in vitro*; ECM components from FE2 and FBS are available at the basal side of the cell, which would therefore stimulate an *in vivo*-like phenotype.

Nevertheless, even the most positive apical-basal phenotype that was observed *in vitro* did not reflect the state that is normally seen *in vivo*. This may be due in part to the fact that the cell line used in these studies, L127D6, was of a relatively high passage number (above 30) and may thus have undergone a degree of de-differentiation as a result, lacking certain molecules important in determining polarity. Indeed, from the findings detailed in Chapter 3, it seems likely that some CAMS can be selectively lost from the imaginal cell surface. Either the transmembranal molecules normally found at the cell-cell and cell-substrate boundaries or intracellular molecules involved in a signalling response could be missing from these established cell lines.

Imaginal cells *in vitro* evidently share some of the cell-cell and cell-substrate molecules that are found *in vivo*, such as the PS integrins, but some molecules are known to be absent, such as the soluble protein which is encoded by the *wingless* gene. In Cl. 8+ cells that had been induced to express *wingless*, by transfection with an expression construct, there was a dramatic

increase in the level of the adherens junction protein, armadillo (van Leeuwen *et al.*, 1994). It is not clear whether *wingless* is being expressed in imaginal cells grown on fibronectin-coated membranes. Armadillo is also known to be expressed in wild-type imaginal cells in vitro (D. Peel, pers. comm.) althouhg there has been little morphological evidence to suggest the existence of any adherens junctions.

Another possible reason for the lack of an apical-basal phenotype is that the *in vitro* cell environment does not faithfully resemble the *in vivo* environment. It could be that either the insoluble components here, in the form of the fibronectin-coated membrane, or the soluble components, in the form of the culture medium, or both, are lacking a factor which is normally present *in vivo*.

The basement membrane of imaginal discs is known to contain type IV collagen, laminin and sulphated proteoglycans, and is essentially similar to what is found in vertebrate cells (Fessler and Fessler, 1989). Mesodermal cells are also believed to be important in influencing the development of epidermal cells and are absent from these assays, although much of the morphogenesis of insect epithelia seems to proceed independently of the mesenchyme (Fristrom, 1988).

Cells of the imaginal disc epidermis produce their own extracellular matrix but also seem to depend upon mesodermal cells for some matrix products (Wigglesworth, 1973). Vertebrate mesodermal cells are known to express fibronectin and collagen types I, II or V (Fristrom, 1988). A consideration for a future study would be the co-culture of imaginal cells with a basally situated mesodermal cell line, such as the *Drosophila* wild-type and *air8* neoplastic overgrowth mutant blood cell lines (Peel, pers. comm.). Such a basally situated cell population may work in concert with the epithelial cells in producing an apical-basal phenotype which more closely resembles the *in vivo* state.

6.1   Introduction

Cell adhesion molecules (CAMs) are known to play a major role in the regulation of the outgrowth and guidance of neuronal growth cones in the central nervous system of *Drosophila* (for reviews, see: Jessell, 1988; Harrelson, 1992). These molecules, in vertebrates, are also known to belong to a specific family of neural adhesion molecules, be it either the immunoglobulin (Ig) superfamily, which includes L1 and neural cell adhesion molecule (N-CAM) (Schachner, 1993; Rutishauser, 1993) or the cadherins, which includes N-cadherin (Takeichi, 1988).

During the 1980s, a number of investigations at the molecular level were carried out to discover suitable candidate guidance molecules for axon outgrowth in the CNS of *Drosophila* and the grasshopper, *Schistocerca*. These investigations, utilising monoclonal antibody screens, initially identified three candidate molecules expressed on the surface of axon bundles, which were thus broadly referred to as the fasciclins, although they are not necessarily structurally or functionally related. Fasciclin I and fasciclin II were originally identified in *Schistocerca*, and later in *Drosophila* (Bastiani *et al.*, 1987) and fasciclin III only in *Drosophila* (Patel *et al.*, 1987; Snow *et al.*, 1989). Other molecules have been identified, using similar methods, which are known to have developmentally significant roles in *Drosophila* CNS development. One such molecule is neuroglian, which is known to be structurally related to fasciclin II (for review, see: Hortsch and Goodman, 1993).

Each of these molecules, fasciclins I, II and III and neuroglian (see Figure E), are transmembrane glycoproteins, each is Ca^{2+} independent in function and all except for fasciclin I are members of the immunoglobulin (Ig) superfamily. Neuroglian and fasciclin II have multiple Ig domains followed by multiple fibronectin type III domains. Fasciclin I, on the other hand, has a more unique structure made up of four tandem domains (Hortsch and

Goodman, 1993). Of the fasciclins, fasciclin III is unique in that it can be expressed outwith the CNS and this was the basis for embarking on a study to determine if it was expressed in imaginal disc cell lines.

Fasciclin III was originally identified, as with many other neural cell adhesion molecules, by a monoclonal antibody screen, as being expressed on a subset of commissural axon fascicles and other axon pathways in the embryonic central nervous system (CNS) of *Drosophila* (Patel *et al.*, 1987). This molecule is also expressed in some other tissues, and at other stages of development, including the visceral mesoderm, the luminal surface of the salivary gland epithelium and the larval eye-antennal imaginal disc (Brower *et al.*, 1980; Zipursky *et al.*, 1984; Gauger *et al.*, 1987).

Fasciclin III is apparently unrelated to any known vertebrate immunoglobulin, having three divergent extracellular Ig domains (Grenningloh *et al.*, 1984). At least two different forms of fasciclin III have been detected through the use of monoclonal antibodies, with alternate molecular masses of 80 and 66 kDa (Patel *et al.*, 1987; Snow *et al.*, 1989). A full length cDNA which encodes the longer, 80 kDa, form was generated and was used to predict the sequence and structure of the molecule (Snow *et al.*, 1989). A cDNA for the 66 kDa protein has also been generated , and indicates that both proteins are identical in their extracellular structure but have different cytoplasmic domains (Snow *et al.*, pers. comm.).

An interesting feature of the fasciclin III structure is that there is a tyrosine residue within the cytoplasmic region of the molecule (Snow *et al.*, 1989). Such a structure is known to attach to adapter proteins and may mediate internalisation of fasciclin III into coated pits; a phenomenon that has been observed during the interaction of neural growth cones with other cells (Bastiani and Goodman, 1984).

Another interesting feature of the intercellular region of fasciclin III is that there are two potential sites for phosphorylation at a serine by protein kinase C which is suggestive that this molecule may act in transducing signals (Snow *et al.*, 1989). Phosphorylation of this molecule may, thus, modulate the function of fasciclin III throughout development.

Fasciclin III was later found capable of mediating homophillic, Ca^{2+} independent, adhesion (Snow *et al.*, 1989; Elkins *et al.*, 1990). This was determined first by inserting the complete fasciclin III cDNA into the normally non-adhesive S2 cells and inducing expression by heat-shock. These cells, which normally grow as single cells in suspension, were then observed to undergo pronounced cell-cell adhesion; forming aggregates containing anything from tens to hundreds of cells in each. The correlation of aggregation being due to fasciclin III was corroborated by immunocytochemical staining with anti-fasciclin III polyclonal antibodies (Snow *et al.*, 1989).

In a later series of experiments, S2 cells which had similarly been transfected with fasciclin III were mixed with cells which had been transfected with fasciclin I cDNA (Elkins *et al.*, 1990). Here, following heat-shock, cells were seen to aggregate, as before, indicating that fasciclin I was also capable of mediating cell-cell adhesion. It was also noted that, by the end of the assay, this initially heterogeneous batch of cells had sorted into homogenous aggregates with each group of cells expressing either only fasciclin I or fasciclin III (Elkins *et al.*, 1990). These findings, that fasciclin III and fasciclin I can mediate cell-cell adhesion and sorting out *in vitro*, are suggestive that these molecules can direct selective cell-cell adhesion *in situ*. This is indeed reflected in the developing CNS, where the pattern of expression of each is spatially restricted.

A recent study has further elucidated a role for fasciclin III as a homophillic CAM in *Drosophila* development. During embryonic

neuromuscular development, fasciclin III is expressed on the RP3 motor neurone and on its synaptic targets, muscle cells 6 and 7 (Chiba *et al.*, 1995). It was found that muscle cells that mis-express fasciclin III were incorrectly targeted by RP3, therefore fasciclin III was identified as a synaptic target recognition molecule. However, the correct target cells in *fasciclin III* null mutants, that failed to express this CAM, also managed to form synapses with RP3, thus it seems that the absence of fasciclin III can be compensated for by another molecle, or another group of molecules (Chiba *et al.*, 1995).

As mentioned before, fasciclin III is unique within this particular group of neural adhesion molecules in that it can be expressed outwith the developing central nervous system of *Drosophila*. Within the developing CNS, where expression is predominant, fasciclin III is found on a subset of embryonic neurons and axon pathways; on the axons in five commissural fascicles, although not neccessarily on the entire cell surface (Patel *et al.*, 1987). Outwith the CNS, at the end of germ band extension, fasciclin III is expressed as a series of repeated stripes across all the body segments. This expression then spreads so that by the time of head involution all the cells of the epidermis express the molecule to some degree (Patel *et al.*, 1987). Elsewhere, fasciclin III is expressed on the visceral mesoderm and the luminal surface of the salivary gland epithelium (Patel *et al.*, 1987; Gauger *et al.*, 1987).

Of greater relevance to this particular study, however, is the expression of fasciclin III in imaginal discs. By using monoclonal antibodies to highlight elements of pattern formation during *Drosophila* eye morphogenesis, Zipursky *et al.*, (1984) identified some sites of expression of a novel glycoprotein which seemed to be developmentally significant. This molecule was later proved to be fasciclin III (Patel *et al.*, 1987), and these sites of expression were indeed distinct from what was seen in the CNS. In the eye-antennal disc, fasciclin III is expressed on the cone cells which surround the clusters of eight

photoreceptor cells, which themselves are deficient for fasciclin III expression (Zipursky *et al.*, 1984).

The possibility that fasciclin III may be expressed in imaginal cells *in vitro* seems entirely plausible, if one consders this expression outwith the developing CNS (Patel *et al.*, 1987) particularly the expression in imaginal tissues during larval development (Brower *et al.*, 1980; Zipursky *et al.*, 1984).

Drosophila imaginal disc cells, as with cells of the embryonic CNS, are known to sort out into homologous groups *in vitro*, according to the disc of origin (Fehon and Schubiger, 1985; Fausto-Sterling and Hsieh, 1987; Peel, 1991). Therefore it is seems likely that cells from different discs diferentially express certain cell surface molecules. The members of the fasciclin family, with their proven ability to direct sorting out between heterogeneously transfected S2 cells *in vitro* (Elkins *et al.*, 1989) seem like the ideal candidates to yield just such a molecule.

We therefore considered that this molecule, or some related form, could be involved in certain adhesive events in *Drosophila* imaginal cell lines *in vitro*. To test this, I first set about screening various cell lines for the expression and localisation of this molecule by using immunocytochemical techniques, centring on the use of polyclonal and monoclonal antisera which were supplied by Peter Snow of the State University of New York at Albany, New York (Patel *et al.*, 1987). I then carried out some Northern blot analyses, based on the use of the fasciclin III cDNA as a probe (Snow *et al.*, 1989), during a period of research as a guest at Professor Peter Snow's laboratory in Albany, as a test for the active transcription of this molecule in these cells.

Snow *et al.*, (1989) have previously employed Northern blot analyses, utilising the fasciclin III cDNA as a probe, to screen various *Drosophila* cell lines for the expression of fasciclin III. However, none of the tested cell lines, which included Schnieders line 1, S2 and K_c cell lines, seemed to have any endogenous transcripts encoding fasciclin III.

I also tried to assess the putative role of fasciclin III in certain adhesive and signal transducing events. The role of fasciclin III on embryonic mscle cells as a target recognition molecule for the fasciclin III expressing RP3 motor neuron (Chiba *et al.*, 1995) is suggestive that this molecule may mediate some form of cell surface generated signal during morphogenesis.

Another study which is also included in this chapter, and which is of a greater general relevance for each cell adhesion study mentioned so far, was the visualisation by immunofluorescence of some cytoskeletal components during imaginal disc cell aggregation *in vitro*. Confocal microscopy was utilised to study the distribution of microtubules and filamentous actin in these cells which was then interpreted in the context of imaginal cell movement *in vitro* and the expression of various CAMs.
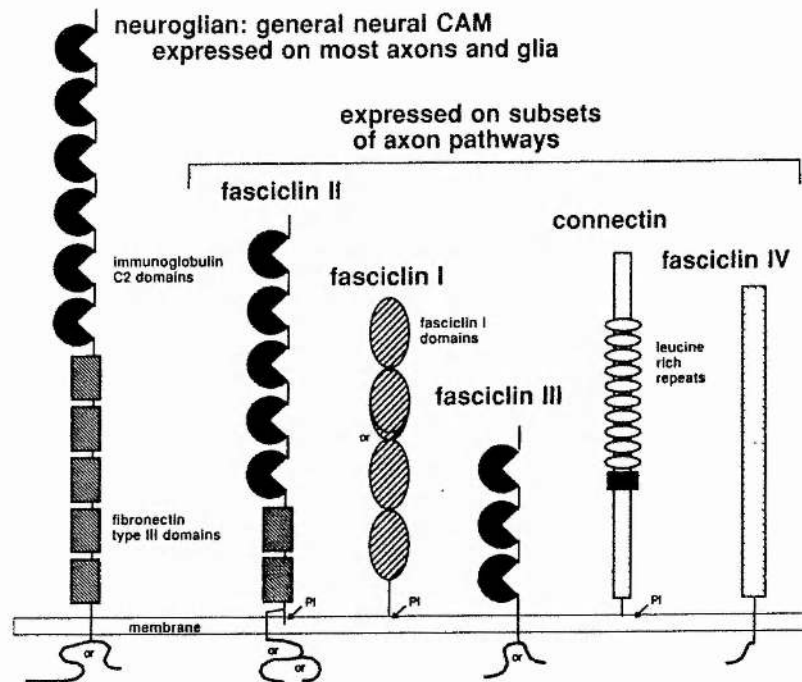
Figure E   A schematic diagram showing the structures of six axonal

glycoproteins in insects. Three of these molecules are members of the

immunoglobulin (Ig) superfamily: neuroglian, fasciclin II and fasciclin

III. Both neuroglian and fasciclin II have multiple Ig domains whereas

fasciclin III has three more divergent domains. (From Goodman and

Doe, 1993.)

6.2   Materials and Methods

6.2.1   Immunocytochemistry

Cloned wing and leg cell lines were initially tested for the expression of fasciclin III by immunofluorescently labelling them using antibodies raised against fasciclin III. This protocol also lists the method used for the immunofluorescent labelling of tubulin in imaginal disc cells.

Cells were plated onto sterile coverslips, at a density of 1×10^5 cells per ml, and were allowed to grow on these for two days. The aggregating cells, adhering to the glass, were washed once in PBS and then fixed in methanol for 5 minutes at -20°C. The coverslips were given three 10 minute washes in PBS with 1% BSA followed by a 30 minute incubation in a blocking solution of PBS with 5% BSA. The coverslips were then incubated in a solution of the primary antibody either overnight at 4°C or for 1 hour at 25°C. The antibody used here was a rat polyclonal antiserum (F3T) directed against the extracellular, truncated, region of fasciclin III, diluted 1:500 in PBS with 1% BSA. For the tubulin-staining assay, the procedure was the same except that the cells were instead incubated with monoclonal anti-α tubulin (Sigma) at this stage. The coverslips were washed and blocked again and were then incubated, for 1 hour at 25°C, in a solution of the secondary antibody: either goat-anti-rat IgG conjugated to FITC (Sigma Immunochemicals) for fasciclin III or biotinylated anti-mouse IgG, each diluted 1:200 in PBS with 1% BSA. Those cells which were to be stained for tubulin were given an extra wash and an incubation in tertiary antibody, FITC linked avidin (Sigma), which was diluted 1:200 in PBS with 1% BSA. Finally, the coverslips for each set of samples were washed for four 10 minute periods in PBS with 1% BSA and were mounted on slides using a solution of 90% glycerol and 1% propyl-

gallate, at pH 9, and viewed using a Leitz/Biorad Confocal Laser Scanning microscope.

Alternatively, cells were allowed to reaggregate by suspending cells, which had been harvested after 2 days growth, at a density of 1×10^6 cells per ml of PBS, in a 24 well plate placed on a shaker. The resulting aggregates were then spotted onto a glass slide, smeared and allowed to air-dry. The cells were then fixed, washed, stained and mounted as before and viewed using a Leitz Diavert inverted fluorescence microscope.

6.2.2 Fasciclin III as a homophillic cell adhesion molecule

(i) Reaggregation assays (Short term cell-cell adhesion)

An assay was devised, based on a protocol used by Fausto-Sterling and Hsieh (1983), later modified by Peel (1991) and used elsewhere in this thesis, which would quantify the rate of cell-cell adhesion in imaginal cell lines incubated either in the presence or absence of antibodies to fasciclin III, to test the role of this molecule in short-term reaggregation. Similar protocols are detailed in Chapters 3 and 4.

Cells were allowed to grow for 3 days under normal culture conditions, to ensure that they were in an active, proliferative, state. The cells were then loosened and removed from the tissue culture plastic by flooding with CSM squirted from a Pasteur pipette which were collected and subsequently spun down at 1000 rpm (500 g) for 5 minutes and resuspended in 1 ml of D=. A sample was taken from this suspension for cell counting, using a haemocytometer. For each sample, an aliquot was taken from the original supernatant of a volume that was estimated to contain 4×10^6 cells, and this was resuspended in 1ml of a particular test solution and was pelleted down as before. The solutions on test here were solutions of PBS containing

either 5 µg/ml of rat anti-fasciclin III polyclonal antiserum (F3T) or an equivalent amount of pre-immune serum (gifts from Peter Snow). Next, the cells were resuspended in 1 ml of their respective test solutions in 10 ml centrifuge tubes. The tubes were then placed on a Spiramix roller (Denley), at a low angle, allowing the cells to reaggregate in suspension, and aliquots were taken at 0, 30, 60, and 120 minute intervals for counting. Quadruplicates of each sample were run simultaneously. The rate of reaggregation was plotted in each sample by measuring the percentage of single cell units present, using a haemocytometer, in each sample over this two hour period, with the number of single cells at time zero taken as representing 100%.

(ii) Aggregation assays (Long term cell-cell adhesion)

To test the function of fasciclin III in long term cell migration and adhesion, or aggregation, imaginal cells were grown in the presence of anti-fasciclin III antibodies. Cells were plated out onto Petri dishes as for a routine cell subculture (see General Methods section, Chapter 2), except that dishes being tested had 5 µg/ml of F3T antiserum added to the culture medium. An equivalent amount of pre-immune antiserum was added to all the other dishes. The cells were then left to grow over a 7 day period and were photographed using a Leitz Diavert inverted microscope with a Wild Photoautomat attachment.

6.2.3   Western Blotting of whole-cell and immunoprecipitated cell lysates

A selection of cloned wing and leg cell lines were tested for the expression of fasciclin III by Western blot analysis. Various techniques were employed for the preparation of samples for this analysis, but I shall only outline the one that seemed the most successful.

Dishes of cells were drained of medium and were rinsed once with D=. The cells were removed from the dish by scraping with a silicone "policeman" or by squirting with D= from a pipette, depending on how strongly they were adhering to the dish, and removed to a 1 ml centrifuge tube. The cells were pelleted at 1000 rpm (500 g) for 5 minutes at 4°C and the pellet was resuspended in 1 ml of D=, and counted with a haemocytometer.

Next, the cell number of each sample was standardised, to 1×10^7, so that a roughly equivalent mass of protein was loaded into each well. For more detailed analyses, the protein concentration in each instance was determined by using the Bradford Test (see General methods section, Chapter 2) and 50 µg of protein was loaded per lane. The cells were spun to a pellet, as before, and were solubilised in 500 µl of lysis buffer on ice for 30 minutes. The lysis buffer, known as IPB, contained: 10 mM Tris, 1% NP-40, 0.2% deoxycholate, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 1 µg/mg aprotonin and 0.5 mM dithiothreitol, pH 8.2. The lysate was then spun at 10,000 g for 10 minutes at 4°C and the pellet was solubilised in 100 µl of Laemmli sample buffer, containing: 0.5 mM Tris, 0.4% SDS, 25% glycerol and 10% mercaptoethanol, pH 6.8. The samples were then boiled for a minimum of 3 minutes prior to loading onto the gel.

For immunoprecipitation of the cell lysates, the procedure is similar to the one noted above. The method, however, first entailed the forming of a complex of the specific antibody with a secondary antibody. This complex was subsequently utilised for the immunoprecipitation of the protein of interest, that is fasciclin III. The antibodies were mixed at a given ratio and allowed to form a visible precipitate. A ratio of 1:5 for undiluted polyclonal antisera, F3T, to undiluted goat-anti-rat IgG (5 µl to 25 µl) seemed to give the best results (monoclonal antibodies, which were derived from tissue culture supernatants were also tried but were used at a ratio of 1:2 to the secondary antibody). The mixture was incubated at 4°C for a minimum of 4 hours on a

rotator, and the complexes collected by spinning at 10,000g for 15 minutes. The complexes were washed once and were resuspended, and stored, in 25 µl of IPB. After solubilising the cell pellet in IPB for 30 minutes on ice, the lysate was spun at 10,000 g for 15 minutes and the supernatant was then incubated with 5 µl of the specific preformed complex for a minimum of 4 hours, on a rotator at 4°C. The resulting complex was collected by centrifugation at 10,000 g for 10 minutes and was then washed and pelleted in IPB for four times, changing the tube each time. The samples were then solubilised in sample buffer and boiled as before.

The samples were then loaded onto a 10% SDS-PAGE gel, resolved and transferred to nitrocellulose by electrophoresis using a Biorad Mini-Protean II dual slab gel kit (see General Methods section, Chapter 2 for details). After blocking in Tris buffered saline (TTBS) plus 1% non-fat dried milk, the nitrocellulose was incubated in a solution containing the polyclonal antibody: rat-anti-fasciclin III (F3T), diluted 1:1000 in TTBS, for 1 hr at 25°C or overnight at 4°C. The membrane was then washed and blocked as before and was then incubated in a solution containing the secondary antibody: goat-anti-rat IgG (Sigma) diluted 1:2000 in TTBS, for 30 minutes at 25°C. Next, the membrane was given at least six brief washes in the blocking solution and one 5 minute wash in PBS with 15 mM Tris, pH 7.5. The membrane was then stained using a standard horseradish peroxidase / 3,3'-diaminobenzidine substrate reaction kit (supplied by Vector), rinsed in H_2O and left to dry on absorbent paper. Molecular weight standards were marked on the membrane immediately after blotting, prior to blocking, by staining with a solution of Ponceau S, for 5 minutes and then rinsing in PBS. (Ponceau S stock solution: 2% Ponceau S (Sigma), 30% trichloroacetic acid and 30% sulfosalicylic acid in H_2O. This was then diluted 1:10 in H_2O for a working solution).

6.2.4   RNA isolation and northern blotting

To further test for the presence of fasciclin III or a related molecule in imaginal cells *in vitro*, RNA was isolated from an established cloned wing cell line and hybridised with radiolabelled probes made from fasciclin III cDNA.

Cells to be used, existing as a confluent monolayer in T-25 flasks (Corning) firstly had their medium removed by aspiration and were then blasted off of the tissue culture surface using a Pasteur pipette in 1 ml of ice-cold PBS. These cells were then pelleted down in a 1 ml centrifuge tube at 500 g for 5 minutes at 4°C, aspirated, resuspended in 1 ml of ice-cold PBS and pelleted again. The supernatant was removed again and 500 µl of RNA extraction buffer A. was added to the pellet. The tube was then vortexed on high for a few seconds and was then placed on ice. This step lyses the cells and breaks up the chromosomal DNA. Both RNA extraction buffers A and B were made up in 0.1% DEPC water, which partially inhibits the action of RNAases,

RNA extraction buffer A.
2% SDS
200 mM Tris HCl
1 mM EDTA   pH. 7.5

150 µl of ice-cold RNA extraction buffer B. was then added. The lysate was then vortexed on high for 10 seconds and was placed on ice for 2 minutes; allowing for the precipitation out the DNA and any proteins present.

RNA extraction buffer B.
4 M potassium acetate, $(KC_2H_3O_2)$
10% acetic acid, $(C_2H_4O_2)$

The lysate was then centrifuged at 10,000 g for 5 minutes and the supernatant was twice extracted in 300 µl of a mixture of chloroform, iso-amyl

alcohol and phenol, (at a ratio of 24:1:25), to remove any remaining proteins. The aqueous and organic phases were separated by centrifugation at 10,000 g for 5 minutes. The aqueous phase was then removed to 650 µl of ice-cold propanol and left to precipitate on ice for 10 minutes. The RNA was then recovered by centrifugation at 10,000 g for 10 minutes at 25°C. The supernatant was discarded and the pellet was washed with approximately 500 µl of cold 70% ethanol and was then allowed to dry at room temperature for 5 minutes. The pellet was then redissolved in 25 µl of DEPC H_2O and an aliquot was taken to determine the concentration of RNA. The RNA could then be frozen and stored at -20°C, either in DEPC H_2O or in 70% alcohol. The concentration of the RNA can be determined by measuring the $OD_{260/280}$ of the taken aliquot: 2 µl diluted in 98 µl of DEPC H_2O, by using the following equation :

$$\frac{OD_a \text{(absorbance at given wavelength a)} \times 40 \text{ (constant)} \times 0.1 \text{ (original sample)}}{0.002 \text{ (sample taken)}}$$

which is equivalent to µg/ml total RNA present. To determine what proportion of the sample is RNA the OD_{260} is divided by the OD_{280}. If this value is equal to or greater than 2, one can assume that the sample is composed of a sufficient level of RNA, rather than DNA and protein, for detection by Northern analysis.

The RNA samples were then run, by electrophoresis, through a formaldehyde gel. The running buffer was prepared as follows: 390 mls distilled H_2O, 20 ml CHURCH (0.5 M N_2HPO_4, pH 7) and 90 ml of formaldehyde. The gel itself was prepared by melting 0.25 g of agarose in 12.5 mls DEPC H_2O and mixing with 2x FORMAL buffer, (7 ml DEPC H_2O, 1 ml CHURCH and 4.5 ml formaldehyde) which was cast and allowed to set for 30

minutes at 25°C. Each sample was prepared by drying 10 μg of RNA in a microfuge tube and dissolving it in 10 μl 2x FORMAL buffer, 10 μl formamide and 0.25% bromophenol blue. The samples were incubated at 60°C for 5 minutes prior to loading. The gel was then resolved, submerged in running buffer and powered at 75 v for approximately 150 minutes.

The RNA was transferred to a nylon membrane (Nytran, Schleicher and Schuell, Keene NH) by capillary elution. This was achieved by placing the gel flat on a box-support on Whatman 3MM paper in a reservoir of buffer, with a sheet of Nytran, two sheets of Whatman paper and a stack of paper towels placed on top of the gel. Buffer was thus drawn from the reservoir, passing through the gel and was absorbed into the towels. A weight was added to the top of the paper towels to ensure a tight connection between all the layers of material used in this transfer. The buffer used for this transfer was 10x SSC: 1.5 M NaCl and 150 mM $C_6H_5Na_3O_7$, pH 7. The transfer was thus allowed to proceed for at least 8 hours at 25°C. The membrane was then removed from the apparatus, rinsed gently in 10x SSC and then placed in a Stratagene UV crosslinker - which causes the irreversible binding of any RNA molecules to the nylon membrane. The membrane was then ready for hybridisation with the desired probe.

For this stage in the experiment, double stranded probes for RNA hybridisation were prepared by using the 'Oligolabelling' method of labelling DNA restriction fragments to a high specific activity with an [α-^{32}P] dCTP analogue (Amersham). The cDNA to be used for the probes were known as F3, an Asp fragment of the long-form (80 kDa) cDNA of fasciclin III and 0.8, a 0.8 kb R1 fragment from the short-form (66 kDa): both derived originally from a full-length cDNA encoding the long-form of fasciclin III, (Snow *et al.*, 1990). 25 ng of the appropriate DNA was dissolved in 16 μl of TE buffer: 0.5 M Tris-HCl, 100 mM EDTA, pH 7.4. The DNA was denatured for 3 minutes at 100°C and was immediately placed on ice. The following were then added to each

tube, still on ice: 5 μl Reagent Mix, 2.5 μl [α-^{32}P]dCTP at 25 μCi activity and 1 μl of Klenow Fragment. These were then mixed gently and incubated at 37°C for 1 hour. The probes were then made up to a volume of 100 μl each with TE and passed, by centrifugation, through 2 cm Sephadex G-50 columns to remove any non-incorporated nucleotides. The probes were then ready for hybridisation after heating at 100°C for 3 minutes and placing immediately on ice. An RNA standard probe was made at the same time. Individual strips cut from the membrane, corresponding to each lane, were then placed in an excess of PREHYB solution: 500 ml of which contains 250 ml CHURCH, 35 g SDS and 250 ml H$_2$O, at 65°C for 15 minutes. Next, the strips were transferred to 50 mls of PREHYB to which the probe was added, and were left to hybridise at 65°C overnight. The strips were then given three 20 minute washes, at 65°C, in a rinsing solution: 500 ml of which contained 20 ml CHURCH, 5 g SDS and 480 ml H$_2$O. The strips were then lightly dried on Whatman paper, wrapped in cling-film, placed in a cassette for exposure to X-ray film, (Kodak X-OMAT AR) and placed at -70°C for 24 hours; after which time the film could be developed and examined.

6.2.5 Signal transduction experiments; fasciclin III as a cell surface receptor

To test whether or not fasciclin III, or a related molecule, was acting as a surface receptor in imaginal cells *in vitro*, whereby the binding and subsequent activation of the molecule led to an intracellular signal, three separate sets of experiments were tried. The first of these involved the measurement of free intracellular calcium ions upon activation of fasciclin III, by employing the use of the calcium indicator, fura-2. The second method was designed to identify candidate cytoskeletal molecules binding to fasciclin III: either by immobilising radiolabelled cell lysate components onto a solid phase adsorbed antibody (SPIT) or by immunoprcipitation by a preformed

antibody complex, as before. The third study that was considered was the dual, immunofluorescent, staining for actin and fasciclin III, the co-localisation of these molecules would be further suggestive of a role for fasciclin III as a signal transducer.

6.2.5 (i) Fura 2 labelling

For this study, Cl. 8+ cells were grown at a concentration of 2.5×10^5 cells/ml on coverslips placed in a 6-well plate in 3 mls of CSM, and were allowed to grow for 2 days. A 5 mM stock solution of the acetoxymethyl (AM) ester, fura-2 (Molecular Probes, Inc.) was prepared in DMSO, which could then be aliquoted and stored at -20°C. An aliquot of this stock solution was then diluted 100-fold in PBS, to give a working concentration of 5 μM fura-2. To facilitate loading of the cells with this dye, the detergent Pluronic F-127 (BASF Wyandotte), was also included in the stock solution at 5% (w/v), as was fetal bovine serum, added to the final buffered preparation at 2%. The aqueous dispersion of fura-2 prepared from the DMSO stock was vortexed immediately prior to adding to the cells, to assure uniformity of loading. The coverslip, with adhering cells, was then washed briefly in PBS, removed from the plate and mounted onto a customised perspex support-slide that was designed so that a square coverslip, bathed in medium, could be viewed using an inverted microscope. To the coverslip, 500 μl of the aqueous fura-2 dispersion diluted in 500 μl of CSM was then added. The cells were incubated, thus, at 25°C for 30 minutes in the dark. The cells were then briefly washed twice in PBS prior to visualisation. At this stage, F3T was also added, at a dilution of 1:200, and left to incubate for 30 minutes in the dark. Negative controls used for this assay were Cl. 8+ cells treated in the same way as in the experimental samples but were not exposed to F3T.

The cells were then viewed using a Nikon inverted fluorescence microscope operating Nomarski optics. At low concentrations of intracellular fura-2, use of the 340/380 wavelength excitation ratio allows accurate measurements of intracellular Ca^{2+} that are essentially independent of the amount of dye in the cell. Thus the cells were subjected to a 340/380 excitation ratio, and the resulting fluorescence in a group of cells was recorded, and quantified using a computer operating 'Metafluor' (Universal Imaging Corp., PA), a program specifically designed for such physiological measurement.

Once the incubation slide was in place on the microscope, a small group of cells had been selected and preliminary recordings were taken, a 10 µl aliquot of goat-anti-rat IgG antibody was added to the preparation (giving a final ratio of 1:200) taking care not to shift the cells being viewed. The purpose of this being to bind to the primary antibody in such a way that groups of the ligand, in this case being the polyclonal antibody, are presented for binding to the receptor, the fasciclin III molecule. A series of recordings of the resultant fluorescence were then taken at 2 minute intervals for a total period of 60 minutes. Any rise in the emitted fluorescence in a given field of cells could then be interpreted as a rise in the free intacellular concentration of Ca^{2+} there.

6.2.5.(ii) Solid Phase Immunoisolation Technique, (SPIT)

This method was based on a technique devised by G. Tamura *et al.*, (1984) for the isolation of molecules recognised by either monoclonal and polyclonal antibodies.

Coating of plates with antibody:

Firstly, 96-well microtiter plates were coated with the antibody of interest, in this case 2D5, a monoclonal antibody to fasciclin III, by adding 100 µl per well of a 100 µg per ml solution of purified 2D5. Control wells were coated with a similar amount of monoclonal antibody directed against fasciclin I, known as 3B11. The dishes were then incubated overnight at 4°C. The 96-well plates were then briefly washed in PBS . Non-specific sites were then blocked by incubating 200 µl per well of PBS + 5% goat serum for 1 hour at 4°C and were washed as before.

Preparation of labelled cell lysates:

Cl. 8+ cells were plated out in 2 wells of a 24 well plate, at a density of 2.5×10^5 in 1 ml of CSM and left to grow for two days. The cells were then incubated in methionine-free medium for 1 hour after the removal of the CSM. The cells were then incubated in 500 µl of methionine-free medium containing a ^{35}S label (Tran^{35}S-label, ICN Biomedicals Inc.) at a concentration of 400 µCi per ml, for 8 hours at 25°C. The cells were then washed briefly in PBS and removed from the dish by squirting PBS from a Pasteur pipette. The cells were pelleted in a 1 ml microfuge tube by spinning at 500 g for 5 minutes and were resuspended in 100 µl of lysis buffer, D-IPB, consisting of the following: 0.1% SDS, 0.1% deoxycholate, 1% NP-40, 100 mM NaCl, 10 mM Na_2HPO_4 and 1% digitonin plus protease inhibitors, pH 7.1. and placed on ice for 30 minutes.

The lysates were vortexed at high speed and were pelleted at 10,000 g for 5 minutes. The supernatants were then removed and added to the wells which had been coated with antibody, as described above, and were incubated overnight at 4°C . The wells were then given four 5 minute washes in D-IPB. Then, 80 µl of Laemmli SDS sample buffer, plus 2% mercaptoethanol, was added to each well and the dish was left at 60°C for 30

minutes. The samples were then removed, boiled for 5 minutes, and were ready for SDS-PAGE analysis.

A gradient gel was used to run these samples (plus high and low molecular weight standards) being cast in a mixing-reservoir so that the concentration of acrylamide at the top, stacking, end was 5%, and the concentration of the gel at the bottom end was 15%. This method of gel casting enables the analysis of proteins of widely varying weights, but is otherwise run as before, (see General methods section, Chapter 2). The gel was run overnight at 100v and was then boosted to 250v until the dye front reached the bottom of the apparatus. The gel was removed and stained, for 30 minutes in Coomassie solution, rinsed briefly in water and was then placed in Destain solution overnight. Next, the gel was placed in a gel-drier, on top of a piece of Whatman paper, connected to a vacuum pump and set at 100°C, for 90 minutes. In this way, the gel was dried onto the paper and was next placed in a cassette directly under a sheet of photographic film for 4-5 days at 25°C. The film was developed as before.

6.3.5.(iii) Co-localisation study

The dual staining of Cl. 8+ cells for fasciclin III and f-actin, employed the use of F3T and phalloidin-FITC respectively. Cells were processed for immunofluorescent staining as before, however, the secondary antibody used in this instance was goat-anti-rat IgG conjugated to rhodamine. After washing, the cells were stained in phalloidin conjugated to FITC diluted 1:200 in PBS with 1% BSA for 30 minutes at 25°C, and were then washed and mounted as before. The cells were then viewed at the appropriate wavelengths for that stain and sites of staining were compared.

6.3   Results

6.3.1   Immunocytochemistry

Staining patterns, using the anti-fasciclin III antiserum, F3T, suggest that fasciclin III is being expressed on the plasma membrane at all stages of aggregation. During the early stages of growth in culture, when the cells exist as a monolayer and there is very little cell-cell contact, certain individual cells stain strongly, especially those that are forming cell processes and seem to be migrating. Figure 6.A is a merged phase contrast and fluorescent image which illustrates a hugely elongated, Cl. 8R cell, which seems to be migratng, that was fixed 4 hours after plating. This cell exhibits a strong peripheral staining for F3T with a few bodies within the cytoplasm which also show some staining. Figure 6.B shows separate phase contrast and fluorescent images of a group of Cl. C9 cells again at an early stage of growth, where the central, stellate, cell has stained very strongly. A similarly shaped cell is pictured in Figure 6. E, with pronounced staining at the periphery. Figure 6.C shows a fluorescing distal region of a W20C6 cell at an early stage after passage traversing a background of weakly staining cells. This cell terminates in a highly branched process, which may play an adhesive, anchoring, role.

Figure 6.D.1 details a set of four fluorescent images, representing different optical sections of the same material taken at 1 μm intervals. These are Cl. C9 cells 2 days after plating out as a monolayer and which are at an early stage of aggregation. The cell at upper left is extending a process which has come into contact with what appears to be a dividing cell at the lower right. In each of these cells there are brightly staining areas within the cytoplasm, possibly corresponding to coated pits or secretory vesicles. Figure 6.D.2 illustrates in greater detail one of the groups seen in Figure 6.D.1. Later on in passage, as the cells proliferate and aggregate into multicellular clumps,

staining is seen, especially at sites of cell-cell adhesion within the aggregates themselves. Figure 6.F is a fluorescent image of an aggregate of Cl. C9 cells fixed after 4 days of growth. The peripheral staining of F3T is again quite evident, but antigen accumulation seems greatest at sites of intercellular contact.

6.3.2   Reaggregation and aggregation assays

The cell lines used in this study were Cl. C9, Cl. 8+, Cl. 13, L127D6, W2 and Cl. 8R. Of all these, the only cell line which seems to show a significant reduction in the rate of cell-cell adhesion as result of the presence of the anti-fasciclin III antiserum, F3T, is Cl. C9; a cloned wing cell line (Figure 6.G). There was also evidence to suggest that two other cloned wing cell lines, W2 and Cl. 8R, were being affected in their rate of cell-cell adhesion; although not quite to the same extent as with Cl. C9. The presence of the fasciclin III antibody seemed to influence the rate of Cl. 8+ adhesion over the first 60 minutes of the assay, but by 120 minutes they have reaggregated to the same degree as the pre-immune serum containing control samples. The presence of anti-fasciclin III serum seems to have no blocking effect on the rate of reaggregation of the other cloned wing cell line, Cl. 13 or of the uncloned leg cell line, L127D6.

The long term aggregation of cells in the presence of F3T, over a 7 day timecourse, did not seem to differ much from the controls where the antibody was absent. The cell lines that were used as subjects in this assay were Cl. C9, Cl. 8+, Cl. 13 and L127D6. Figure 6.H.1 shows Cl. C9 cells at 5 days after plating in CSM that contains an aliquot of pre-immune serum. Figure 6.H.2 shows an equivalent population of cells that have had F3T added, at a concentration of 5 µg/ml. In each sample, aggregation appears to proceed at the same rate. This result is typical of the responses of each imaginal cell line

that has been tested to date, with there being very little morphological variation between the control and the experimental samples.

6.3.3   Western blot analyses

Western blotting was originally carried out using whole-cell lysates probed with the polyclonal antibodies directed against fasciclin III. The results from this suggested that a species of around 120 kDa (not shown) was being detected. However, subsequent analysis, specifically by the use of a control cell line that was known to be fasciclin III non-expressing (3B11, transfected with a baculovirus to express fasciclin I), suggested that this result was probably due to a cross reaction of the antiserum with a cellular component which is unrelated to fasciclin III. This may be due to the fact that some antisera have not been affinity purified and may contain antibodies to species other than fasciclin III, such as bacterial antigens. Therefore I attempted to run a Western blot of detergent solubilised cell lysates that had been immunoprecipitated with the monoclonal antibody, 2D5. From such a blot there is seen a form, or forms, which migrate between 65 and 80 kDa (Figure 6.I), which are absent from the control lanes. These species do not seem to correspond to the 80 kDa embryonic long form since there is no reaction with antisera specific for this form. They do, however, seem to react with an antiserum specific for the 66 kDa short form, but do not co-migrate on blots. In some earlier blots bands were seen at around 29 kDa but are believed to correspond to a proteolytic fragment of fasciclin III, which is known to be particularly protease-sensitive (Peter Snow, pers. comm).

6.3.4   Northern blot analyses

RNA isolation and northern blotting were carried out to further, and more assiduously, assay for the expression of fasciclin III in Cl. 8+ cells. Figure 6.J represents the results of a northern blot of RNA samples that have been probed using both the F3 and 0.8 oligonucleotide probes; the positive control present in lane four was RNA isolated from 9-13 hr *Drosophila* embryos and, thus, known positive for the expression of both forms of the molecule. Lanes two and three represent RNA isolated from 1 day and 7 day post passage cells respectively, representing cells in proliferative and aggregated states respectively. The bands corresponding to the long and short form fasciclin III RNA, seen in lane four are absent from lanes two and three. This result, endorsed by many later blots, suggests that fasciclin III or a related protein is not being expressed in this cell line.

6.3.5   Signal transduction

6.3.5 (i) Fura 2 labelling

This set of experiments was a brief attempt at assaying for the rise in intracellular Ca^{2+} in antibody activated cells, and served as a useful preliminary for similar future studies. The interpretation of the assays that were attempted suggested that F3T bound cells did not display any significant increase in intracellular Ca^{2+} when compared to control cells (the data from these experiments are unavailable).

6.3.5(ii) Solid Phase Immunoassay Technique (SPIT)

Any bands which were exposed by the experimental lanes (labelled lysates which had been plated onto plastic pre-coated with monoclonal anti-fasciclin III antibody) but were absent from control lanes (those coated with monoclonal anti-fasciclin I antibody) were to be considered as representing possible candidate molecules which were either co-precipitated with or bound to fasciclin III. Two such bands were seen at around 94 and 120 kDa, which are absent from the control lanes.

6.3.5(iii) Co-localisation study

Actin expression

Aggregating imaginal cells stain very strongly for f-actin at cortical sites just beneath the surface of the plasma membrane. Figures 6.K and 6.L are fluorescent confocal images of Cl. C9 and Cl. 8+ cells respectively, fixed and stained with phalloidin FITC at 2 and 4 days after plating. This pattern of expression is simlar in distribution to that of fasciclin III, supporting the possibilty that the two could be functionally linked, either in signal or force transduction.

Tubulin expression

Tubulin expression in imaginal cells throughout the process of aggregation, like that of f-actin, seems equally abundant and ubiquitous. Figure M shows a group of aggregates, fixed 7 days after plating. Here, there seems to be a brightly staining network of cell extensions and filopodia within the aggregates themselves, although the brightest staining is often seen

toward the periphery. Figures 6.N.1 and 6.N.2 are phase contrast images of Cl. 8+ cells photographed at a similar stage of aggregation.

Both Figures 6.O and 6.P are details of Figure 6.M, the latter generated using a confocal laser scanning microscope. In both images, elongated cell processes joining two aggregates can be seen (with especial clarity in the confocal image) which serve to connect connecting the aggregates like guy-ropes. Sometimes bipolar cell bodies are also seen in this interstitial space (Figure 6.Q), contacting neighbouring aggregates by sending out processes from either end. Sometimes, fascicles of cells can be seen extending between aggregates (Figure 6.N.2). Some of these processes extend over what appears to be a great length and are packed with microtubules. The cell processes pictured in Figures 6.P and 6.Q seem to have converged on a common point, forming connections over the shortest available distance. Those processes which have come into contact with their targets appear taut, but sometimes processes can be seen which seem to have failed to establish contact and are either bent or coiled in appearance.

Figure 6.A A merged phase contrast/immunofluorescent image of a single large Cl. 8R cell fixed and stained for fasciclin III 4 hours after plating. The peripheral red colouration represents sites of fasciclin III expression. This cell, which is hugely elongated, appears to be migrating, extending three growth cone-like structures from the cell body, each of which is strongly stained for the antigen. Pseudocolour has been added to this image, as with some of the following Figures.

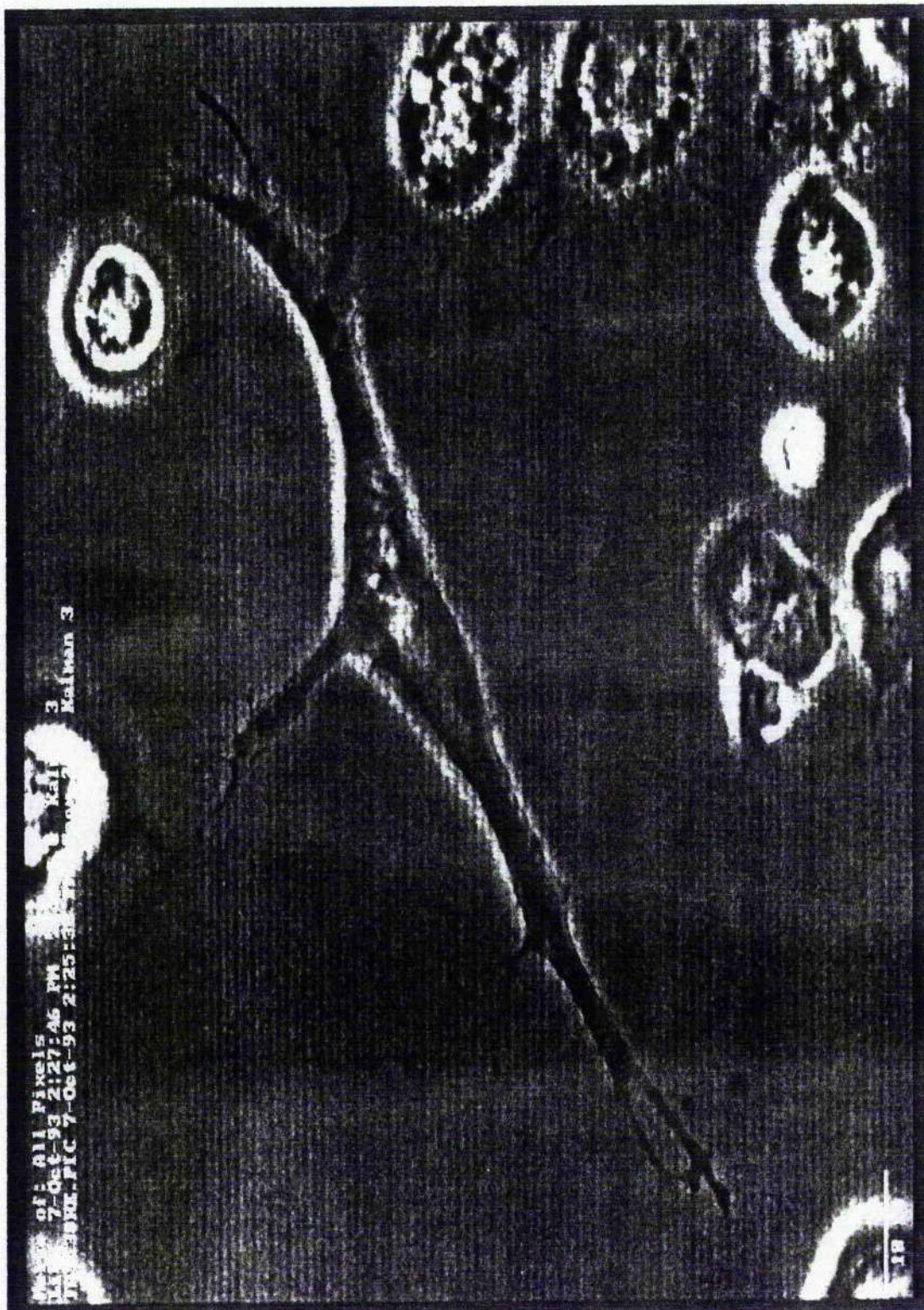*Scale bar represents 10 μm*

Figure 6.A

Figure 6.B   A dual image of a group of Cl. C9 cells fixed and stained for

fasciclin III expression 4 hours after plating, the image on the left

being phase contrast and the image on the right being

immunofluorescent. Here, the stellate cell in the centre of this image is

staining very strongly against a background of weakly staining cells. A

peripheral expression pattern of antigen can be seen, with evidence for

vesicular staining within the cytoplasm.

Scale bar represents 10 μm

Figure 6.C   A highly branched distal portion of a large, possibly migratory,

W20C6 cell at an early stage of passage terminating in a field of weakly

staining cells. This well stained structure is, as in Figure 6.A, reminiscent

of a growth-cone and may signify that the cell from which it stems is

motile.

Scale bar represents 10 μm
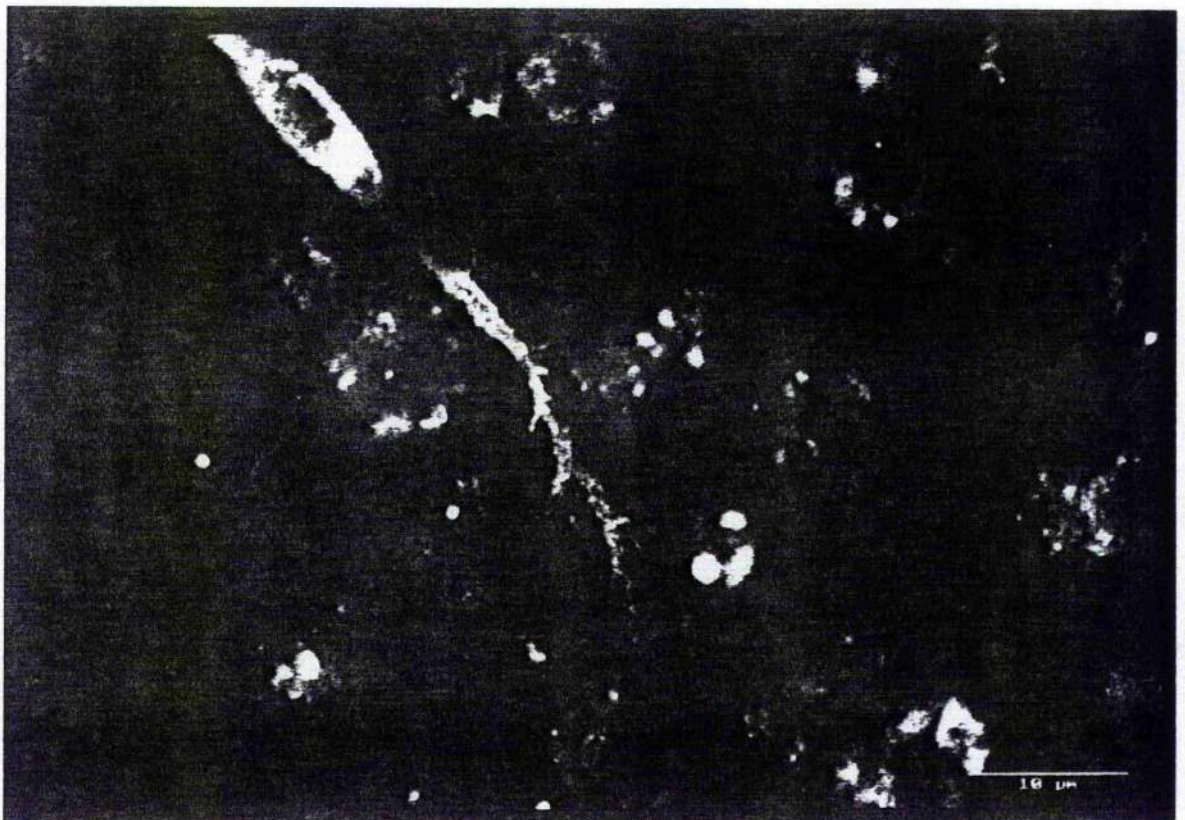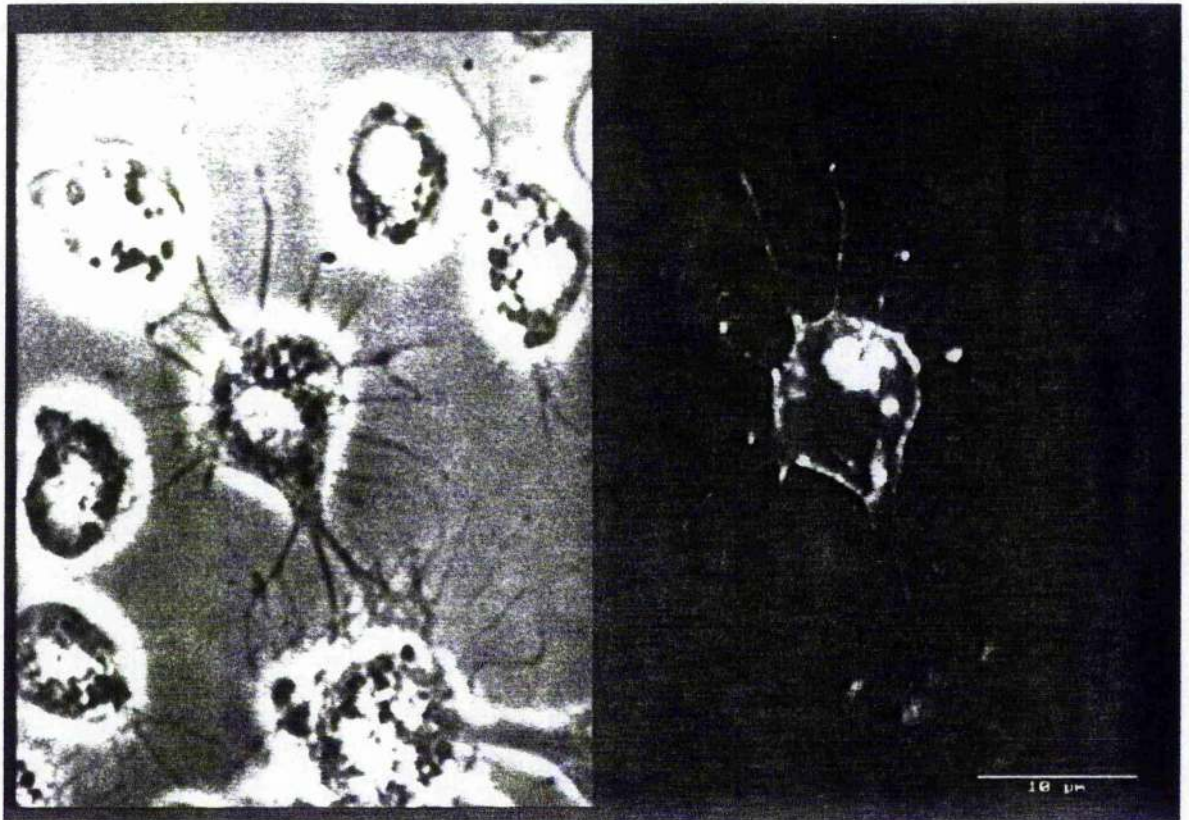
Figure 6.D   The first of these two images, 6.D.1, is a composite image representing four different optical sections, taken at 1 μm intervals using a confocal laser scanning microscope, of a small group of Cl. C9 cells stained for fasciclin III expression. The cell at upper left is extending a process to the cell at lower right, which seems to be undergoing division. Each cell has strong peripheral and vesicular staining. Figure 6.D.2 shows in greater detail one of the images ("Number 3 of 7") of this group of cells, which are at an early stage of aggregation. The brightly staining bodies or vesicles within the cells are marked with arrows.

Scale bars represent 2 μm
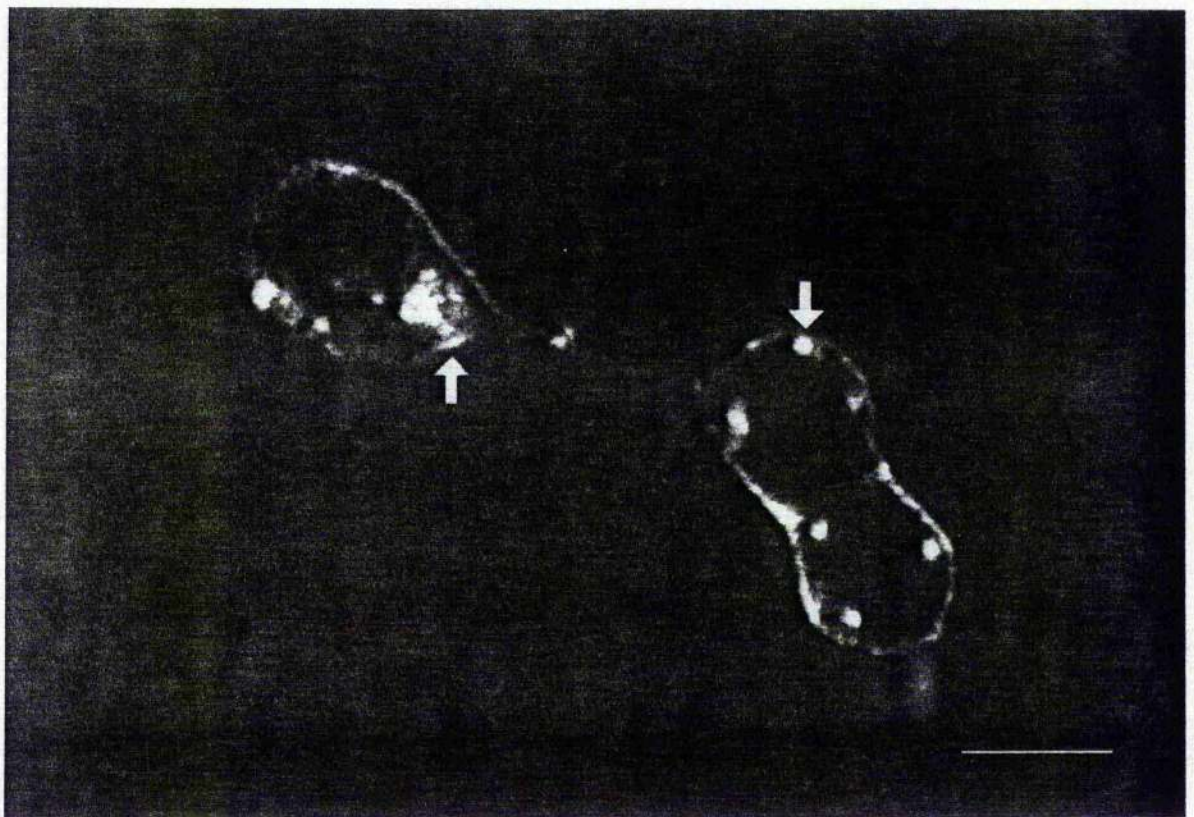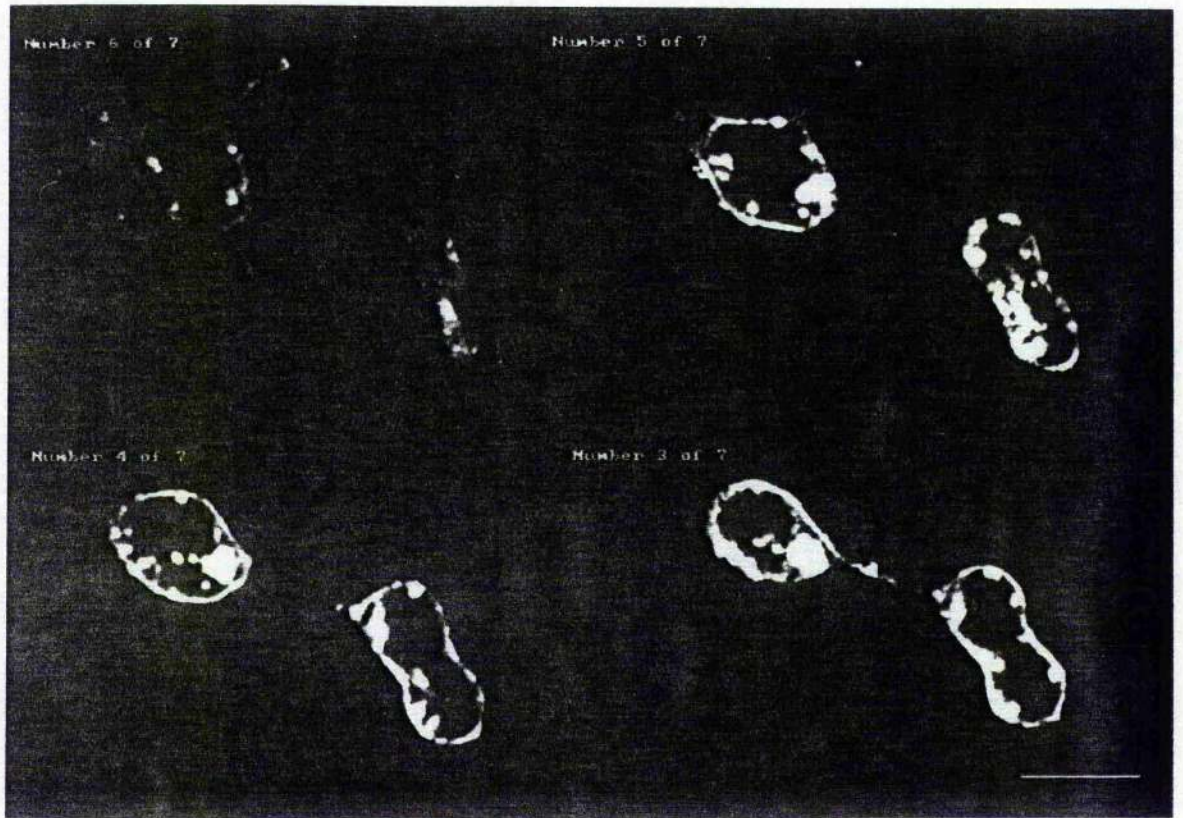
Figure 6.D

Figure 6.E   A cloned wing cell (W20C6 line) stained for fasciclin III. This arborised cell is staining very strongly for fasciclin III, which seems especially pronounced at the cell surface, in sub-cellular vesicles and on the large process-like extensions.

Scale bar represents 10 µm

Figure 6.F   Fasciclin III expression in aggregates of imaginal cells. This micrograph illustrates fasciclin III expression within a clump of Cl. C9 cells fixed 4 days after plating. Antigen expression is again evident at the surface of each cell but is especially strong at cell-cell contact sites

Scale bar represents 10 µm
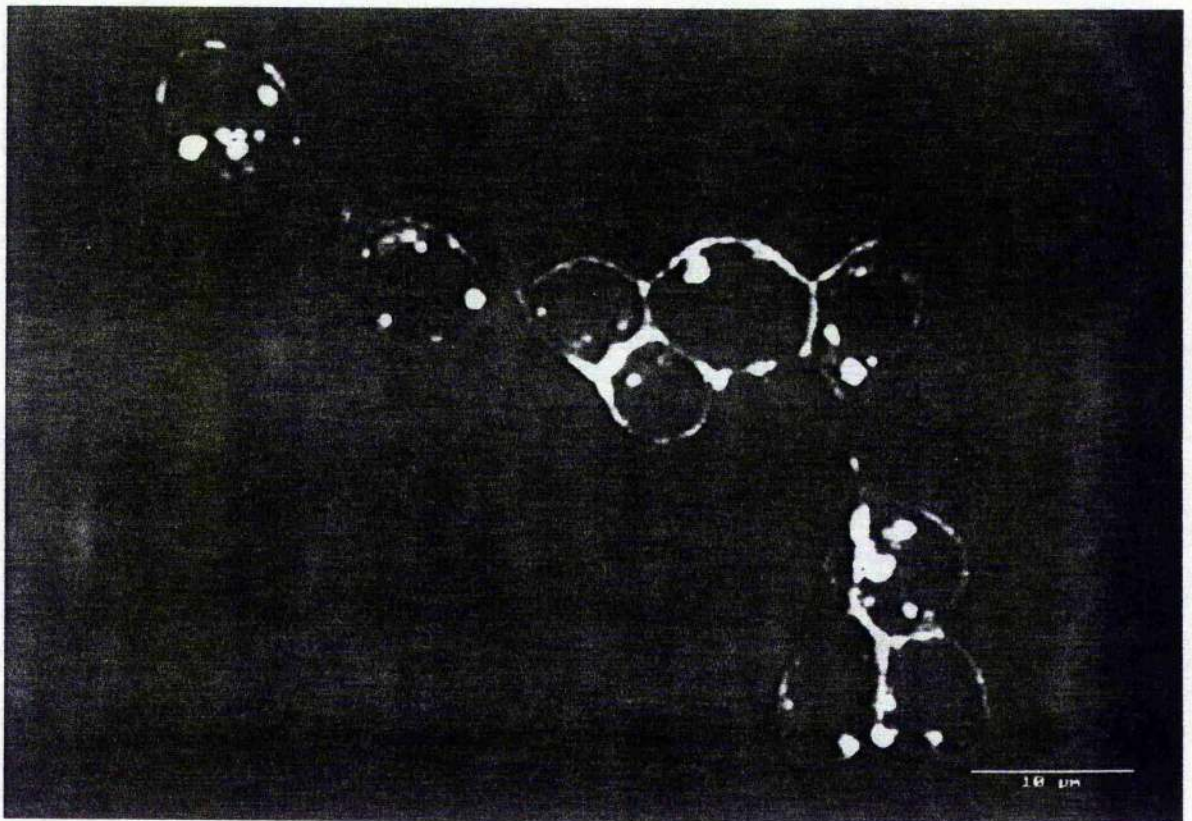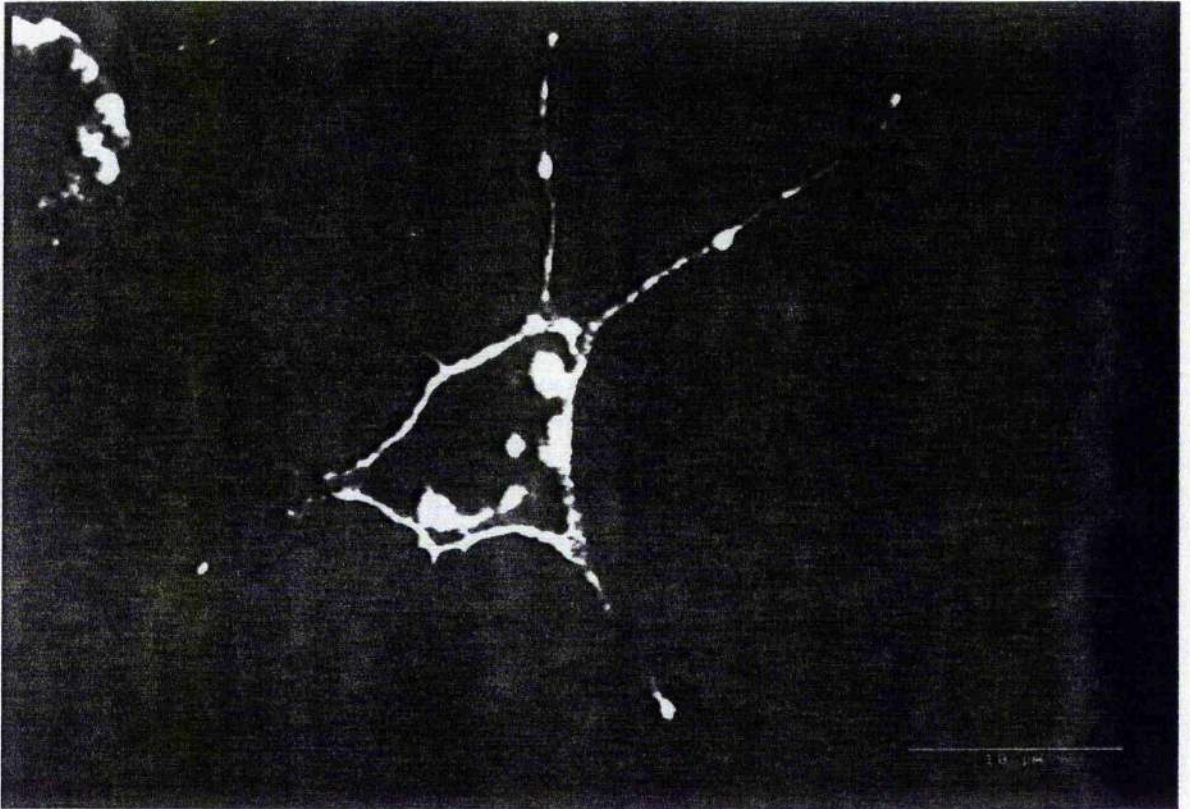
Fig. 6.G. Cloned wing cell line reaggregation: F3T
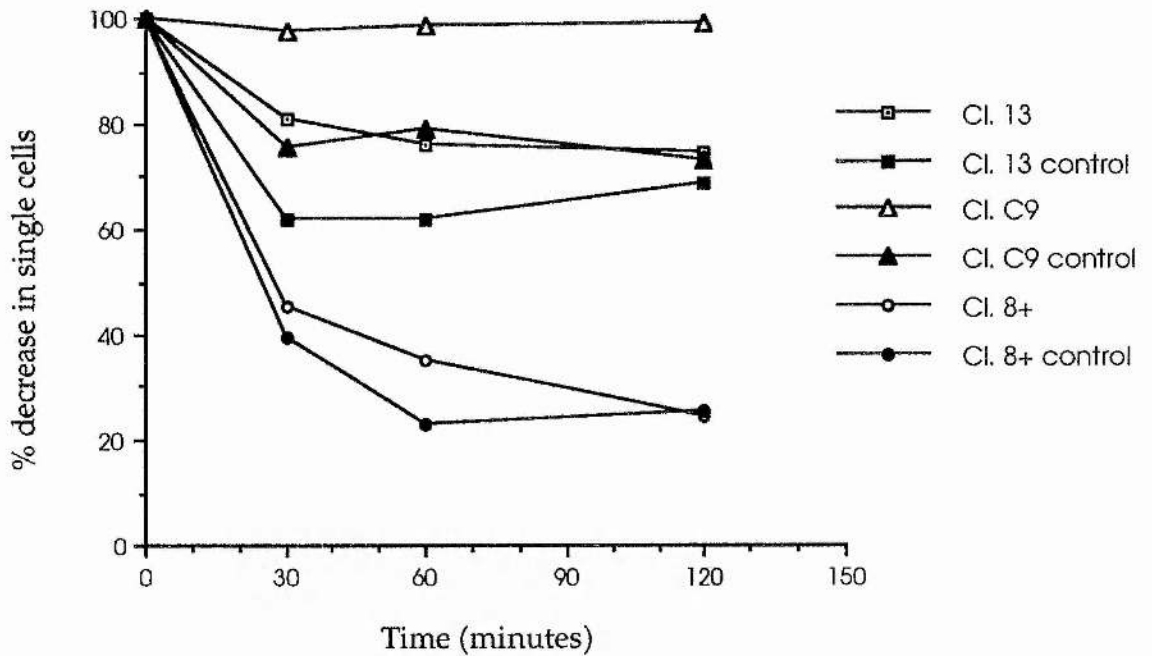
Figure 6.H. The rate of reaggregation of three imaginal disc cell lines, Cl. 13, Cl. C9 and Cl. 8+, either in the presence or absence of the anti-fasciclin III antibody, F3T. The only line where the rate of reaggregation seems to be significantly diminished by the presence of the antibody is Cl. C9, the other two reaggregating to more or less the same degree in both the control and experimental samples.

Figure 6.H   Effect of the anti-fasciclin III antibody, F3Ton long-term

imaginal cell aggregation. Phase contrast images of two cultures of Cl.

C9 cells pictured 5 days after plating, (1) being the control and (2) being

the test culture, containing an anti-fasciclin III antibody. There seems to

be little morphological variation between the two samples, both

apparently in the process of aggregation.

Scale bars represent 50 μm

Figure 6.H

1.
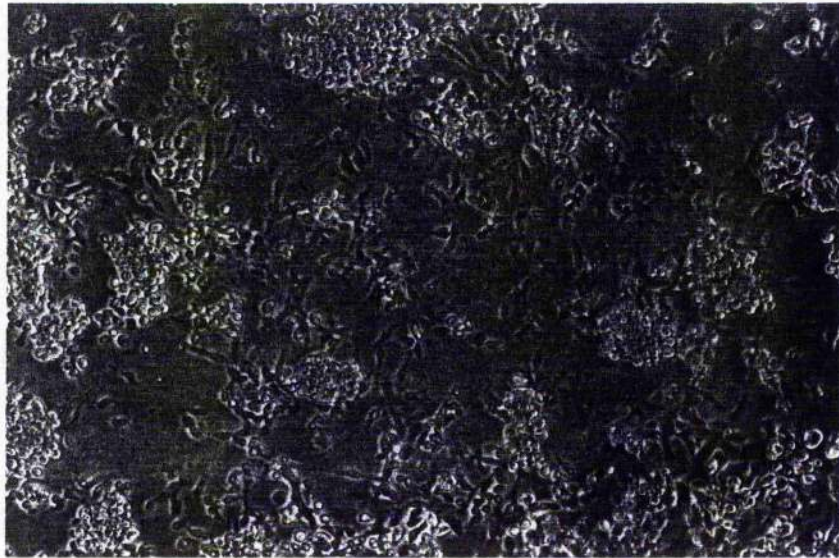
2.
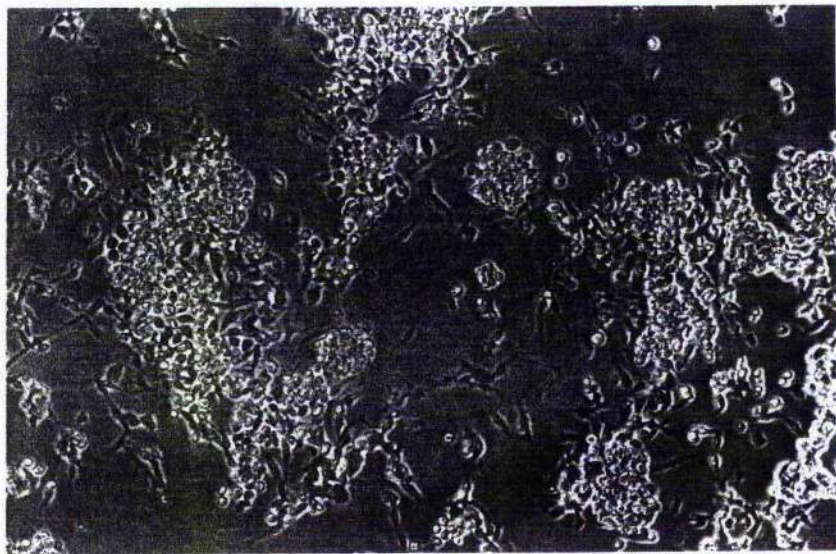
Figure 6.I   A Western blot of cell fractions probed by immunoprecipitation
using a monoclonal antibody to fasciclin III (2D5) and analysed on a 10%
polyacrylamide gel. The different samples probed here are: S2 cells
transfected with a baculovirus encoding fasciclin III (Lane 1), Cl. 8+
imaginal disc cells at 3 days growth (Lane 2) and grasshopper 3B11 cells
expressing fasciclin I (Lane 3). In Lane 2 some bands are seen which are
known to migrate between 65 and 80 kDa (asterisk). The 80 kDa long
form of the molecule can be seen in the positive control in Lane 1 (arrow).

Figure 6.J   The results of a Northern blot, probing Cl. 8+ cells for fasciclin
III-encoding RNA molecules. RNA was isolated from 2 day (Lane 2) and
7 day (Lane 3) Cl. 8+ cells which were then resolved on a 1%
formaldehyde agarose gel and were analysed using a probe generated
from a fasciclin III cDNA (Snow *et al.*, 1989). Weight markers are shown
in Lane 1. The 2.5 kb species corresponding to fasciclin III in Lane 4,
taken   from 9-13 hour embryo RNA and representing the positive
control, is   indicated with an arrow. No bands can be seen in either Lanes 2
or 3.
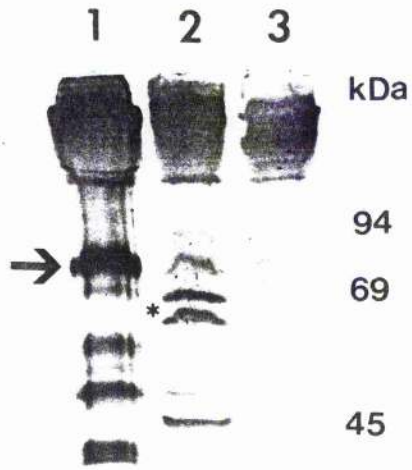
Figure 6.K   This figure is a confocal laser scanning micrograph of a field of Cl. C9 cells which have been stained with phalloidin-FITC to indicate, by fluorescence, sites of F-actin expression. These cells are at 2 days growth and are already beginning to move into aggregates, a small clump of cells being seen at lower left. Each cell is clearly stained for F-actin at sites near the cell surface, including a bipolar, fibroblast-like cell (f) which is spanned between two groups of cells.

Scale bar represents 10 μm

Figure 6.L   A confocal micrograph of an aggregate of Cl. 8+ cells, stained for f-actin expression as in Figure 6.K. These cells are at a more advanced stage of growth, being fixed 4 days after plating and have adhered together to form a chain of closely linked cells. F-actin expression is particularly evident at some sites of cell-cell contact (arrow).

Scale bar represents 10 μm
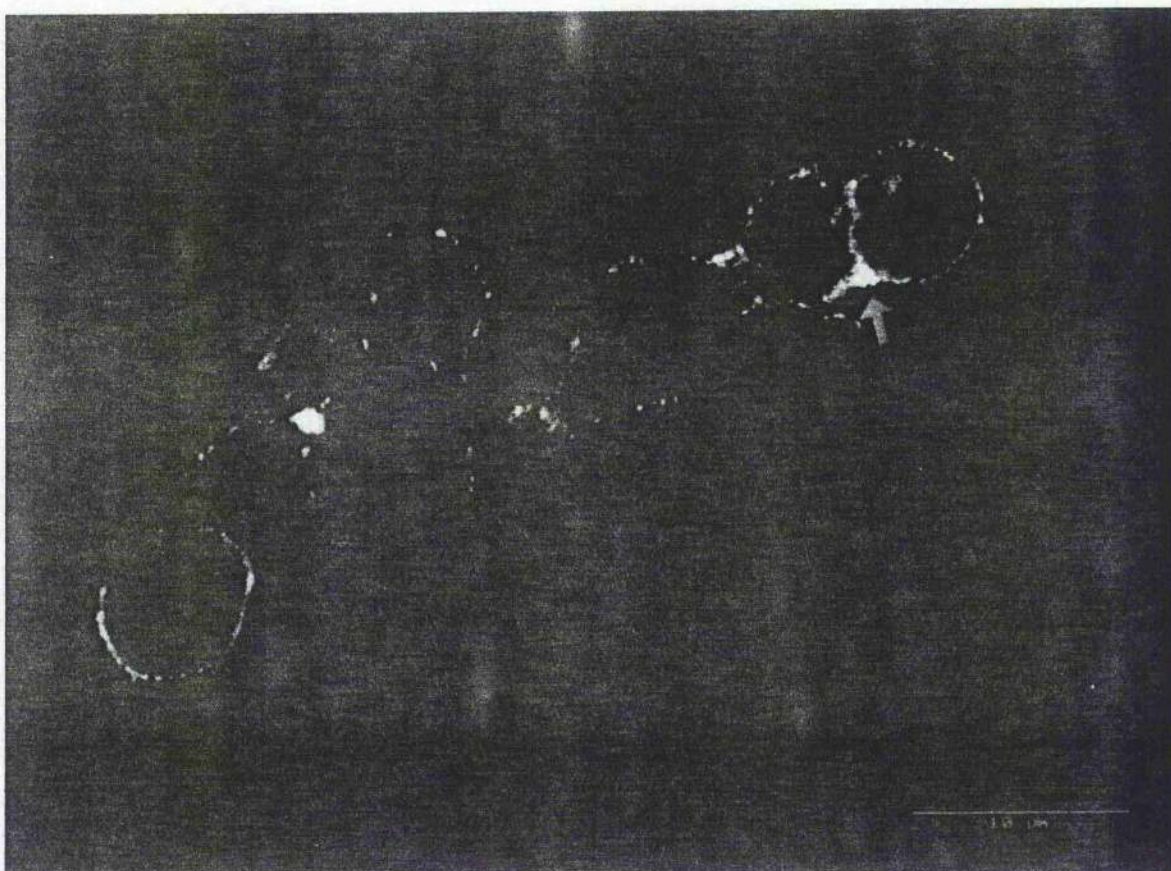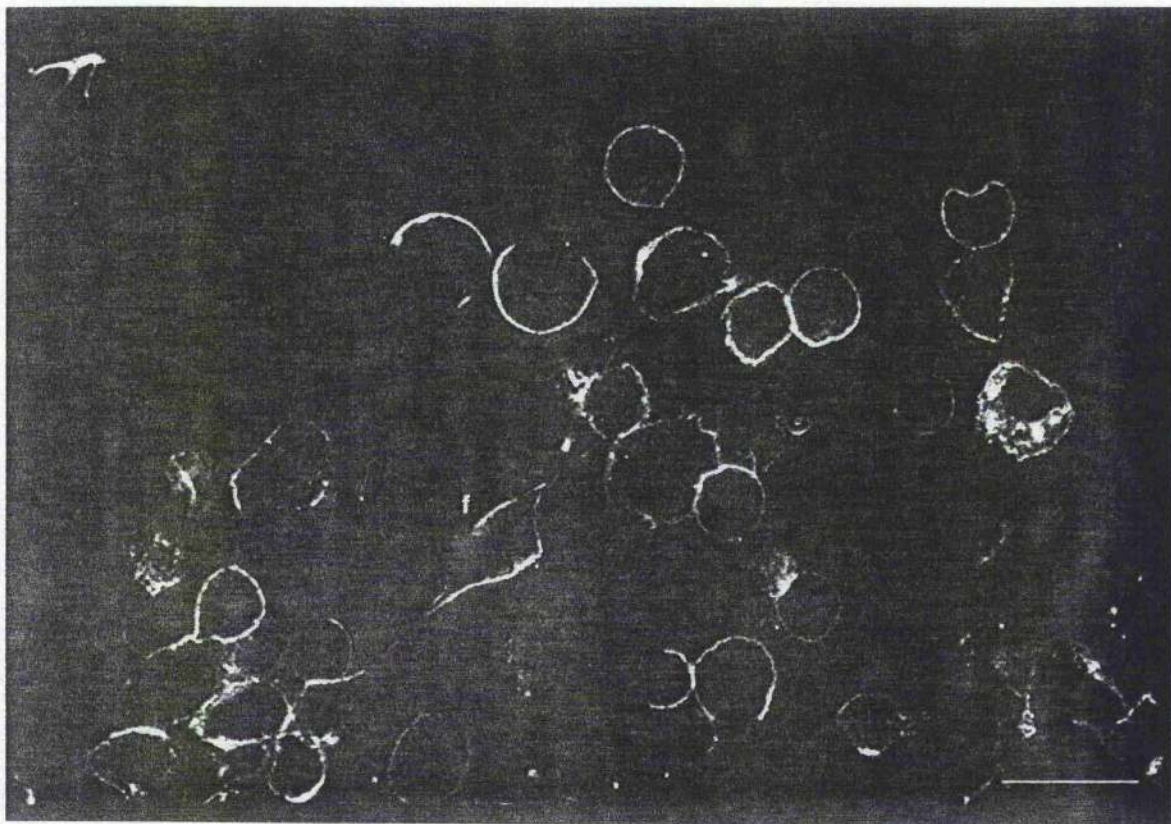
Figure 6.M   This micrograph details the expression of tubulin in a field of
aggregated Cl. 8+ cells fixed 7 days after plating. Tubulin expression in
cells throughout growth, *in vitro*, is ubiquitous. Here tubulin expression
is found in cells throughout each aggregate, where there seems to be a
complex network of cell processes.

Scale bar represents 50 µm

Figure 6.N   Phase contrast images of live aggregates of Cl. 8+ cells,
photographed at a similar stage of aggregation as in Figure 6.M. These
micrographs illustrate the standard morphology of aggregated imaginal
disc cells. The aggregates in Figure 6.N.1 are joined, as in Figures 6.M,
6.O and 6.P, by single cell processes which seem to extend over a finite
distance between proximal aggregates. Occasionally, elongated fascicles
of cells (Figure 6.N.2) can be seen bridging the gap between more distant
aggregates, especially in cultures grown on fibronectin (see Chapter 4).

Scale bar represents 15 µm
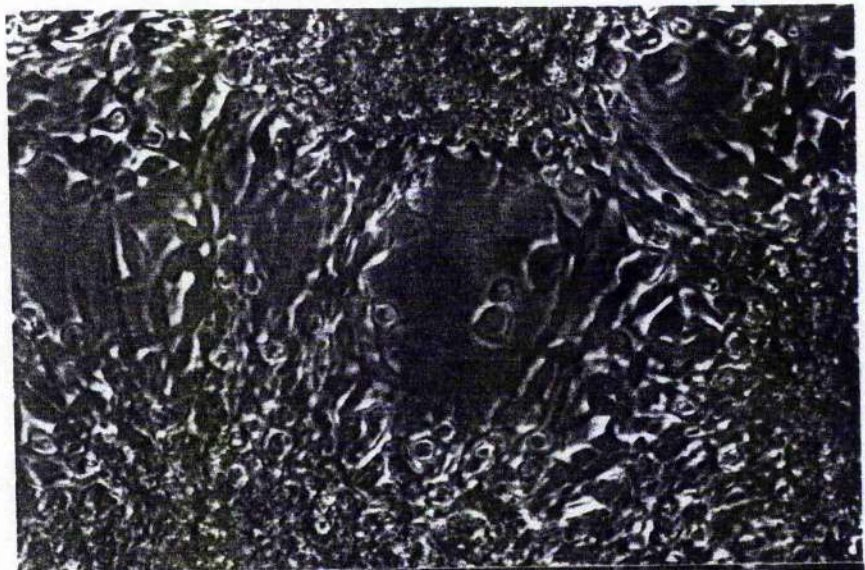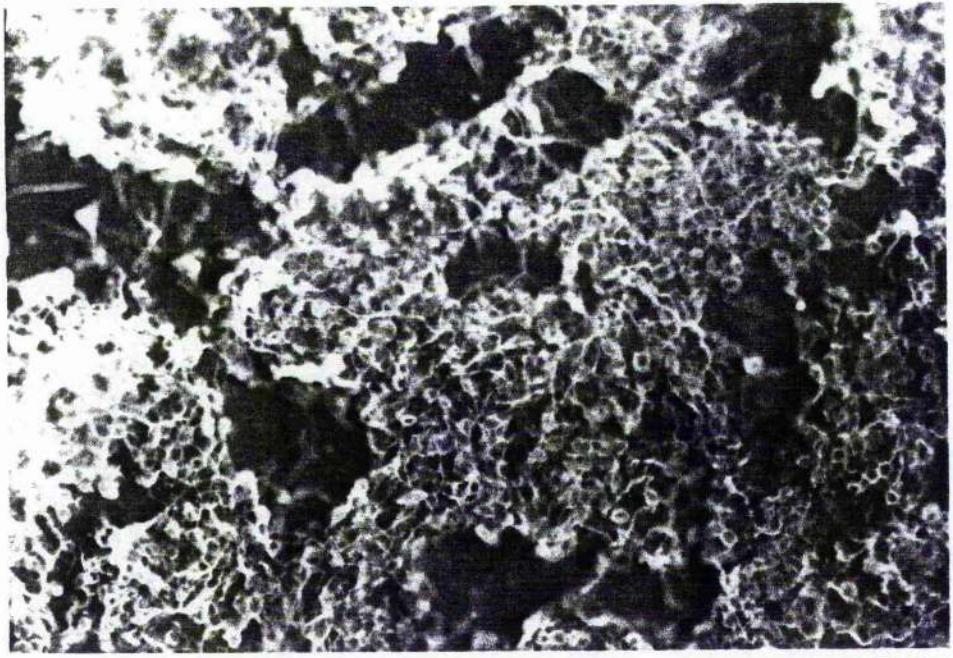
1.

2.

Figure 6.O   A fluorescent image of tubulin expression in a group of

aggregated   Cl. C9 cells (direct image, photographed using a 35 mm camera

attachment). Here, and in Figure 6.P, elongated cell processes (p) and

bipolar fibroblast-like cells (f) can be seen extending bridging the space

between aggregates.

Scale bar represents 20 μm

Figure 6.P   A digitised image of a detail of the field of cells pictured in Figure

4.O, recorded using confocal laser scanning microscopy. Again, as in

Figure 6.O, Fibroblast-like cells (filled arrows) and cell processes (open

arrows) are seen extending between the aggregates at top and bottom. A

few of these processes seem to be converging on a common point, and

some appear to be forming fascicles with other processes.

Scale bar represents 10 μm
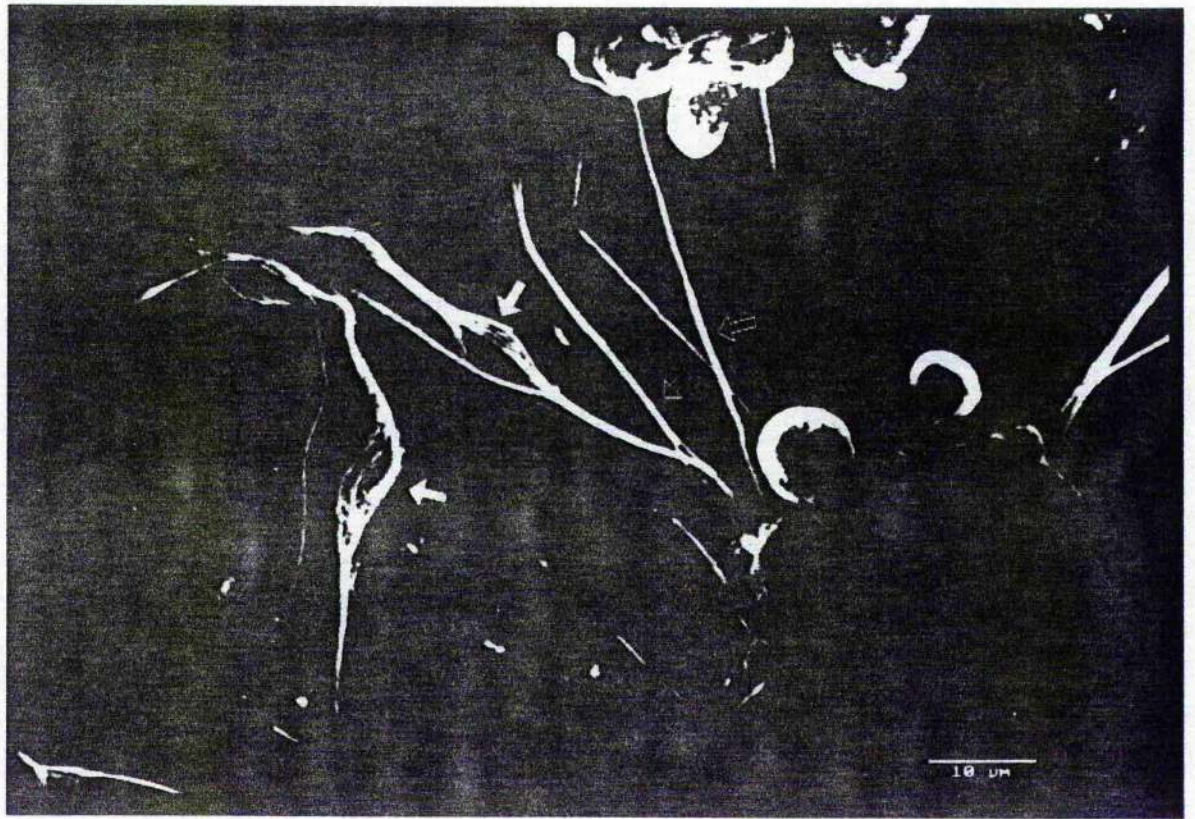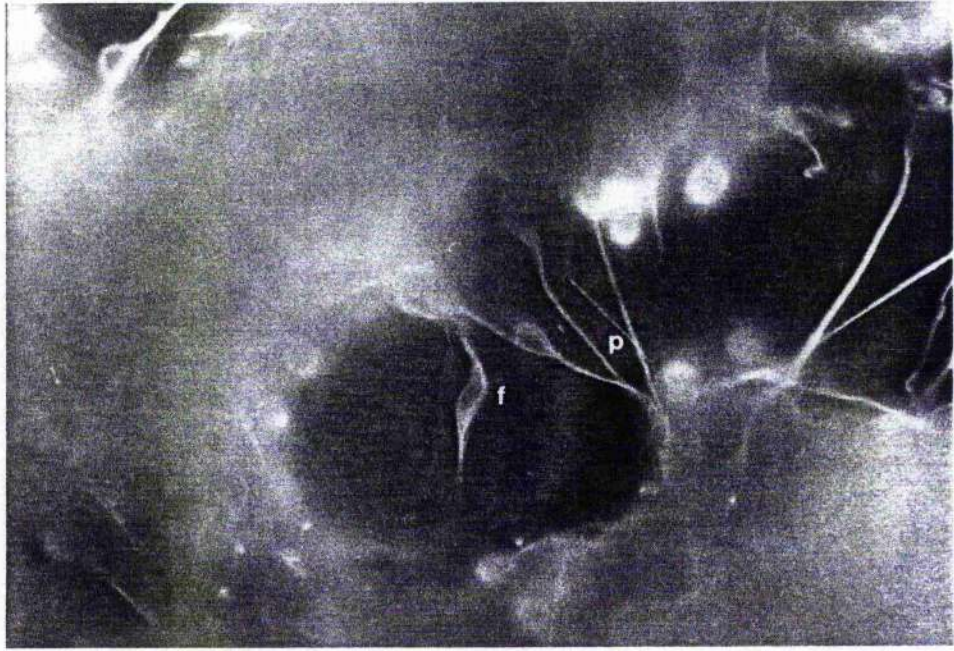
6.4   Discussion

6.4.1   Immunocytochemistry

On the evidence of the immunocytochemical studies it appears that fasciclin III is being expressed on the surface of all tested imaginal cell lines throughout morphogenesis with an accumulation of antigen at cell-cell contact sites. Staining was also seen in vesicles at points close to the cell surface. The antibodies used stained cells at each stage of morphogenesis, from single, migratory cells to cells that were found within aggregates.

The role of fasciclin III as a cell adhesion molecule (CAM), working in a homophillic, Ca^{2+} independent manner, was originally assigned on the evidence of studies whereby a plasmid encoding the molecule was transfected into normally non-adhesive S2 cells which then, on heat-shock activation, led to pronounced cell aggregation (Snow *et al.*, 1989). Thus, it seems possible that fasciclin III is being expressed on the surface of imaginal cells during morphogenesis *in vitro* to perform roles in cell migration, cell-cell adhesion and perhaps sorting-out. The observed staining patterns are suggestive that this molecule is involved in cell-cell adhesion with the antigen tending to accumulate at sites of cell-cell contact. Fasciclin III is present only on those single cells which appear to be 'dynamically' interacting with their environment, in cells which are either migrating or are extending cell processes. Those cells which are rounded up, prior to division, do not stain for fasciclin III, reinforcing the implication that this molecule functions in migration and adhesion.

6.4.2   Adhesion assays

Short and long term cell-cell adhesion assays were designed with the intention of measuring the rate of cell-cell adhesion whilst blocking fasciclin III binding sites with polyclonal antibodies. The results of these experiments lent some evidence to suggest that the presence of the fasciclin III polyclonal antibody, F3T, limited the rate of reaggregation in at least one cell line, Cl. C9, during the 2 hour assay, lending some evidence to suggest that this molecule is involved in cell-cell adhesion. There was little evidence, however, to suggest that the presence of fasciclin III antibodies had any deleterious effect on long term aggregation in the imaginal cell cultures tested, with samples of cells tending to aggregate the same either in the presence or absence of these antibodies.

However, the findings of such assays may be open to interpretation due to the existence of other, functioning, CAMs which are unblocked by these assays, namely the PS integrins (Leptin, 1987; Hynes 1992) and the cadherins (Takeichi, 1988) which are not accounted for and are known to function in imaginal cell growth *in vitro*. An informative assay of fasciclin IIIs adhesive capacity would require either the blocking or deletion of these molecules.

6.4.3   Western blotting

Western blotting studies provided strong evidence that fasciclin III, or some related form, was being expressed in these cells during aggregation. It may seem surprising that this isolated species, which migrates between 65 kDa and 80 kDa, did not seem to correspond to either the known long, 80 kDa, or short, 66 kDa, forms. However, fasciclin III is known to exist in at least four highly related forms of which the 80 kDa and 66 kDa forms have

been cloned and sequenced (Patel *et al.*, 1987). Therefore, it seems perfectly feasible that a new, variant, form exists in imaginal cells and was being recognised by the 2D5 monoclonal antibody due to the molecule sharing a common extracellular domain with the known forms. Whether these forms represented a distinct isoform generated by alternative splicing, or were due to some sort of post-translational modification such as glycosylation or phosphorylation was not clear at this stage. Fasciclin III is known to undergo alternative splicing to create multiple forms of each protein with either different cytoplasmic domains or different membrane attachments (Patel *et al.*, 1987; Snow *et al.*, 1989). There has been no evidence found to suggest that variable forms of the extracellular site of fasciclin III can be produced by alternative splicing (Grenningloh *et al.*, 1990), so it seems likely that if this species represents a variant form of fasciclin III then it may differ in its cytoplasmic domain.

6.4.4   Northern blotting

Interpretation of the Northern blotting experiments carried out to date served to contradict the findings of the earlier immunocytochemical and Western blot analysis. Results from these assays seemed to clearly indicate the presence of fasciclin III, or a related species, with suitable sites of expression and even a similar molecular weight. However, Northern blot analysis suggested that fasciclin III was not being expressed in the test cell lines at any stage of growth. This finding may be due to the nature of RNA analysis, which is notoriously difficult due to the chemical and biological lability of this molecule, compounded by the nucleolytic activity of a variety of resilient and ubiquitous ribonucleases (RNase) meaning that any samples of purified RNA, under a broad range of experimental conditions, can be rapidly and irrevocably lost. However, the technique employed for RNA isolation was

tested by successfully co-isolating RNA from S2 cells which had been transfected for fasciclin I expression and running in tandem with the imaginal samples but it is possible that isolation from imaginal cells could be more problematic than S2 cells perhaps due to the increased presence of RNases there.

Another interpretation of this result is that fasciclin III is indeed being expressed in these cells but at a very low rate of turnover at the cell surface, which would explain the lack of detected mRNA in these cells, and brings into question the functional importance of such a molecule if it is evidently transcribed so little.

The other, admittedly less welcome, interpretation of these findings is that fasciclin III is not expressed at all in the tested cell lines and that the findings of the immunocytochemical and Western blot experiments was due to the cross reaction of the fasciclin III antibodies with an unknown molecule of similar molecular weight and pattern of expression, unlikely though this might seem. Until further analyses can be carried out, the reason for this finding cannot be sufficiently answered. However, many new imaginal disc cell lines are in the process of being established (D. Cottam, pers. comm.) including the first lines from the eye-antennal disc, thus there may yet be found an imaginal cell line which unambiguously expresses fasciclin III, or a related form, *in vitro*.

6.4.5 Signal transduction

There was little evidence from these preliminary analyses to suggest that fasciclin III may be acting as a molecule involved in signal transduction in imaginal cells *in vitro*. Co-localisation studies suggest that f-actin is expressed at similar sites of fasciclin III expression, immediately adjacent to the cell surface. From this result it seems possible that a fasciclin III molecule

may act as an organising centre for an actin network and a site for transducing signals to the cell's interior, although such a structure may also signify a mechanical support as a focal contact either in adherent or single cells (Burridge *et al*, 1988).

The results from the fura-2 experiments indicated that bound fasciclin III, if present, when homophilically bound at the cell surface by a polyclonal antibody, did not elicit any detectable rise in the concentration of free intracellular Ca^{2+} which would suggest that it does not function as a signal transducer. However, there was an absence of a suitable positive control with which to calibrate the response of the test sample thus it is possible that there was a lack of experimental finesse which may have masked a true response, which was compounded by the preparatory nature of these novel assays.

There was also evidence from the radio-labelled immunoprecipitation studies (SPIT) that some cytoskeletal species, of weights around 94 and 120 kDa, were being co-precipitated with fasciclin III. The identity of these species are as yet, unknown but this technique may yet prove to be of use in future assays of this type.

Actin and tubulin expression: immunocytochemistry

Filamentous (f-) actin expression is very abundant in aggregating imaginal cells, being found at the periphery of each cell at a subcellular cortical site. In some groups of aggregating cells, this expression is particularly abundant at sites of cell-cell contact. This apparent accumulation of antigen at sites of cell-cell adhesion could be an indication that junctional complexes, perhaps lacking a complexity that may be seen *in vivo*, are forming there. These putative junctions may operate in a similar fashion to their homologous counterparts *in vivo*. This lack of complexity is reflected in the

ultrastructural studies of imaginal cells *in vitro* carried out to date, as mentioned in Chapter 5.

Microtubules are found in vast quantities in imaginal disc cells throughout aggregation, and especially in the extended cell processes that are seen to link adjacent aggregates. Single cell processes are seen to extend between aggregates that are fairly proximal, whereas aggregates that are more removed tend to have multicellular fascicles joining them, suggesting that cells can only extend to a critical length to contact another aggregates otherwise they have to form a scaffold with their fellow cells to form an inter-aggregate network. Cells that come into contact with their target seem to form an established connection, marked by an accumulation of cytoskeleton and cell adhesion molecules, whereas processes that fail to contact their targets appear to retract back into the cell body, suggesting that single cell processes can extend only over a finite length.

This abundance of both actin and microtubules in imaginal cells *in vitro* strongly suggests a role for these cytoskeletal elements in cell movement and aggregation. It is entirely feasible to contemplate that f-actin could directly interact with tubulin, through microtubule associated proteins (MAPs) within the cell and integrins at the cell surface, in a force transducing role which may bring about cell movement (Griffith and Pollard, 1978; Bogaert *et al.*, 1987; Wilcox *et al.*, 1989). The possibility of actin and tubulin having a role forming a putative tension-transmitting junction has been discussed in Chapter 4 in comparison to the transalar arrays that are found during pupal wing morphogenesis (Mogensen and Tucker, 1988).

The growing aggregates themselves seem to consist of a rich network of processes which stain strongly for tubulin, which may be functioning in directing the formation of close intercellular contact between the cells and maintaining the cohesion of the aggregate. The homology in structure and function between imaginal cell process *in vitro* and epidermal feet *in vivo*

(Locke and Huie, 1981; Locke 1985), acknowledged in Chapter 3, may therefore be particularly apt.

The interpretation of the findings of Chapters 3 and 4 was that imaginal cells *in vitro*, on being plated out as a monolayer of single cells, undergo a period of cell-substrate interaction as the cells adhere and migrate on the tissue culture surface. Later in passage, there appears to be a subtle shift in the imaginal cells adhesive strategy, as the cells tend to adhere more to their neighbours than to the substratum. The ubiquitousness of both f-actin and tubulin in these cells throughout this process strongly implicates their funtional involvement at all stages of imaginal cell growth.

It might be informative to treat imaginal cells during aggregation with drugs which are known to disrupt the polymerisation of both actin and tubulin, such as phalloidin and colchicine respectively, to test if these molecules have specific roles in driving aggregation. Thus, aggregation *in vitro* may be a subtle temporal shift between "traction-induced" aggregation in the early stages followed by cell-cell induced later on.

7.1   Introduction

This chapter is a brief account of two unrelated sets of experiments that were carried out which centred on the use of imaginal disc cell lines. The aim of the first set of experiments was to observe the morphological response, if any, of imaginal disc cells to a rise in intracellular cyclic AMP (cAMP). The second set of experiments was designed to determine the response of imaginal disc cells when presented with the larval serum protein, arylphorin, in the culture medium, specifically to test if this protein could be endocytosed or not.

7.1.2   Morphological response of animal cells to an intracellular rise in cAMP

Many groups of animal cells in culture are known to undergo pronounced shape changes in response to an intracellular rise in cyclic AMP (cAMP) which might affect various processes such as membrane movements, signal transduction and intracellular transport. These include fibroblasts, smooth muscle cells, osteoblasts, astrocytes and epithelial cells (for review, see: Edwards *et al.*, 1988). These cell shape changes can be bizarre, with the cells appearing as either stellate or arborised.

cAMP induced cell outgrowths can be similar to the neurites seen extending from neuronal cells. Lamb *et al.*, (1988) observed that by raising the intracellular cAMP level, fibroblasts adopted a rounded morphology, with a concomitant loss of actin microfilament bundles. Mouse neuroblastoma cells extend neurites and cease division when intracellular cAMP was raised in cells incubated in serum free medium (Miller and Ruddle, 1974) and rat neonatal sympathetic and embryonic neurones respond to a rise in cAMP again by neurite extension as well as long-term cell survival. Dog thyroid epithelial cells respond to the intracellular rise in cAMP by cell retraction and

rounding up, caused by the disruption of actin microfilaments (Roger *et al.*, 1988). Perhaps one of the most marked morphological change induced by a rise in intracellular cAMP is seen in cultured iris epithelial cells of the adult newt, *Notophthalmus viridescens*. Here, the cells undergo a pronounced cell shape change, known as 'stellation', the broad undulating membrane of the cell being converted to branching strands (Ortiz *et al.*, 1973).

Cells from the established lines, BHK21, NIH-3T3 and CHO are also known to undergo bizarre cell shape changes when in the absence of serum and when cAMP is elevated (Edwards *et al.*, 1993). Normally, both BHK21 and NIH-3T3 are fibroblastic in shape whereas CHO are epithelioid, but adopts a fibroblastic-like phenotype when cAMP levels are increased (Freshney, 1987). The arborisation and stellation seen in these cultures was reversible by the addition of vertebrate serum (FBS) to the culture medium (Edwards *et al.*, 1993).

7.1.3   Insect Larval Serum Proteins

Holometabolous insect larvae are known to synthesise massive amounts of proteins referred to as "larval serum proteins" (LSPs) or "storage proteins", within fat body cells that accumulate within the larval haemocoel, notably during the last larval instar prior to metamorphosis (for general introductions see: Levenbook, 1983; Riddiford and Law, 1983; Patrinou-Georgoula *et al.*, 1983). Some of these proteins have a high content of aromatic amino acids, in the form of tyrosine and phenylalanine, and have, thus, collectively become known as the arylphorins (Telfer *et al.*, 1983). The arylphorins, which constitute the main fraction of late larval haemolymph proteins (up to 80% in *Calliphora* larvae) but which are absent from the adult form, are thought to function primarily as storage proteins and as a source of

amino acids and energy for the synthesis of new proteins during metamorphosis (Wyatt and Pan, 1978; Schenkel *et al.*, 1983).

The first arylphorin to be characterised was found in the blowfly, *Calliphora vicina* and was thus named calliphorin. Calliphorin is a 500 kDa hexamer composed of six closely related 78 kDa polypeptides which are encoded by a large multigene family, which are tandemly arranged on chromosome 2 (Markl *et al.*, 1992; Scheller, 1983). This multiplicity and clustering of arylphorin genes is not reflected in *Drosophila* where the arylphorin, LSP-1, composed of α, β and γ subunits (Lepesant *et al.*, 1982), is encoded by only three related and unlinked genes. Similar proteins are also known to accumulate in the haemocoels of hemimetabolous insects prior to moulting.

Other than acting as storage proteins, arylphorins are believed to perform specific roles in development (for review, see: Scheller, 1983; Peter and Scheller, 1991). There is known to be a close correlation between the titres of arylphorins and developmental events. Arylphorins are ubiquitous in the tissues of dipteran insects during the different stages of growth, from the first larval instar to the adult. The synthesis of arylphorins commences during the last larval instar, and they are secreted by the fat body into the haemolymph at a high concentration. This titre of arylphorin reaches a maximum during the feeding phase but then declines during the wandering phase and pupariation, the lowest titre being recorded during the adult stage. The arylphorins are removed from the haemolymph back to the fat body cells, by receptor mediated endocytosis (RME), where they are stored in the form of large protein granules (Peter and Scheller, 1991; Wyatt and Pan, 1978).

20-HE is known to have some effects on arylphorin expression and endocytosis, but the exact relationship remains obscure. In *Calliphora*, activation of the arylphorin genes begins at the beginning of the last larval instar but is not affected by 20-HE (K. Scheller, pers. comm.). However, the

termination of gene activity and the end of the translation of stable arylphorin mRNAs is known to conicide with a small rise in ecdysteroid titre at the end of the feeding stage and the there seems to be a causative link here (Burmester *et al.*, 1995). 20-HE is clearly involved in the receptor mediated uptake of arylphorin by larval fat body binding proteins both *in vitro* and *in vivo* (Burmester and Scheller, 1995; Burmester *et al.*, 1995).

Arylphorins are also known to bind to ecdysteroids; a role which may have functional implications during development. Enderle *et al.*, (1983) demonstrated that calliphorin, both *in vitro* and *in vivo*, could act as a carrier for ecdysteroids. Here, the protein was demonstrated to bind to these steroid hormones with a low affinity, although the huge abundance of this protein (e.g. constituting 7 mg of a *Calliphora* individual of a total body weight of 120 mg) would compensate for this (Wyatt and Pan, 1978). The arylphorins are believed to function in insect maturation by binding to ecdysteroids and removing them from the haemolymph during stages of development that require low titres of these hormones. Experiments centring on the use of viable *Drosophila* mutants, deficient in the expression of LSP-1, suggested that the removal of ecdysteroids from a mid-pupal peak by LSPs was necessary for the maturation of male and female flies (Roberts, 1987).

One further role which the arylphorins are known to play in dipteran insects is one of cuticle formation and sclerotisation (König *et al.*, 1986; Peter and Scheller, 1990). This assumption has been substantiated experimentally through the use of radiolabelled arylphorins, cuticle immunochemistry which indicated the involvement of arylphorin in the cuticle (König *et al.*, 1986) and by the crosslinking of arylphorin, by sclerotisation elements, *in vitro* (Agrawal and Scheller, 1987). Such a function for arylphorin originally seemed likely due to the high content of aromatic amino acids in these proteins. The *Calliphora* arylphorins are known to be transported through the epidermal cells and are incorporated into the cuticle where they interact with cuticle

proteins, drive the sclerotisation process and function as an essential structural component. This incorporation of calliphorin to the cuticle of both the integument and the trachae of *Calliphora* larvae was significantly enhanced by the presence of 20-HE (König *et al.*, 1986; Agrawal and Scheller, 1987).

Imaginal discs of *Calliphora* and *Drosophila* are capable of re-absorbing arylphorin, furthermore, there is evidence to suggest that three arylphorin-binding proteins exist in the wing and leg imaginal discs, (Klaus Scheller, pers. comm.). The involvement of the ecdysteroid hormones in influencing the uptake of arylphorins into imaginal and fat body tissues from the haemplymph is now well documented, but the exact molecular relationship between the two remain unclear.

Most imaginal disc cell lines *in vitro* have displayed marked morphological and physiological responses to 20-HE, manifested as senescence, cell-death and chitin synthesis. These cell lines were also found to express functional ecdysteroid receptors much as they would *in vivo* (Peel and Milner, 1992b). Thus, it would be reasonable to assume that imaginal cells *in vitro* may be able to actively take-up arylphorin which may then be subsequently incorporated into secreted cuticle. In this way one would then have a suitable model for studying receptor mediated endocytosis *in vitro*, with which one could further elucidate modes of protein uptake and arylphorin incorporation into the cuticle at the molecular level.

It was with this in mind that I began a series of experiments, initially as a short collaboration with Professor Klaus Scheller (of the Theodr Boveri Institute, Universty of Würzburg), to determine if imaginal cells *in vitro* could actively endocytose exogenous arylphorin, if this process was dependent on ecdysteroid hormones and, if so, how this was accomplished.

7.2 Materials and Methods

7.2.1 Morphological response of imaginal disc cell lines to rise in cAMP

The cells that were used in these assays were taken from the cloned wing lines, Cl. 8+, Cl. C9 and W20C6 along with the uncloned leg cell line L127D6. Cells taken from 3 day, proliferative, cultures were given a routine passage and were pelleted twice in serum free medium (MM3). The cells were then plated as single cells onto 24-well plates (Nunc). The substrates used for cell growth in these assays were either tissue culture plastic or tissue culture plastic which had been coated with human fibronectin, equivalent to 25 µg/well (each well being 1.76 cm^2 in diameter) using the procedure detailed in the methods section (4.2) of Chapter 4. The cells were plated on these surfaces at a density of 5 x 10^5 cells/well, in 1 ml of MM3 containing 1% bovine serum albumin (BSA). The cells were then incubated in a CO$_2$ humidified incubator set at 25°C for 1 hour.

Once the cells had adhered to their respective substrates, half were treated as experimental samples and the other half were treated as negative controls for an increase in intracellular cAMP. The experimental cultures were incubated in MM3 medium plus 1% BSA which contained the cAMP protease inhibitor, 3-isobutyl-1-methylxanthine (IBMX: Sigma) and N^6, 2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (dibutyryl cAMP: Sigma) both at a concentration of 0.4 mM and latterly at a concentration of 0.8 mM.

The cultures were then monitored and photographed at timed intervals after culture initiation using a Leitz diavert inverted microscope operating phase-contrast optics.

7.2.2   Arylphorin: Immunocytochemistry

Cells to be fixed for antibody staining were grown at a seeding density of 5×10^5 cells/ml in 12 ml of medium on sterile glass slides in 90 mm Petri dishes. For this study the cells were to be stained to test for either the presence of endogenous arylphorin and/or for the uptake of arylphorin from the culture medium over 20 minute and 1 hour time courses. For the test of endogenous arylphorin staining no 20-HE was present in the culture medium, however for the exogenous uptake test 20-HE was added at a concentration of 5 ng/ml (equivalent to half of the amount needed to mediate a full morphological and physiological effect on the cells according to the findings of Peel and Milner [1992b]).

The slides, with the cells adhering to them, were removed from the Petri dishes after 2 days of growth and were rinsed gently in PBS plus 1% goat serum for 5 minutes. The cells were then fixed in methanol at 0°C for 5 minutes, followed by a rinse for 30 minutes and placed in a blocking solution of 10% goat serum in PBS for 1 hour at 20°C. The cells were then rinsed and incubated overnight, at 4°C (or for 3 hours at 20°C) in a solution of the primary antibody, anti-rabbit-calliphorin-IgG, which was diluted 1 : 200 in 1% goat serum in PBS (giving a final concentration of 2.5 µg of protein per ml of PBS) or an equivalent amount of pre-immune serum for the controls. Next, these cells were rinsed as before, for 1 hour, then blocked for 1 hour in a 10% goat serum in PBS solution, followed by an incubation for 1 hour, at 20°C, in a 1 : 200 solution of biotinylated ant-rabbit IgG (Sigma) diluted in 1% goat serum in PBS, to give a final concentration of 5 µg antibody per ml of PBS. The cells were again rinsed and blocked and incubated for 1 hour, at 20°C, in a 1 : 250 solution of avidin-FITC (Sigma) diluted in 1% goat serum in PBS to give a final concentration of 5 µg protein per ml of PBS.

The cells were given a final 30 minute rinse on a shaker, and were mounted in a solution containing 70% glycerol, 30% 0.1M TRIS, (Ph. 9) and 2% propyl-gallate (coverslips were added and were sealed with dental wax). Each slide of cells was observed using a Leitz microscope with a fluorescent light source and were photographed with a 35 mm camera attachment.

7.2.2   Western blotting

Cells were grown for four days at a density of 6×10^5 cells/ml in 5 ml of CSM in Petri dishes. In these dishes, at timed intervals prior to sample preparation, the cells were exposed to varying levels of exogenous arylphorin either in the presence or absence of 20-HE (at a concentration of either 5 or 10 ng/ml). 100 µg of lyophilised, labelled protein was dissolved into 5 ml of medium (MM3 with 2% FBS) in 50 mm Petri dishes at the start of each assay (equivalent to 1×10^5 cpm/dish for the ^{35}S-linked probe or 8.8×10^3 cpm for ^{3}H-arylphorin. Both probes were a gift from K. Scheller).

The cells were initially rinsed in 1 ml of D= and were then scraped from the dish using a "policeman". The cells were then spun in a Beckman centrifuge to form a loose pellet and were resuspended in 1 ml of D=. Next, the samples were equilibrated so that each tube contained the same number of cells. Cell densities were determined by using a haemocytometer and protein concentrations were determined by using the Bradford method (see General Methods section, Chapter 2) in this way each sample was equilibrated for uniform loading of protein onto the resolving gel. 3×10^6 cells were taken from each sample and were added to 0.5 ml of CSM which was then sonicated for 10 seconds. To this lysate, 25 µl of 0.5 % SDS was added and kept on ice. Protein from these samples were extracted by adding 0.5 ml of phenol-chloroform-isoamyl alcohol mix (ratioed 25 : 24 : 1 respectively) to the sample in an Eppendorf tube. The tubes were then vortexed briefly to

emulsify the fractions and then spun for 2 minutes at 13,500 rpm in a Microcentaur centrifuge. The heavier organic phase, which contained the proteins, was then removed and diluted in 3 volumes of ethanol, which was then placed at -20°C for 3 hours. The precipitated proteins were then pelleted in a centrifuge (13,500 rpm for 5 minutes) and were solubilised in 150 μl of double-strength Sample Buffer (100 mM Tris.Cl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 10% mercaptoethanol and 0.2% bromophenol blue) and were boiled for 3 minutes.

The samples were loaded onto a 7.5% SDS polyacrylamide gel (see General Methods section, Chapter 2) and were resolved by electrophoresis (powered by a Mini-Protean II dual slab cell, Biorad). The proteins were then blotted onto a nitrocellulose membrane (PVDF membranes, Biorad) using the Mini-Trans-blot module of the same electrophoretic apparatus (see General Methods section for buffers).

After blotting, the nitrocellulose membrane was removed from the electrophoresis cell and each lane was cut out as an individual strip and separately washed in TTBS buffer (0.1% Tween 20, 100 mM Tris, 0.9% NaCl: pH 7.5) and was blocked overnight in this solution plus 1% non-fat dried milk at 4°C on a shaker. The membranes were rinsed in 3 to 4 changes of TTBS buffer followed by incubation for 30 minutes at room temperature in a solution of the primary antibody (anti-rabbit-calliphorin-IgG diluted 1/500 in TTBS buffer: giving a working concentration of 5 μg/ml). The membranes were rinsed, as before, in TTBS buffer and were then added to a solution of the secondary antibody (biotinylated anti-rabbit-IgG (Sigma) diluted 1/200 in TTBS buffer: giving a working concentration 5 μg/ml). Next, the membranes were rinsed in TTBS and transferred to a solution of Vectastain ABC reagent, an avidin based detection reagent made up in TTBS, for 30 minutes at room temperature on a shaker. The membranes were rinsed again and transferred to a 3, 3'-diaminobenzidine (DAB) substrate solution for peroxidase detection:

consisting of 200 µl DAB stock solution (40 mg/ml diaminobenzidine tetrahydrochloride) plus 30 µl of 3% H_2O_2 (freshly prepared from a 30% stock) in 10 mls of 100 mM Tris, pH 7.5. The reagent was washed off with fresh water when the bands had darkened sufficiently, stopping the reaction and the membranes were left to air-dry. Pre-stained molecular weight markers (Sigma) were then used to determine the weights of the bands seen on the experimental lanes.

7.2.3 Autoradiography

Another technique employed to assay for the active uptake of arylphorin from the culture medium and to determine whether or not this process was dependent on the presence of ecdysteroid hormones, was to incubate the cells with varying titres of radiolabelled arylphorin and to measure, by recording scintillation activity, rates of incorporation of the probe into the cells.

In early assays, radiolabelled arylphorin probes (linked to ^{35}S and ^{3}H) were added to the culture medium of various cell lines at different stages of growth either in the presence or absence of 20-HE. Latterly, competitive binding assays were set up whereby radiolabelled probes were co-incubated with an excess of unlabelled competing protein in the supernatant to determine if arylphorin was being actively endocytosed by the cells. Another approach to assay for the uptake of arylphorin was to co-incubate the radiolabelled probes with antibodies directed against arylphorin, to try and block the specific uptake of the protein.

Different imaginal disc cell lines, usually at 2 or 7 days of growth, were incubated with varying levels of radiolabelled arylphorin (partially purified *Calliphora* arylphorin which was labelled, *in vivo*, with ^{3}H-phenylalanine, the incorporated activity of which was 88 cpm/µg protein or with ^{35}S-methionine

at 1×10^3 cpm/µg, which were added to 50 mm Petri dishes at a concentration according to the method used for Western blotting) as a preliminary experiment to determine which cell line, if any, would act as a suitable candidate for further studies. Unlabelled purified calliphorin, isolated from adult *Calliphora* haemolymph (supplied as a gift from K. Scheller), was used in competitive binding studies at a concentration of 2 mg/ml (or 10 mg/dish; 100 times more concentrate than the probe). Anti-calliphorin IgG antibodies were added at an equivalent concentration of 10 µg/ml (or 50 µg/dish) in relevant blocking studies.

Cells were harvested after incubation with the particular test medium at 25°C, MM3 medium plus 2% FBS which contained radioactive probe and/or competing protein and were rinsed and pelleted as usual. The pellets, in 1 ml centrifuge tubes (Eppendorf), were thoroughly washed twice in PBS (pH 7.4) and were lysed by the addition of 100 µl 0.5% SDS in PBS. 10 µl of each sample was then taken for protein determination (using the Bradford method, see General methods section, Chapter 2), each sample then being standardised to contain an equivalent amount of protein. The protein in each sample was precipitated by incubating each with 500 µl of trichloroacetic acid (TCA: Sigma) for 30 minutes on ice. Next, the samples were warmed to 25°C and were adsorbed to cellulose filter paper (Whatman) which were rinsed three times in an excess of 5% TCA and once in ethanol. The filters were then dried and placed in scintillation vials to which 2 ml of a scintillant cocktail (Sigma, Cat. No: S-3898) was added. The samples were then counted for incorporated radioactivity using a liquid scintillation analyser (Model 1600 TR, Packard).

7.3   Results

7.3.1   Morphological response of imaginal disc cells to rise in cAMP *in vitro*

In each assay undertaken, and with each cell line tested, there was no observable morphological change in the shape of imaginal disc epithelial cells when the intracellular concentration of cAMP was increased. Figure 7.A illustrates the typical response of an imaginal disc cell line when incubated in MM3 plus 1% BSA to which was added dibutyryl cAMP and the cAMP protease inhibitor, IBMX (the concentration of both being 0.8mM). Figure 7.A.1 shows Cl. 8+ cells 24 hours after plating in MM3, appearing phase dark and rounded whereas Figure 7.A.2 shows Cl. 8+ cells, at a similar stage, plus cAMP and IBMX, with little obvious morphological variation being seen between the two.

Some of the cells in the experimental culture, pictured in Figure 7.A.2 and detailed in Figure 7.A.3, do appear as if they may be undergoing some alteration in their shape, becoming either arborised or stellate. However, this shape change seemed to be restricted to a minority of the cells in these cultures and was also seen in some imaginal cells that had been grown under control conditions. The cell pictured in Figure 7.B, from an earlier study, was grown on a human fibronectin substrate, similarly in MM3 with 1% BSA, and was stained for f-actin with phalloidin-FITC (using the protocol detailed in Chapter 6). This cell, which has a strong cortical distribution of f-actin, has a very marked branching morphology sending out thin processes, which are apparently packed with microfilaments, uniformly from the cell surface.

7.3.2   Arylphorin expression: Immunochemistry

Using the anti-calliphorin IgG probe for immunochemical analysis, it seemed that arylphorin was present in the cytoplasm of all cultured imaginal cells, regardless of long or short incubations with exogenous sources of protein, or in the presence of 20-HE. The cells shown in the following figures are all from the Cl. 8+ line. Figure 7.C represents a control sample, imaginal cells which have been stained using the pre-immune serum whereas Figures 7.D to 7.F have been specifically probed for the presence of arylphorin. The cells in Figure 7.D have not been incubated in the presence of any exogeous calliphorin or 20-HE prior to fixing, but fluoresce as well as cells which have. Calliphorin, without 20-HE, was present in the incubation medium of the cells pictured in Figure 7.E, whereas the cells pictured in 7.F were incubated with both for 1 hour prior to fixing.

Any variance in staining intensity between test samples is undetectable here, this analysis lacking a precise quantitative aspect. However, there does appear to be some difference in staining intensity between these cultures and the control, albeit a slight one. The cells in the test samples tend to stain throughout the entire cell, including cell processes, whereas those in the control seem to stain only within the main body and at a reduced level. The staining in the test samples does not seem to be restricted to any specific part of the cell, although in Figure 7.E there is evidence of an accumulation of staining at a site just beneath the plasma membrane.

7.3.3 Western blotting

Figure 7.G illustrates a Western blot of resolved Cl. 8+ cell lysates which has been probed using the anti-calliphorin IgG antibody. There are three test samples shown here. Lane 1 represents cells without any treatment.

The cells for Lane 2 had been pre-incubated with calliphorin (500 µg/ml) for 20 minutes prior to lysis whereas the cells in Lane 3 had been preincubated wtih both calliphorin and 20-HE (5 ng/ml) for 20 minutes. In lane 1 there is strong banding at around 35 kDa and in all three lanes there is some very faint banding at around 80 kDa.

7.3.4 Autoradiography

The results of the Western blot analysis suggested, as with the immuncytochemical studies, that arylphorin was present in imaginal cells. However, to clarify if this species represented an endogenous source of protein or if it was incorporated from the medium, a series of experiments centring on the use of radio-labelled calliphorin were performed. Here, radiolabelled calliphorin (either ^{3}H-phenylalanine or ^{35}S-methionine, both supplied by K. Scheller) was incubated with live cells, either by itself or in the presence of a 'cold' competitor, being unlabelled calliphorin. Later, to determine if this uptake was active, putative receptor sites were blocked by co-incubating the radiolabelled probe with the anti-calliphorin IgG antibody.

Figure 7.H represents the respective response of three different imaginal cell lines, Cl. C9, L127D6 and Cl. 8+, to incubation with the tritiated probe by itself. The recorded levels of activty for each cell line suggest that the probe was being incorporated into the cells and especially during the first 30 minutes of culture. It seems, from this analysis, that the cloned wing cell lines, Cl. C9 and Cl. 8+ are more succesful than the uncloned leg cell line, L127D6, at internalising the protein.

Some assays were then performed to try and determine if the steroid hormone, 20-hydroxy ecdysone (20-HE), enhanced the uptake of arylphorin by these cells. Figure 7.I details the response of Cl. 13 cells to incubation with tritiated arylphorin either in the absence or presence of 20-HE (present at a

concentration of 5 ng/ml). In both samples there was a pronounced upward shift in radioactivity, and suspected protein internalisation, during the first 3 hours of culture which had decreased by the 24 hour stage, therefore the presence of 20-HE does not seem to significantly enhance the uptake of arylphorin here. Indeed, Cl. 8+ cells tended to incorporate a greater amount of radioactivity when in the absence of 20-HE (Figure 7.J). After 3 hours of assay, cells which had been incubated with 5 ng/ml 20-HE displayed a significantly lower level of radioactivity than cells which had been incubated in the absence of the hormone.

To test whether the suspected uptake of protein was active, such as by receptor mediated endocytosis (RME), the cells were incubated with a fixed concentration of arylphorin probe in the presence or absence of an excess of non-radioactive arylphorin protein. If arylphorin was being specifically incorporated into the cells then the presence of unlabelled arylphorin would serve to outcompete the labelled protein to the surface receptor sites which would be manifested as a drop in radioactivity. This, indeed, seemed to be true with cells of the Cl. 13 cell line when incubated with the ^{35}S labelled arylphorin and competitor (Figure 7.K), which was present at a concentration 100 times greater than that of the probe (10 mg/ml). Here there was a clear indication that the presence of the unlabelled competitor had caused a reduction in the uptake of radiolabelled protein from the culture medium, there being a significant reduction in activity from the 'probe only' sample and the sample which contained both the probe and competitor.

However, a separate assay, centring on the competitive uptake of arylphorin by Cl. 13 cells, but this time using the ^{3}H-labelled arylphorin probe, suggested instead that this incorporation was non-specific (Figure 7.L). This experiment, which was carried out over a similar time period, indicated that by 25 hours of assay those cells which had been incubated with both the probe and the competitor had a higher level of incorporated radioactivity (i.e.

that the presence of the "cold" competitor was actually enhancing the uptake of the labelled protein). Both of these assays were carried out using a minimal sample size due to the availability of radiolabelled arylphorin.

Latterly, assays were performed which substituted anti-calliphorin IgG antibodies as the competitor for co-incubation with the radiolabelled probes (not shown). The intention here was, if the uptake of arylphorin from the medium was mediated by receptors then the antibodies would bind to the available protein and prevent it from being incorporated into the cells.There has been little evidence to date, however, to suggest that the presence of these antibodies have had a preventative influence on arylphorin internalisation in imaginal disc cells.

Figure 7.A   The morphological response of Cl. 8+ cells to an intracellular elevation of cAMP. The control cells in Figure 7.A.1 are pictured 4 hours after plating onto tissue culture plastic, as are the cells in the experimental cultures where the an intracellular elevation of cAMP has been induced (Figures 7.A.2 and 7.A.3, overleaf). There does not seem to be much variation in cell shape between the control and experimental samples, both groups of cells appearing mostly rounded and phase-dark with little evidence of arborisation or stellation.

Scale bars represent 25 μm

1.
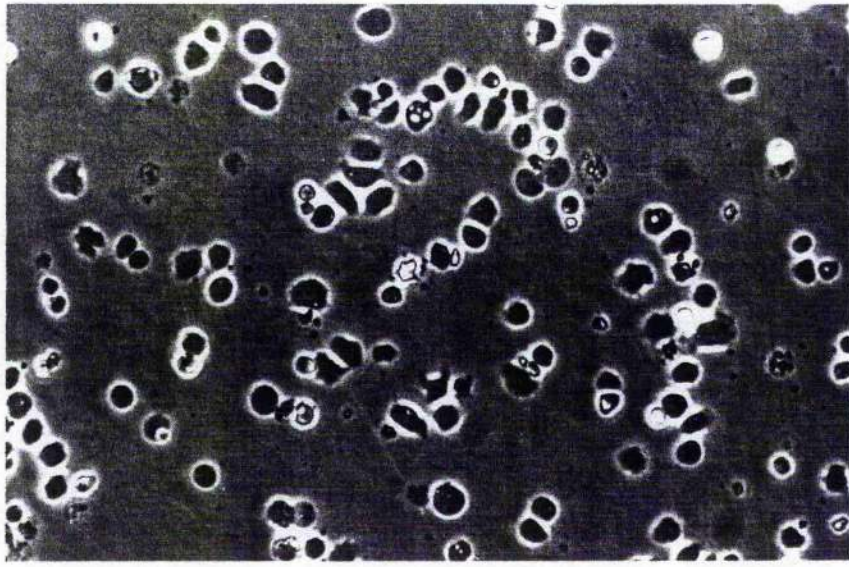
2.
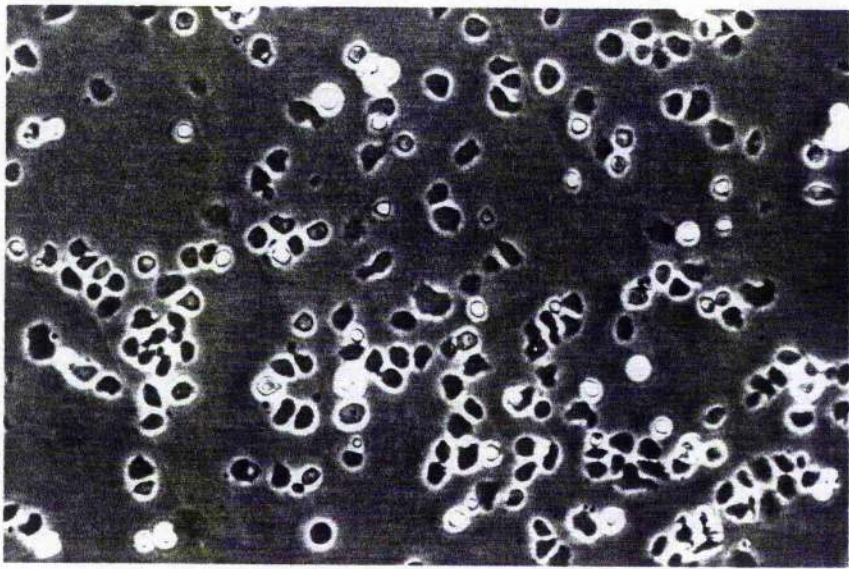
Figure 7.A.3 This micrograph is a detail of Figure 7.A.2, which is of Cl. 8+ cells 4 hours after plating under conditions that induce an intracellular elevation of cAMP. Occasionally, the odd cell can be found which has an unusual shape, such as the stellate cell (arrow) indicated in this micrograph.

Scale bar represents 15 μm

Figure 7.B A confocal micrograph of a small group of Cl. 8+ cells which have been grown on a substrate of human fibronectin under conditions identical to those in the control culture pictured in Figure 7.A.1 and fluorescently stained for F-actin. Here, in the absence of elevated levels of intracellular cAMP, the central cell has adopted a strikingly arborised morphology, extending thin processes which are staining strongly for F-actin. There also seems to be a subcellular cortex of F-actin, from which the cell-process microfilaments seem to extend.

Scale bar represents 10 μm

3.
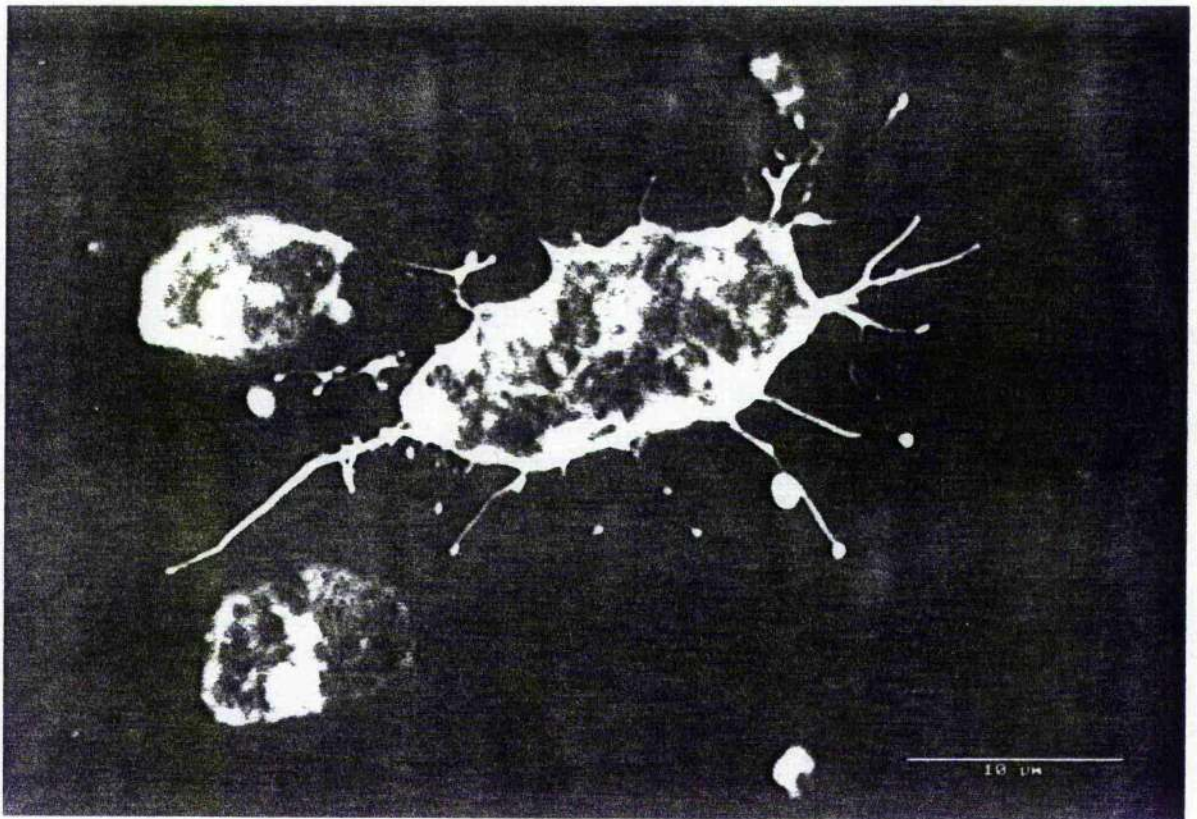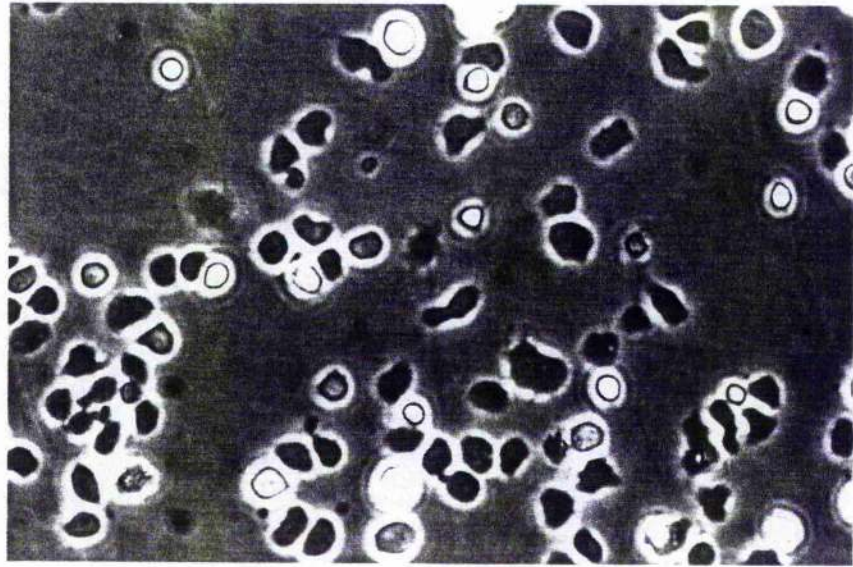
Figures 7.C - 7.F   This set of figures details the staining pattern of four different samples of Cl. 8+ cells, some of which have been probed using an anti-calliphorin polyclonal antibody. Figure 7.C represents a negative control which displays little staining in comparison to the other samples, (however, the intensity of staining in this sample has unfortunately been enhanced during the production of these prints.) Figure 7.D represents cells which have not been incubated with any endogenous arylphorin or 20-HE prior to fixing. The cells in Figures 7.E and 7.F represent cells which have been respectively incubated with arylphorin and arylphorin plus 20-HE for 1 hour prior to fixation.

Scale bars represent 15 μm
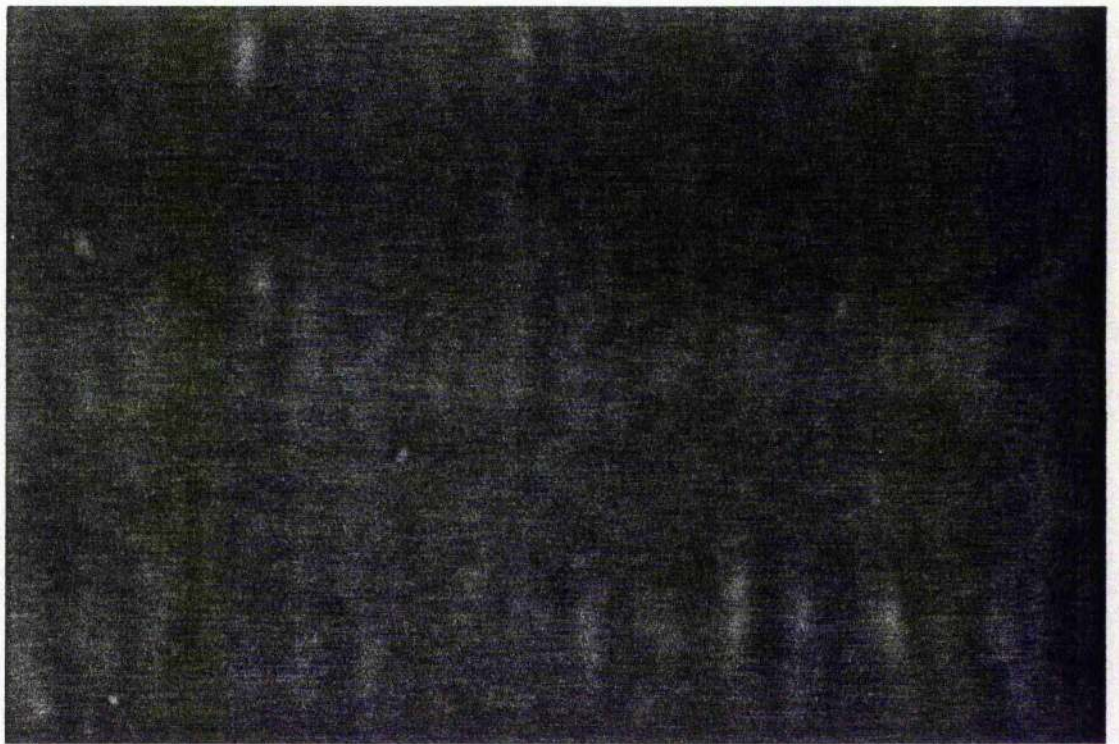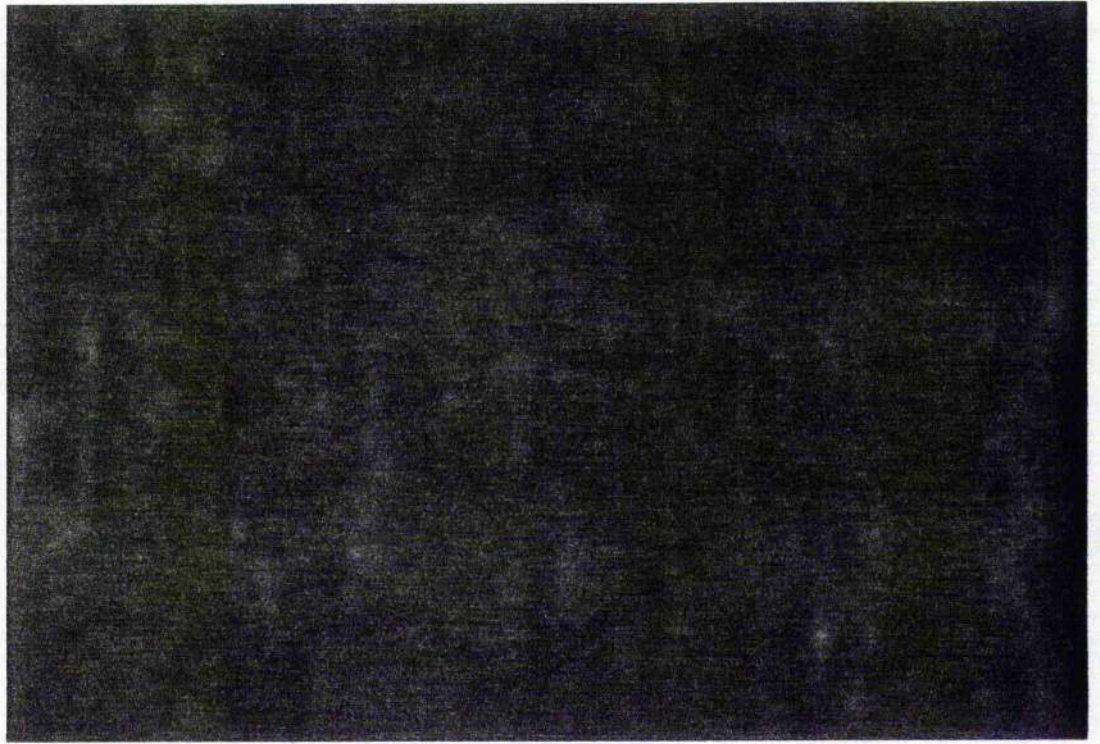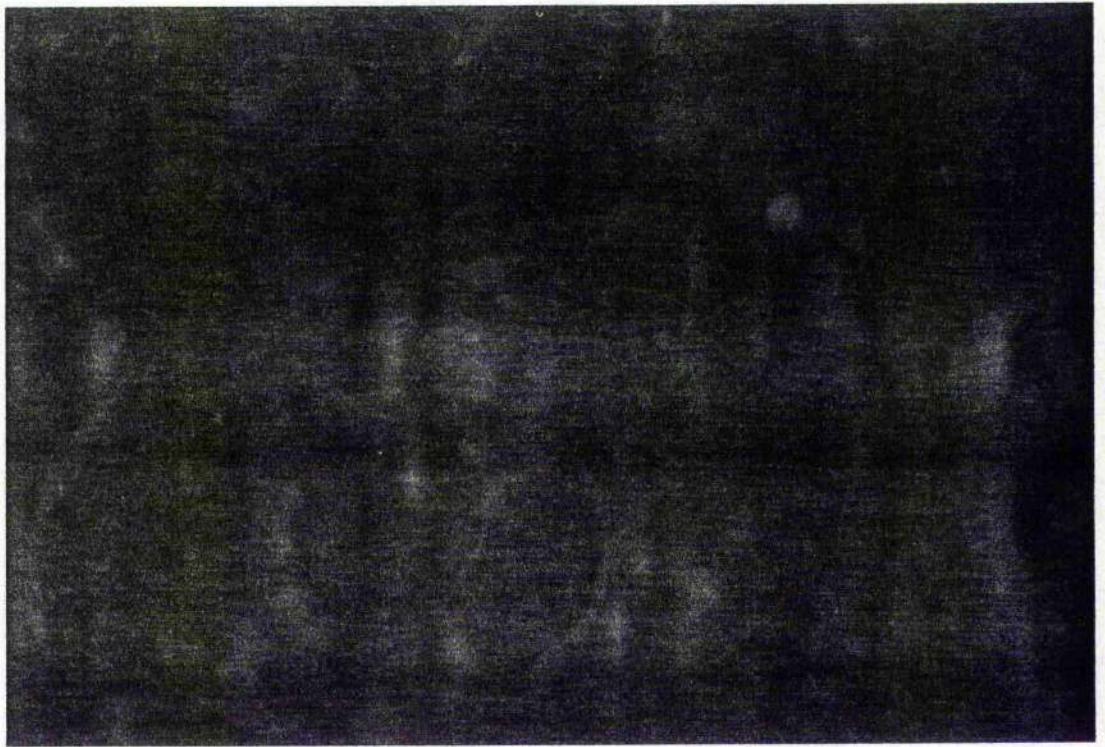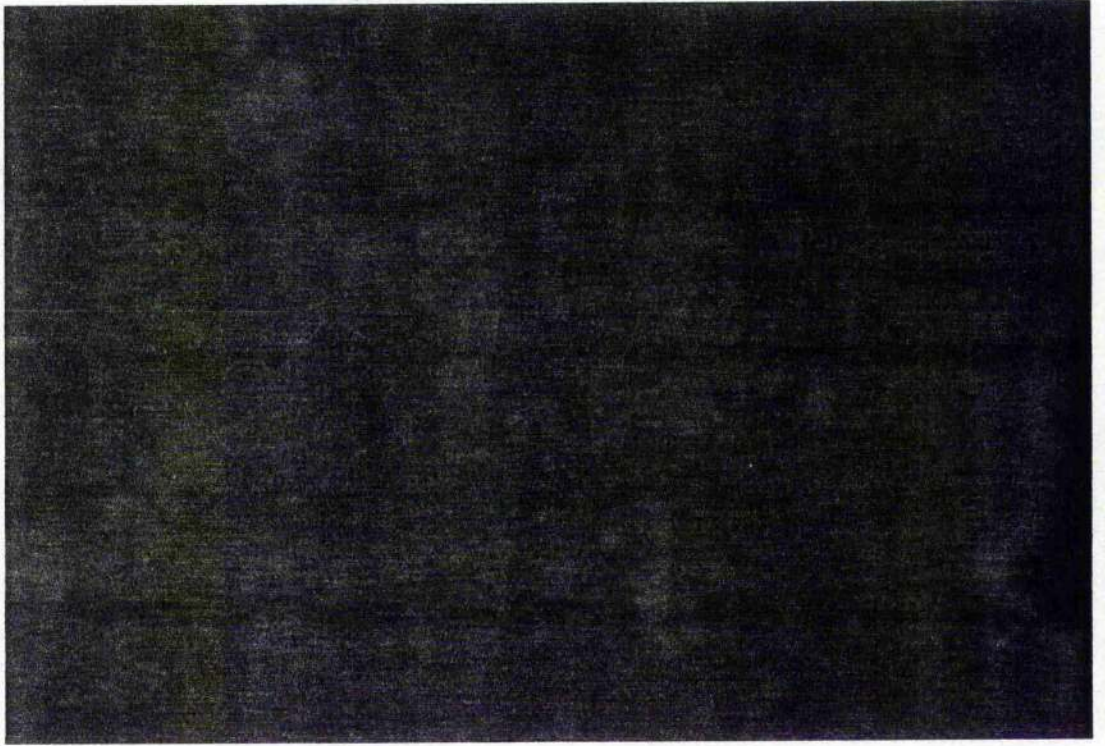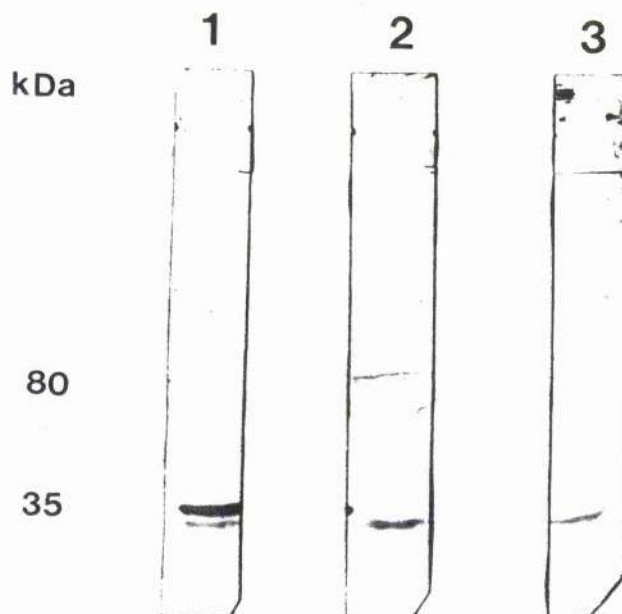
Figure 7.G

Figure 7.F   A Western blot of Cl. 8+ cell lysates which have been probed
using an anti-calliphorin polyclonal antibody. The samples in each lane
represent cells which have been incubated in the presence or absence of
arylphorin and 20-HE prior to lysis. **Lane 1** represents cells which were
untreated, **Lane 2** represents cells which were preincubated with
arylphorin for 20 minutes prior to lysis, whilst those cells in **Lane 3** were
pre-incubated with both arylphorin and 20-HE for the same period. In
each lane there is a faint banding of stain corresponding to a protein
species of around 80 kDa. There are also stronger staining bands in each
of around 35 kDa, especially in Lane 1, but these may be attributable to
proteolysis.

Uptake of a tritiated arylphorin probe in three imaginal cell lines.
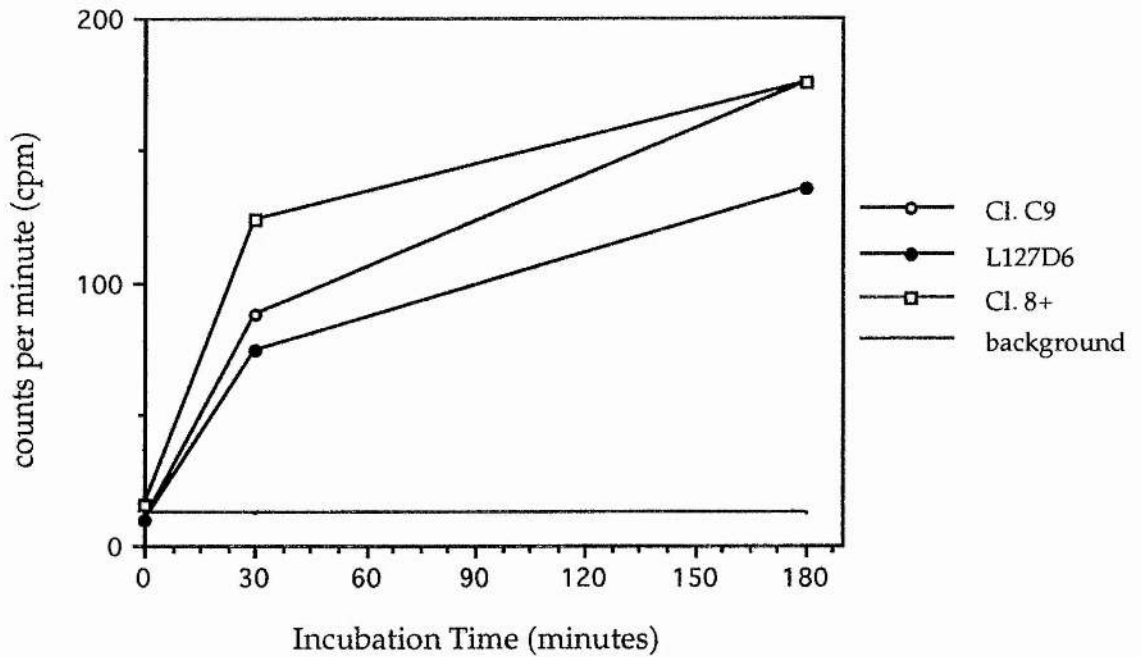
Figure 7.H. The response of three imaginal disc cell lines to incubation with a ^{3}H-labelled arylphorin probe. Here, cells in culture were exposed to the probe on its own, for three different timed periods: 0, 30 and 180 minutes. A control batch of cells, where the probe was absent, was used to define a background level of activity. In each cell line under test, there appears to be a rapid increase in incorporated probe during the first 30 minutes of assay, which then decreases during the next 150 minutes, suggesting that arylphorin has been internalised from the culture medium.

**Uptake of tritiated arylphorin probe
in Cl. 13 cell line: +/- 20-HE.**

Legend:
- Cl. 13 + 20-HE
- Cl. 13 - 20-HE

X-axis: Time (hours)

Y-axis: counts per minute (cpm)
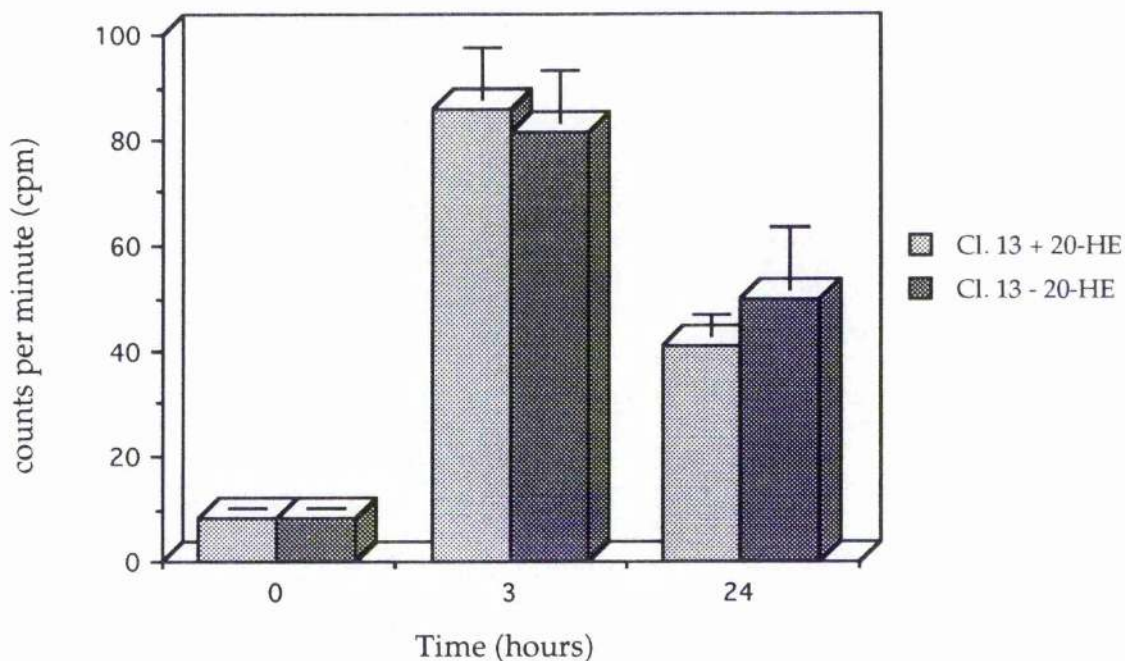
Figure 7.I. The uptake of radiolabelled arylphorin from culture medium by
Cl. 13 cells incubated in either the absence or presence of 20-HE (at a
concentration of 10 ng/ml). In both samples there appears to be a drop
in the level of incorporated protein after the first 30 minutes. By the
end of the assay the greater level of incorporation seems to have been
achieved in cells where 20-HE is absent.

**Uptake of tritiated arylphorin
in Cl. 8+ cell line: + 20-HE.**
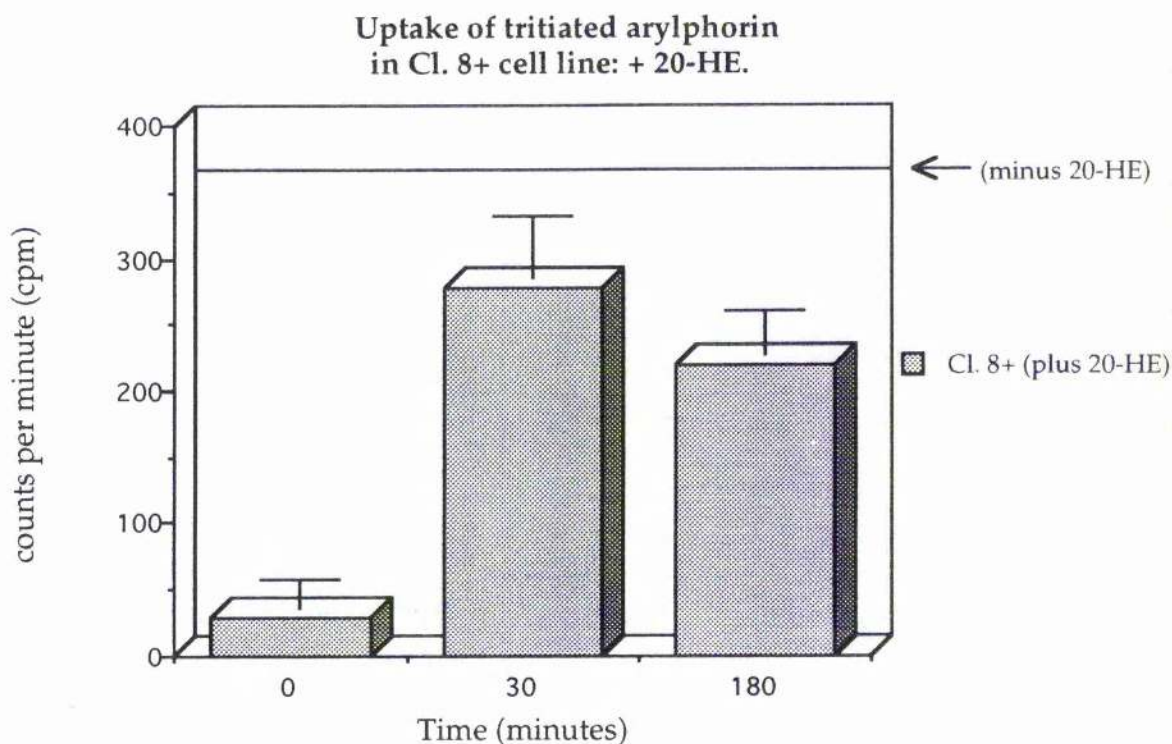
Figure 7.J. The uptake of radiolabelled arylphorin from the culture
medium by Cl. 8+ cells in the presence of 20-HE (at a concentration of 5
ng/ml). The arrow represents the level of incorporated activity in cells
which have been incubated for 180 minutes with an equivalent amount
of radiolabelled protein but in the absence of 20-HE. In cells incubated
with 20-HE, there is a drop in the level of incorporated protein after the
first 30 minutes. The greates level of incorporation of radioactivity,
however, has been achieved in the absence of any 20-HE.

Uptake of 35S-labelled arylphorin in
Cl. 13 cell line: + Competitor
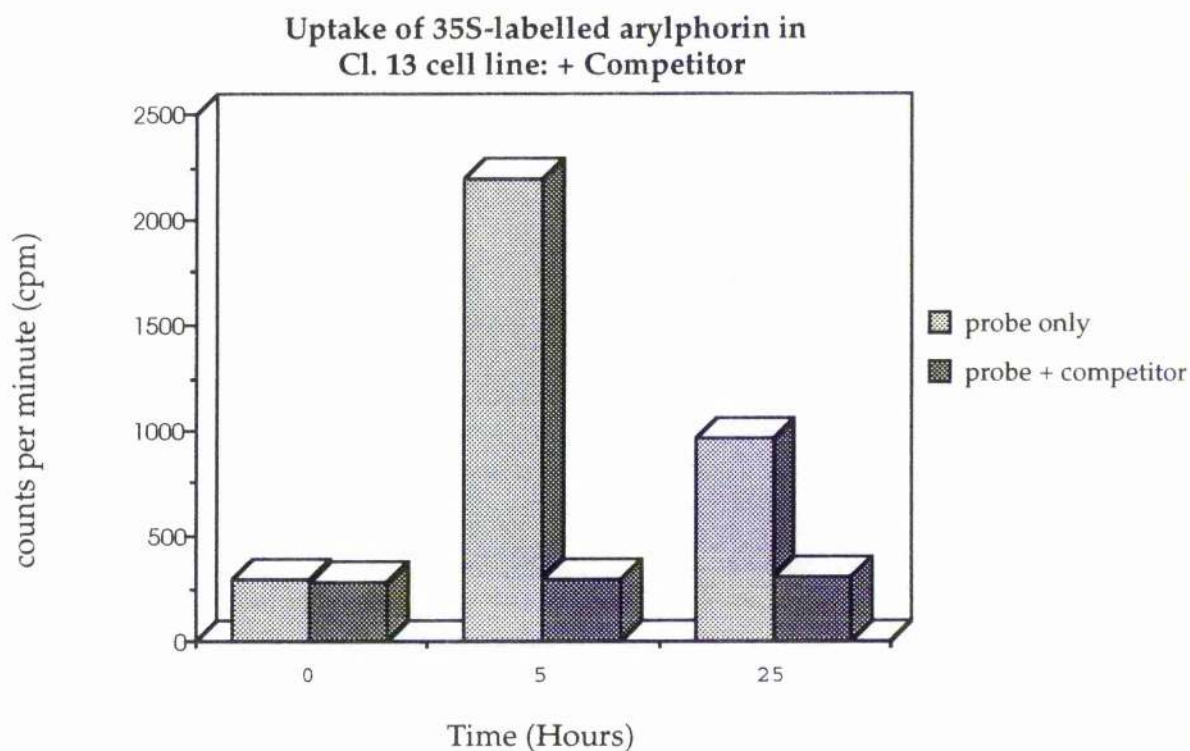
Figure 7.K. The uptake of an arylphorin probe by CL. 13 cells, as with Figure

7.L., either in the absence or presence of a "cold" competitor. The probe in

this particular instance has been labelled with ^{35}S linked to methionine,

which has a higher specific radioactivity than the ^{3}H probe. However, in

this assay the presence of the unlabelled competitor has apparently

reduced the uptake of the probe suggesting that the incorporation of

arylphorin by these cells is specific. This result appears to contradict the

findings illustrated in Figure 7.L., that Cl. 13 uptake of the probe is

enhanced by the presence of the competitor.

Uptake of tritiated arylphorin in
Cl. 13 cell line: + Competitor.

counts per minute (cpm) (y-axis: 0, 20, 40, 60, 80, 100, 120)

Time (hours) (x-axis: 0, 6, 24)

Legend:
▨ probe only
▨ probe + competitor
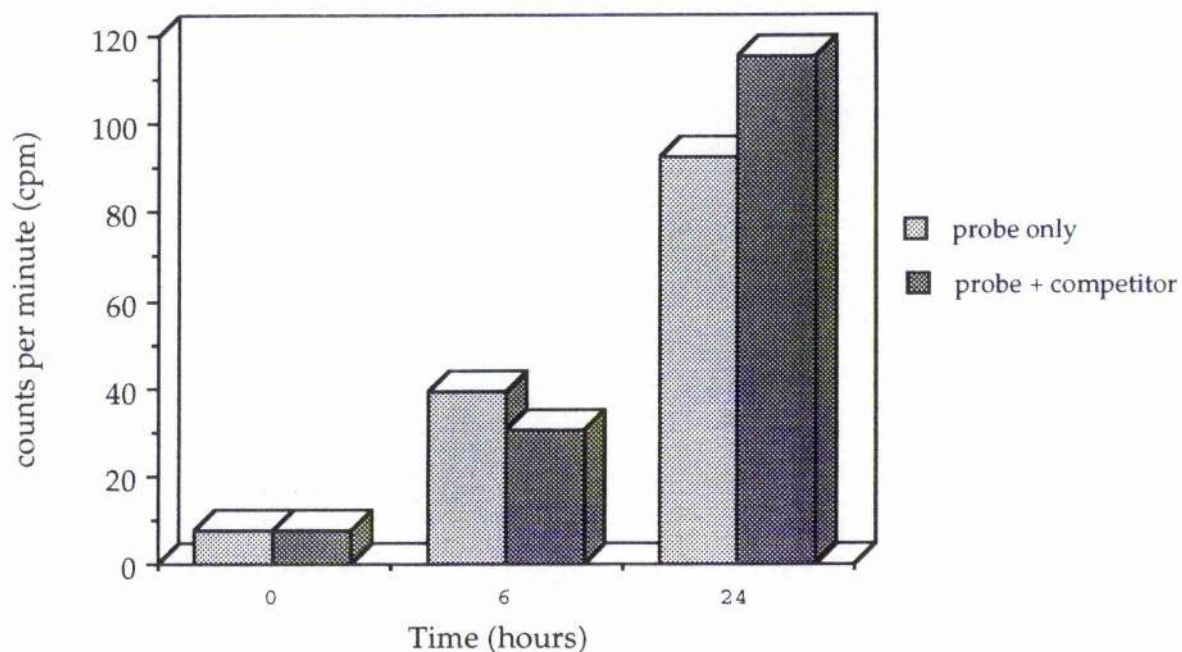
Figure 7.L.   The uptake of tritiated arylphorin by Cl. 13 cells either in the absence or presence of a "cold" competitor: unlabelled calliphorin at a 50-fold concentration. The results of this assay suggest that the cells can incorporate more of the probe when in the presence of the competitor, rather than the absence, as may be expected (see Figure 7.K). It could be the case that the concentration of labelled arylphorin here is too low to fill up the receptor sites required for incorporation, and that the extra protein promotes this uptake.

7.4   Discussion

7.4.1   cAMP induced cell shape change

There did seem to be a limited degree of cell shape change taking place in cells where the intracellular concentration of cAMP had been raised (Figures 7.A.2 and 7.A.3) but this variation in morphology was also seen in control cultures. The phalloidin stained cell pictured in Figure 7.B has a highly branched morphology, which it has adopted without any induced increase in the intracellular concentration of cAMP, therefore the cell shape changes seen in those cultures where cAMP and IBMX were present cannot be attributable to an increase in the abundance of this second messenger.

As mentioned elsewhere in this thesis, imaginal disc epithelial cells *in vitro* can undergo cell shape changes reminiscent of the *in vivo* phenotype. Imaginal epithelial cells *in vivo* extend long thin processes, known as epidermal feet, from their basal ends to form an interlacing network of cell extensions between the cells and the basal lamina which is believed to have a significant function in driving epidermal morphogenesis (Locke 1985; Fristrom, 1989). These sort of protuberances are also seen *in vitro* and are believed to play a role in the aggregation of imaginal cells (Peel *et al.*, 1990). Thus the occasional appearance of a stellate or arborised imaginal cell in culture is perhaps unsurprising.

Not all cells respond to elevated levels of cAMP, as with fibroblasts and neurones, by changing shape in an elaborate and radical manner. Raising cAMP causes CHO cells to elongate and form parallel arrays while 3T3 cells merely flatten (Edwards *et al.*, 1993), a morphological response that would have gone unnoticed in these particular assays where the control cells had already flattened. Therefore, it is feasible that the elevation of cAMP could in

fact be having a morphological effect on imaginal cells but that these changes are too subtle to be detected with this particular assay.

The spreading and outgrowth of the processes in BHK21 cells, studied by Edwards *et al.* (1993), seems dependent on the presence of microtubules as determined by the use of colchicine, which reversed this shape change. Imaginal disc cells display an abundance of microtubules (see Chapter 6) during movement *in vitro*. These researchers also concluded that cAMP-induced stellation resulted from a selective depletion of microfilaments, with f-actin, especially in the form of stress fibres, being broken down in the cell cortex but with this protein instead being strongly expressed at the tips of the growing processes. Such a selective depletion and expression of f-actin could create a cytoskeletal system leaving the cell in a pliable state and able to undergo radical shape changes. cAMP may affect actin by the dephosphorylation of myosin light-chain kinase (MLCK) perhaps destabilising microfilaments (Lamb *et al.*, 1988; Edwards *et al.*, 1993). It is possible that a rise in intracellular cAMP may be affecting the imaginal cell cytoskeleton in a manner that is not manifested as a gross alteration in cell shape but which could be detected by a detailed immunochemical analysis. Thus the visualisation of f-actin, by immunofluorescence, in cells where cAMP has been elevated may yet yield evidence that this second messenger can influence imaginal cell function. Specific evidence that the concentration of cAMP within the cell is actually being elevated during such assays, and which has not been attempted here, would also be a useful adjunct to a future study, and could be visualised again by immunochemical analyses.

7.4.2   Arylphorin expression and uptake

The presence of the ecdysteroid, 20-hydroxy ecdysone (20-HE), did not appear to significantly enhance the uptake of tritiated arylphorin in either of

the tested cell lines, Cl. 13 and Cl. 8+. Ecdysteroid hormones have been implicated in mediating the transport of arylphorins into fat body in *Calliphora*, both *in vitro* and *in vivo* (Burmester and Sccheller, 1995; Burmester *et al.*, 1995), and through epidermal cells and into secreted cuticle where it is known to function in sclerotisation and as a structural component (König *et al.*, 1986; Agrawal and Scheller, 1987). Although arylphorins have been demonstrated to function in binding ecdysteroids, albeit with a low affinity (Enderle *et al.*, 1983) the uptake of arylphorins in either fat-body or disc tissue in *Drosophila* or *Calliphora* is not necessarily dependent on the presence of these hormones. In *Drosophila*, 20-HE is known to enhance the formation of protein granules in fat-body but is not essential for this process to occur. Moreover, in *Calliphora* ecdysteroids have no known effect on the cellular uptake of arylphorins from the haemolymph (Wyatt and Pan, 1978).

Indeed, the presence of 20-HE even seems to have a deleterious effect on the uptake of radiolabelled arylphorin in some cell lines, such as Cl. 13 and Cl. 8+ (Figures 7.I and 7.J), untreated cells tending to take up more protein. This deleterious effect may be linked to the known morphological response of imaginal disc cell lines *in vitro* to various levels of 20-HE, which is the cessation of cell division and widespread cell death (Peel and Milner, 1992b). The concentration of 5 ng/ml used in these assays was half that of which was considered optimal for eliciting both a morphological and biochemical response (Peel and Milner, 1992b) although any titre above 2 ng/ml is known to cause a significant reduction in cell viability (Cullen and Milner, 1991). Such a loss of viability may account for the lower uptake of labelled protein from the culture medium here. It therefore seems unlikely that 20-HE is influencing the uptake of arylphorin from the culture medium in these cell lines, although there is still a possibility that endogenous expression could be enhanced, which could be tested by further Western blotting analyses.

However, there does seem to be some evidence to indicate that arylphorins, whether produced endogenously or endocytosed from the culture medium, can exist in cultured imaginal disc cell lines. Immunoytochemistry, limited though that approach may seem here, indicated that both instances may be true, which was further endorsed by gel electrophoresis. The results of the Western blot carried out indicated the presence of separate species, of around 30-40 kDa and 80-90 kDa. The lighter species cannot be attributed to the arylphorin monomer, but may instead be the result of proteolysis, the heavier species in each lane, however, may represent the monomer. The physico-chemical properties of arylphorin are known to be complex, the protein tending to dissociate easily into many species of varying electrophoretic mobility (Levenbook, 1983), which could account for the doublet of bands seen in each test lane, or perhaps even the lighter species although this seems unlikely.

The results of the autoradiography analyses suggested that the radiolabelled probes could be incorporated into imaginal disc cells, especially over shorter time periods (of around 3 hours). The competitive binding studies carried out to date, designed to determine if arylphorin uptake was specific, are somewhat ambiguous in that the presence of an excess amount of unlabelled competing protein appears to either reduce or enhance the uptake of a fixed concentration of labelled arylphorin (Figures 7.K and 7.L respectively). The first result (Figure 7.K) satisfies the criteria expected of a system where such uptake was active and may be occurring as receptor mediated endocytosis (RME). Such a mode of protein uptake seems entirely feasible with regard to recent findings indicating the existence of three proteins at the surface of imaginal cells which may be arylphorin receptors (K. Scheller. pers. comm.) The latter assay (Figure 7.L), using the ^{3}H- rather than the ^{35}S-labelled probe, suggests instead that the unlabelled competitor *enhances* the uptake of the probe. One interpretation for this phenomenon

could be that the concentration of labelled arylphorin here is too low to adequately fill up the receptor sites needed for it's internalisation to the cell and thus an extra titre of protein, in the form of the unlabelled arylphorin, can cause greater uptake. Until these assays are repeated this apparent ambiguity of cell response cannot be adequately addressed.

Thus, the results of the assays carried out to date are somewhat persuasive, implying that arylphorins may be produced endogenously by imaginal disc cells *in vitro* and that the uptake of protein from the culture medium is not significantly enhanced by the presence of 20-HE. Some ambiguousness remains, however, as to the specific nature of this uptake which would be relatively simple to address experimentally.

8.1   Conclusions

The purpose of this final section is to bring together the general conclusions from each of the preceding experimental chapters, detailed in the respective discussion sections, and to review these from within the ongoing context of *in vitro* imaginal cell research. Another aim of this chapter being to propose some potential experimental applications for imaginal cell lines which may be addressed in the future.

The *Drosophila* imaginal cell lines which were the subjects of this research were isolated and established by Douglas Currie, Martin Milner and Clive Evans and have now been in existence for approximately 8 years. Some of these lines have now undergone well over 100 passages. Three or four years after culture initiation, my predecessor in St Andrews, David Peel, was to prove successful in cloning new lines from the existing ones. These cloned lines have also attained a similar status of establishment and an even wider usage. The reason for the establishment of continuous imaginal disc cell lines *in vitro* is straightforward; that is, to provide models of a developmentally interesting tissue, the imaginal epidermis, which are amenable for detailed biological analyses. The attraction of which are further enhanced when one considers the extensive genetic records and the numerous sophisticated genetic tools that are available to *Drosophila* researchers.

The need for imaginal disc cell lines was pre-empted in part by studies which were designed to assess the adhesive and sorting-out capacities of these cells (e.g. Garcia-Bellido, 1966; Fehon and Schubiger, 1985). Studies such as these were often thwarted by problems associated with the need for a period of *in vivo* incubation, or more often by damaging techniques used in the dissociation of the disc tissue, as outlined by Poodry *et al.*, (1970) and Fehon *et al.*, (1987). The methods frequently used to disrupt imaginal discs, where the cells in the epidermis are intimately associated through an array of

specialised junctions, required harsh enzymatic and physical shear forces which invariably lead to a loss of cell viability or cell-death (Garcia-Bellido, 1966; Fehon and Schubiger, 1985). The incubation of cells within metamorphosing larvae also prompted the question of whether or not cell proliferation and cell sorting could occur.

Imaginal cells growing *in vitro* differ morphologically from those *in situ* in that they lack the specialised intracellular junctions that serve to intimately adhere and maintain cells within the plane of the epithelium, as discussed in Chapter 5. This accounts for the relative ease with which the cells can be dissociated when in culture and seems synonymous with the lack of any perceived apical-basal polarity.

This loss of polarity is also seen in various neoplastic overgrowth mutants such as *lethal (2) giant larvae* and *discs large* which are tumorous in phenotype (Bryant *et al.*, 1993), and the *fat* (Mahoney *et al.*, 1991) and *crumbs* mutants which are hyperplastic in phenotype (Tepaß *et al.*, 1990). The tumorous overgrowth mutants both appear to encode molecules involved in signal transduction, implying a basic loss in intercellular communication (for review see: Cohen, 1993). On the other hand, the hyperplastic mutants, which maintain the normal characteristics of an epithelial sheet, lack the expression of cell adhesion molecules (CAMs). For example, lethal mutations at the *fat* locus cause a massive overgrowth of *Drosophila* imaginal discs by increasing cell proliferation (Bryant *et al.*, 1988). This locus was later discovered to encode a member of the cadherin superfamily (Mahoney *et al.*, 1991). The absence of such a cell adhesion molecule is perhaps reflected in the ultrastructure of mutant *fat* discs, where the cells tend to be cuboidal rather than columnar and are more loosely packed than in the wild type discs. There were also fewer and less developed intercellular junctions that were seen between these disc cells, coupled with a lack of apical-basal polarity (Bryant *et al*, 1991). It thus seems that contact-dependent cell interactions mediated by

such molecules may play an important role in proper disc development. It certainly seems to be true here that the re-occurrence of proliferation during the culture of imaginal disc cell lines is concurrent with a loss of apical-basal polarity.

These cell lines were generated by culturing imaginal disc fragments in 96 well plates and isolating cells that had disaggregated and migrated from the sheets of epidermis (Currie *et al.*, 1988). Thus it appears that in order to generate a continuously growing population of cells *in vitro*, one is only selecting a certain cell type, or group of types, that are predisposed to disaggregation and migration from the imaginal tissue which may not necessarily represent the typical cell that is found, *in vivo*. The continuous cell culture of a cell line, by its very nature, serves to further select only those cells which are the most successful at growth *in vitro* and it seems likely that by the time that they were cloned (Peel and Milner., 1990) these cell lines probably only consisted of one cell type. Indeed, in imaginal disc cell culture, the fibroblast-like cells seemed to predominate, outcompeting the sickle-shaped and lamellocyte-like cells which subsequently disappeared (Currie *et al.*, 1988).

Cells of the late third instar imaginal epidermis, from which these lines originated, have ceased to divide and remain as a tightly organised polarised epithelium. It may thus be the case that similar cell adhesion molecules (CAMs), encoded by these "tumour suppressor" genes or related molecules involved in a signal transduction pathway, are also absent from the established imaginal disc cell lines, which would account for the unchecked proliferation and lack of epithelial organisation which would normally be seen within the disc.

Another factor which may explain why the typical epithelial phenotype is absent here, and one which is addressed more fully in Chapter 5, is that some of these CAMs lack the proper spatial distribution that would

normally be found *in situ*, with *crumbs* expression being a point in case. It would be of interest to find out if this was indeed the case by determining if the pattern of *crumbs* expression is altered, or if proliferation is decreased, when cells are grown under conditions which seem to favour apical-basal polarity (such as on cell culture inserts). Other than these notable deviations from the *in situ* state, the identity of imaginal cells *in vitro* seems to be very similar to that which would be found in the imaginal disc.

The determination of the nature of adhesion of imaginal cells, both to the substratum and to other cells, was one of the main experimental foci of this project. As with imaginal cells *in situ*, it appears that candidate molecules from each of the three main recognised groups of CAMs, the cadherins (Chapter 3), the PS integrins (Chapter 4) and the immunoglobulin superfamily (of which the fasciclins, including fasciclin III, are members: Chapter 6) are present and functioning during imaginal cell morphogenesis *in vitro*. The earliest indication of the presence and identity of such molecules was provided by the cell-cell and cell-substrate responses of imaginal cells when either in the presence of calcium ions, these CAMs being either Ca^{2+} dependent or independent in function.

Again, there does not seem to be a lack of expression of any one specific CAM in imaginal cells *in vitro*, evidence being provided by the various immunochemical and reaggregation assays outlined in each chapter, but rather a lack of the correct spatial distribution of these molecules which may account for the   different growth characteristics of these cells *in vitro* when compared to those *in situ*.

It seems likely that the molecules present on the imaginal cell surface *in vitro* may be performing similar roles to those found in the imaginal discs themselves. For instance, the expression of PS integrins is intimately linked with different aspects of cell growth and adhesion *in vitro*, appearing to be vital to the "morphogenesis" seen here, much as these molecules are suspected

for the correct morphogenesis of cells *in vivo*. Here the loss of a defined spatial distribution of antigen within the cell is added to by the loss of a restricted spatial distribution between cells, with cloned cells expressing both $\alpha_{PS1}\beta_{PS}$ and $\alpha_{PS2}\beta_{PS}$, rather than one or the other depending on whether they were derived from either the dorsal or ventral wing epithelia, as one might expect. This again reflects a loss of positional information between cells in the *in vitro* state, which is translated into a loss of control of cell proliferation.

It does seem possible, from cloning studies and related adhesion assays, that these CAMs can be selectively lost from the surface of imaginal disc cells, which is similar to the reported loss of ecdysteroid hormone receptors in some cell lines (Peel and Milner, 1992b) reflected by the observation that older cell lines tend to behave differently than newly isolated ones: newer cell lines tending to be more adherent to the substrate, implying a loss of cell-substrate adhesion molecules.

The movement of imaginal cells from a simple monolayer into multicellular aggregates over the course of a passage has been well documented but little understood (Peel *et al.*, 1990; Peel 1991). Imaginal cells seem more likely to adhere to a substrate of fibronectin than to a substrate of either collagen or laminin, which may mimic the response of cells to the *Drosophila* extracellular matrix (ECM) molecule tiggrin, which shares some structural homology with fibronectin (Fogerty *et al.*, 1994). On a substrate of human fibronectin, imaginal cells will adhere and migrate much as they do on tissue culture plastic, but form very dense aggregates which are joined by fascicles of cells. This mode of aggregation seems consistent with the "traction-based system of morphogenesis", which proposes that individual cells 'pull' on the substrate which leads to the formation of condensations of cells. This certainly seems plausible for the movement of imaginal cells *in vitro*, and does not rule out the possibility that aggregation could occur by the transduction of tension directly between cells, via cytoplasmic extensions. As

with the traction model, tension could be transmitted between adjacent cells during aggregation by the interaction of the molecular 'linchpins', the PS integrins on the surface of cell extensions, with components of the ECM being found there. Indeed, during aggregation there appears to be a shift in imaginal cell adhesive strategy from cell-substrate to cell-cell adhesion, accommodating both models for imaginal cell aggregation.

There is little ultrastructural evidence to suggest that any complex junctions are being formed during imaginal cell morphogenesis *in vitro*, with the possible exception of those cells grown under conditions that favour the formation of apical basal polarity (Chapter 5). Nevertheless, even this level of junctional differentiation does not represent the sort of junctions that are found within developing imaginal tissues, such as the transalar array, the complex cytoskeletal apparatus that is found within the evaginating pupal wing (Tucker *et al*, 1986; Mogensen and Tucker, 1987; Fristrom *et al.*, 1993).

If tension is created between aggregating imaginal disc cells *in vitro*, it seems likely that this is being transmitted via the extracellular matrix (ECM), as indicated by the antibody blocking studies, where the β_{PS} subunit antibody, aBG-1 (Hirano *et al.*, 1991) prevented cellular adhesion to human fibronectin. This antibody was also known to block cell-cell adhesion, but this effect was reversed on the addition of foetal bovine serum, further implicating the ECM as playing a role in imaginal cell morphogenesis *in vitro*, and notably in cell-cell adhesion.

Antibody studies have been widely used in the determination of function of many CAMs, being applied for use either *in vitro* or *in vivo* . The work of Masatoshi Takeichi and his colleagues in determining the function of cadherins in vertebrate and invertebrate tissue morphogenesis has utilised many such approaches (for review, see: Takeichi *et al.*, 1989). By perturbing morphogenesis in this way, Takeichi *et al* assigned tentative roles for cadherins in specific cell adhesion, such as the role of E- and P- cadherin in

the development of the epithelial lung primordia *in vitro* (Hirai *et al*, 1989).

However, antibody blocking studies *per se* , are not believed to be the ideal tool for determining the function of these CAMs. For a more defined interpretation of the function of cadherins, Takeichi and his co-researchers relied on other assays; specifically the transfection of cadherin-encoding cDNA into neutral cells, and thereby conferring a gain of function which more conclusively indicated cadherin behaviour (Takeichi *et al.*, 1989). Similar transfection studies were carried out by Elkins *et al.*, (1990), who, by transfecting genes that expressed either fasciclin I or fasciclin III into different populations of the normally non-adherent S2 cell line were able to prove that these molecules bind homophilically by observing that the cells sorted-out. Some researchers, on the other hand, prefer to work *in vivo*, and create transgenic animals using a gene linked to an inducible promoter to specifically encode a molecule, or by injecting antisense RNA, again linked to a specific promoter to facilitate its expression in individual tissues.

Little is known at present about the state of determination of these cells, and whether the developmental fate is altered as a result of *in vitro* culture. The lack of a heterogeneity of cell types and a correct three-dimensional cytoarchitecture, coupled to a possible lack of suitable growth medium, means that these cells cannot undergo true metamorphosis *in vitro*. Imaginal cells are known to synthesise chitin and presumably cuticle under the influence of 20-HE, but none of the cuticular markers associated with imaginal disc morphogenesis *in vivo* are seen (Peel and Milner, 1992b). From some preliminary work, it seems that groups of aggregated cells can undergo metamorphosis when transplanted to late third instar larva prior to pupation, complete with the formation of identifiable markers such as bristles and trichomes. Further analysis of metamorphosed imaginal tissue would indicate the state of determination in these cell lines.

Another area of future interest, and one which I have begun to address, would be to determine the role of certain growth factors in imaginal disc cell lines, specifically the TGFβ -like molecule encoded by the *decapentaplegic* (*dpp*) gene complex.

The *dpp* complex is known to function in the formation of dorsal structures in the developing embryo and in the proper morphogenesis of the imaginal discs (Spencer *et al.*, 1982). The cDNA corresponding to the dpp gene has been cloned and sequenced and has been found to have a strong sequence homology in the C-terminal region to corresponding regions in mammalian proteins belonging to the TGF-β family (Padgett *et al.*, 1987). Any mutation in the disk region of the gene creates a loss of distal parts from all adult cuticular appendages (Gelbart, 1985) caused by massive cell death in the corresponding imaginal tissue (Bryant, 1988). Bryant concluded that some product of the *dpp* gene is needed for cell survival and that this need is greater in the presumptive distal regions of the discs rather than the proximal parts.

It may be the case that the *dpp* encoded protein is important in conferring viability proliferation in cultured imaginal disc cells, much the same as it is evidently required *in vivo*, and that some cells are more dependent on this growth factor than others. Some preliminary work has been carried out by myself in collaboration with a research group in Madison, Wisconsin, headed by Professor F. Michael Hoffmann to determine the role of this molecule *in vitro*. This project involved various technques, such as measuring imaginal cell proliferation following the addition of an exogenous source of *Drosophila* growth factors and devising methods to transfect functional growth factor-encoding genes into imaginal and S2 cells. However, these studies were hampered due to problems encountered with cell culture and remain to be addressed in sufficient detail.

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