

CELL INTERACTIONS AND THE RESPONSE TO
ECOLYSTEROIDS OF 'DROSOPHILA' IMAGINAL DISC
CELL LINES

David John Peel

A Thesis Submitted for the Degree of PhD
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Cell interactions and the response to ecdysteroids of
Drosophila imaginal disc cell lines.

by David John Peel

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I, David Peel hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree of professional qualification.

Signed

Date 21st March '91

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No. 12 on October 1st 1987 and as a candidate for the degree of Ph.D. in September 1988.

Signed

Date 21st March '91

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

Signature of Supervisor

Date

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Abstract

This thesis is a study of the biology of *Drosophila* imaginal disc cells growing as continuous cell lines. Their morphological characteristics and cellular properties were analysed in order to characterise the cells *in vitro*.

Morphological analysis of the cells revealed several cell types within the original cell lines perhaps representing a diversity in the cellular origin of these cells. A new cloning technique was devised which enabled a single imaginal disc cell to give rise to new cell line and indicated that a single cell could give rise to the different cell types seen in culture. This indicates that the diversity in morphology of the cells in culture was an indication of the conditions in culture rather than as a result of cellular diversity.

The original cell lines and the newly derived cloned cell lines were subjected to the insect moulting hormone 20-HE in order to ascertain the degree of differentiation that was possible in culture. The cells showed a dramatic morphological response to hormone, they elongated, began to aggregate and threw out cell processes. This is combined with concomitant biochemical changes in the cell lines, including the induction of chitin synthesis and acetylcholinesterase.

The cells in culture show a characteristic pattern of aggregation which was studied at the ultrastructural level using electron microscopy. These studies and also immunofluorescence of cell aggregates indicated the prevalence of cell processes and a role was postulated for their action in bringing about these aggregates. Aggregation was also

correlated with the expression of PS integrins, which are well characterised *Drosophila* adhesion molecules.

The adhesive properties of the cells were further characterised with reaggregation experiments in different media as a prelude to setting up cell sorting assays between wing and leg cell lines. This proved somewhat inconclusive but pointed to some sorting out occurring between wing and leg cells.

Abbreviations

20-HE	20-hydroxy ecdysone
ABTS	2,2 azino di (3 ethylbenzthiozoline 6 sulfonic acid)
AChE	Acetyl cholinesterase
Acph ⁿ⁻¹¹	Acid phosphatase ⁿ⁻¹¹ null mutant strain
AEC	3-amino-9-ethylcarbazole
AThCh	Acetylthiocholine iodide
BSA	Bovine serum albumin
Ca ²⁺	Calcium
CAM	Cell adhesion molecule
CME	Currie, Milner and Evans, refers to ref (1987)
CPM	Coumarinylphenylmaleimide
cpm	counts per minute
D=	PBS without Calcium or Magnesium
DAB	Diaminobenzidine
DDC	Dopa decarboxylase
DEHP	di-iso-octylphosphate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECM	Extra-cellular matrix
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunoassay
FBS	Foetal Bovine Serum
FCS	Foetal calf serum
GlcN	Glucosamine
H ³ GlcN	Tritiated glucosamine

H ³ Pon A	Tritiated Ponasterone A
HCl	Hydrochloric acid
HEPES	N-[2-hydroxyethyl] piperazine-N'- [2-ethanesulfonic acid]
HRP	Horse radish peroxidase
I.U.	International units
<i>l(2)gl</i>	<i>lethal (2) giant larvae gene</i>
<i>l(2)mbn</i>	<i>lethal (2) malignant blood neoplasm</i>
<i>l(3)mbn</i>	<i>lethal (3) malignant blood neoplasm</i>
Mg ²⁺	Magnesium
NaOH	Sodium hydroxide
O.D.	Optical density
PBS	Phosphate buffered saline
PIPES	Piperazine-N,N'-bis[2-ethane-sulfonic acid]
PMSF	Phenylmethylsulphonylfluoride
Pon A	Ponasterone A
PS	Position specific antigens
R	Ecdysteroid receptor
R. 123	Rhodamine 123
RER	Rough endoplasmic reticulum
RGD	Arg-Gly-Asp tripeptide
RIA	Radio Immune Assay
S.E.	Standard error of the mean
S2	Schneider line 2 embryonic cells
sdh	Succinate dehydrogenase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning Electron Microscope

TBS	Tris buffered saline
TEM	Transmission Electron Microscope
TTBS	Tween Tris buffered saline
UPB	Unspecific protein binding

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"Argumentation cannot suffice for the discovery of new work,
since the subtlety of Nature is greater many times than the
subtlety of argument"

Francis Bacon 1561-1626

Chapter 1

Introduction

Introduction

The fruit fly *Drosophila melanogaster* is a Dipteran insect that has a holometabolous mode of life. This means that it undergoes what is called complete metamorphosis, involving a larval, pupal and adult stage. The pupal stage being devoted to transforming the larva into the adult. The adult cuticular structures (exoskeleton) of the insect are the result of the development of imaginal tissue formed into specific structures called the imaginal discs, which are specialised epithelial infoldings of the larval ectoderm. The different imaginal discs that occur, are determined to form different parts of the adult insect (see Figure 1). Metamorphosis transforms these discs during the pupal stage into their specific adult structure and is under the control of the moulting hormones, primarily involving the steroid hormone 20-hydroxy ecdysone (20-HE). 20-HE causes the imaginal discs to evert and differentiate, producing the cuticular exoskeleton and the various bristles and hairs that are found on the surface of the adult insect.

Imaginal discs have been extensively used experimentally. There are a number of reasons for their use. In this system determination and differentiation are separated, in that determination of each disc occurs during embryogenesis and the larval period, and the final differentiation of that pattern only occurs in response to the moulting hormones at metamorphosis. This provides a separation between determination and differentiation which does not occur in other systems where the two often run concurrently. The

adult cuticle is covered in bristles and hairs which provide a specific spatial pattern marker system enabling the state of determination of the cells to be ascertained after metamorphosis. The imaginal discs are also distinct and easily manipulable and are capable of being cultured *in vivo*.

These facets of this system have resulted in a great deal of information being gathered about the growth, determination and pattern formation of imaginal discs *in vivo* (for reviews see Ashburner and Wright 1978 and Whittle 1990). Combining this knowledge about imaginal discs, together with the sophistication of genetic approaches and wealth of genetic data available in *Drosophila*, means that imaginal discs make an excellent model system for the analysis of developmental and cell biological problems. However contrary to the success of genetic approaches in *Drosophila*, cell biological techniques have been rather limited. These include the use of embryonic cell lines and the ability to *in vivo* culture imaginal discs and short term *in vitro* culture. Imaginal discs or fragments of disc can be cultured *in vivo* either in a metamorphosing larva to allow differentiation of the implant, or in an adult female host to allow further growth. This technique has been widely used and led to the discovery of transdetermination. This occurs when a piece of imaginal tissue repeatedly cultured *in vivo*, changes its state of determination and differentiates to form a different adult structure from its original specification (Hadorn 1978). Other experiments using *in vivo* culture of imaginal disc fragments led to the discovery that when cells from different parts of the disc were experimentally combined, there was extensive

proliferation and intercalation of missing positional values (Haynie and Bryant 1976). Thus the *in vivo* culture technique has allowed quite a lot of interesting experiments to be performed. However the problem with this *in vivo* technique is that once implantation has occurred, nothing is known about the fate of the implanted fragment until it is recovered after it has undergone cuticular differentiation. The other problem is that little is known about the hormonal and nutritional milieu that the implanted cells find themselves in and it is very difficult to manipulate the culture conditions. Some of these problems have been overcome by the use of organ culture where explanted imaginal discs undergo a near normal developmental routine in response to exogenous hormonal stimuli (Milner and Muir 1987). None of these techniques provides the ease of manipulation and the ability to produce large amounts of homogeneous cellular material that cell lines can. However the development of *Drosophila* cell lines in comparison with other techniques is relatively primitive.

In 1981, Sang commented that of all the established cell lines available only one is not from embryonic material (Sang 1981). The first *Drosophila* cell lines were established in 1969 by Echalier and Ohanessian (1970) and Kakpakov *et al* (1969), both from 6-12 hour embryonic material. From this initial breakthrough the much used K_C cell line was established (Echalier and Ohanessian 1970). In 1972 later embryos were used to set up three separate lines which are still much in use today (Schneider 1972). Numerous other cell lines were set up following these initial attempts, but those mentioned above and clones thereof have remained by and large

the only cell lines that have been used extensively. However all these cell lines suffer from one major problem, that is they are made up from embryonic material. These cell lines are established from mechanically disrupting or enzymatically dissociating whole embryos with all the plethora of cell types that exist within them. This is perhaps best illustrated by looking at primary cultures of embryonic material where the cells are allowed to differentiate, hence revealing the cell types present in this material. This was done in a study by Shields *et al* (1975), where primary cultures were obtained by homogenising 6-8 hour embryos. In these cultures, in the first stage nerve, muscle, fat-body, chitin secreting and haemocyte-like cells appear, to be followed later by tracheal, macrophage-like and a variety of fibroblastic and epithelial-like cells. Vesicles of what appeared to be imaginal cells also developed which were shown to develop into adult structures upon implantation into a larva about to undergo metamorphosis (Dübendorfer *et al* 1975). Therefore it can be seen that in such cultures a variety of different cell types appear, some identifiable and some not. It is from such cultures that cell lines eventually develop, usually following a long period of adaptation, before certain cell types grow up and form a line. It is not certain which cell type eventually gives rise to an established line. A great many embryonic cell lines from *Drosophila* now exist, Schneider and Blumenthal (1978) list 31 established cell lines but by now the numbers of established lines must be much larger. The use of these embryonic cell lines has been extensive, and they are still

very widely used today often as a complement to other more genetic studies.

Attempts to initiate a more defined cell type into culture have been somewhat less successful than the establishment of embryonic cell lines. One notable success was the development of blood cell lines (Gateff et al 1980), using the malignant blood cell neoplastic mutants, *lethal (2) malignant blood neoplasm (1(2)mbn)* and *lethal (3) malignant blood neoplasm (1(3)mbn)*. These cell lines were easily established by taking haemolymph samples from mature mutant larvae and transferring them to medium where permanent cultures can be established due to the neoplastic nature of these mutant cells. These cultures contain a range of haemocytes with an over proliferation of proplasmotocytes and plasmotocytes, together with adherent podocytes and lamellocytes. The cells also contain a range of viruses. These cells retain some of their normal characteristics, and are found to contain large amounts of acid phosphatase and undergo phagocytosis of neighbouring cells (Gateff et al 1980). Therefore this technique provides a reliable defined cell culture but of a very specialised type. Further attempts to produce a more usable defined *Drosophila* cell line proved difficult. Davis and Shearn (1977) designed a highly complex serum free medium which was supplemented by insulin, a juvenile hormone analogue and conditioned by larval fat body, called Medium X. This medium is based upon a partial characterisation of larval haemolymph (Begg and Cruikshank 1963), as indeed are most others. Davis and Shearns' Medium X allowed cut discs to regenerate but did not allow proper cell

culture of dissociated disc cells. These regenerating discs were used in transdetermination experiments by transplanting the regenerated fragments and allowing metamorphosis *in vivo*, and showed that transdetermination *in vitro* occurred in a similar way to that *in vivo* (Shearn et al 1978). However there was still no growth of dissociated disc cells and the complexity of Medium X made it difficult to repeat the work done with regenerating disc explants.

In a later study, Wyss (1982) developed a new culture medium that, while devoid of serum, was supplemented by a fly extract, 20-hydroxy ecdysone and a high concentration of bovine insulin (10 $\mu\text{g/ml}$). This medium supported limited proliferation of protease dissociated disc cells to form small colonies of cells, ranging up to about 100 cells. However unfortunately no cell lines could be established from such cultures. What might seem an unusual feature of these attempts to culture imaginal disc cells is the presence in the medium of insulin, usually thought of as a vertebrate hormone. Seecof and Dewhurst (1974) first demonstrated the value of insulin in primary cultures of *Drosophila* embryonic cells. Evidence that insulin or an insulin-like molecule was present in *Drosophila* came with a study by Meneses and De Los Angeles Ortíz (1975) which showed that a protein extract from *Drosophila* showed insulin-like activity in a mammalian bioassay. Further evidence that insulin or insulin-like molecules were present in *Drosophila* came with the identification of a homologue to the mammalian insulin receptor in *Drosophila* which was able to bind insulin and cross-reacted with anti-human insulin receptor antisera (Petruzzelli et al 1985 and Petruzzelli et

al 1986). Also an insulin degrading enzyme was isolated, which was found to be developmentally regulated (Stoppelli et al 1988). Thus insulin or a closely related molecule is obviously present in *Drosophila* and indeed insulin appears to have a widespread role as a mitogen in a variety of systems (Heyner et al 1990).

Whilst difficulties were being experienced in setting up cell lines from *Drosophila* imaginal discs, discs from other species were less problematic. Lynn et al (1982) managed to establish cell lines from imaginal discs of the Lepidopteran *Trichoplusia ni*. These cells grow as multicellular vesicles in a modification of Grace's medium containing 10% FCS (Grace 1962). Other imaginal disc cell lines were established from *Spodoptera frugiperda* and *Plodia interpunctella* (Lynn and Oberlander 1983). Therefore it seems that other insects are somewhat more amenable for the initiation of cell cultures than *Drosophila* (Schneider and Blumenthal 1978). Returning to *Drosophila*, the culture medium devised by Wyss (1982) appeared to have come closest to allowing dissociated disc cells to develop, and so it seemed that insulin, a fly extract and the addition of 20-HE were necessary prerequisites for any successful attempt at establishing cell lines from imaginal discs. However providing the right medium is only half the solution to providing the ideal conditions for the establishment of a cell line, the other important part of the procedure being disc dissociation. In the study of Wyss (1982), protease VIII was used to enzymatically dissociate disc tissue followed by vigorous pipetting to fully dissociate the cells as protease VIII treatment still left the discs in

one coherent piece. No figures are given for the degree of dissociation achieved by this method, although it appeared that dissociation was quite thorough and only a few cells were left as clumps. Following Wyss's study in 1982 two later studies experimented with several techniques and established two different alternative dissociation procedures. In one, Fausto-Sterling and Hsieh (1983) settled on using trypsin in Ca^{2+} and Mg^{2+} free Ringers (García-Bellido and Nöthiger 1976) to partially dissociate the cells, followed by pipetting. This method achieved a 50% cell yield, 80% single cell dissociation with an initial viability on the basis of the exclusion of the vital dye trypan blue of around 80% (Fausto-Sterling and Hsieh 1983). In another study, Fehon and Schubiger (1985) used a more complex dissociation procedure involving collagenase followed by a brief exposure to a citric acid solution of pH 2.5. This method achieved a 65% cell yield with 90% viable single cells.

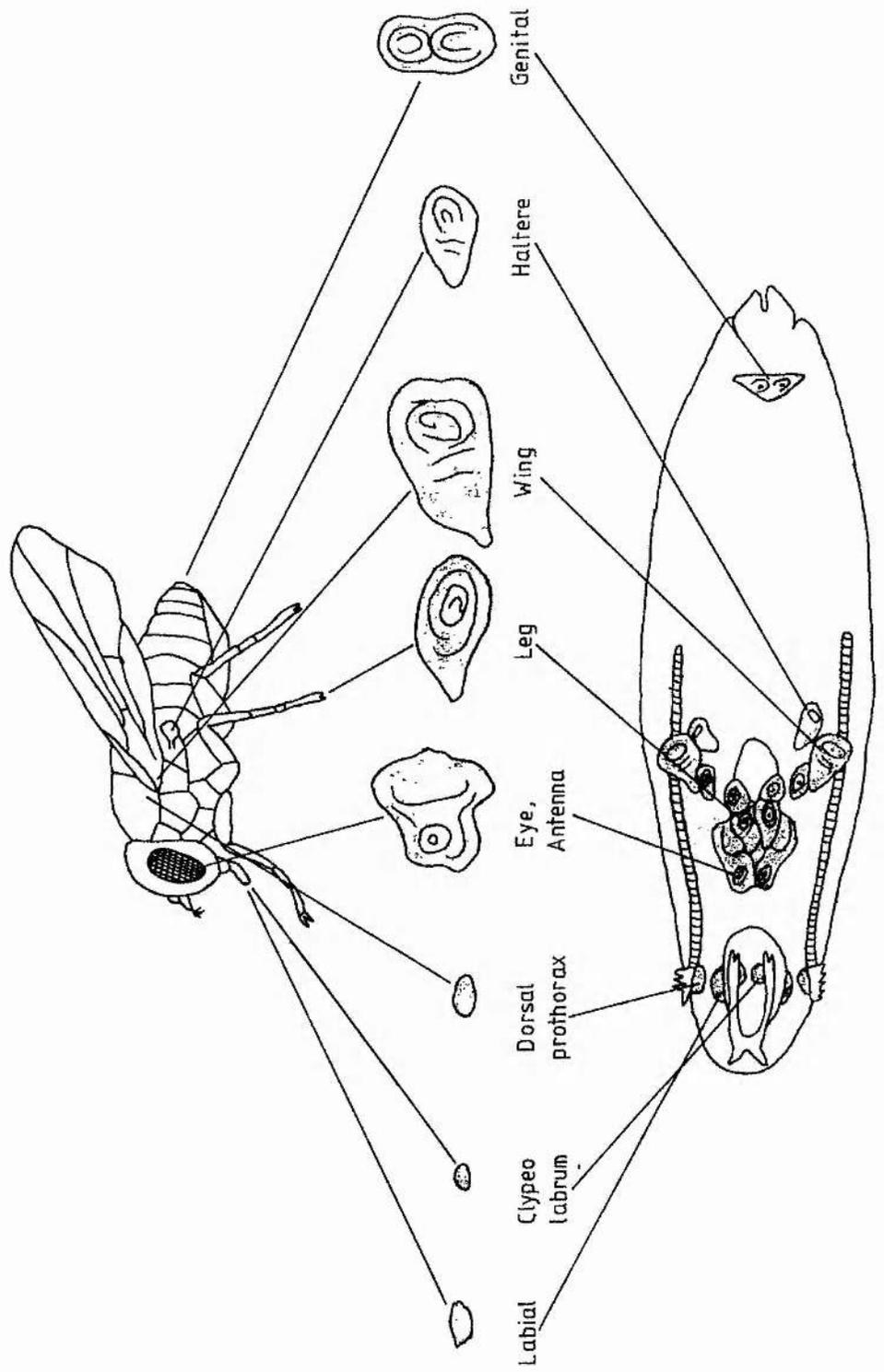
These studies led the way to further attempts to establish cell lines from imaginal discs. These finally proved successful in a technique developed by Currie, Milner and Evans (1988). Here imaginal discs were dissociated by the use of protease VIII (Dispase) in 4 mM EDTA in Ca^{2+} , Mg^{2+} free PBS (D=) followed by a short exposure to trypsin and mechanical disruption by pipetting. The imaginal disc tissue which was not fully dissociated by this treatment but rather consisted of small clumps of tissue together with single cells was then suspended in a new medium based on Shields and Sangs M3 (Shields and Sangs 1977). This new medium consisted of M3 with 2% FBS and the addition of insulin and a fly extract (made

from homogenising adult flies) together with 1 ng/ml of 20-HE. These dissociated cells and partly dissociated clumps developed into primary cultures consisting of sheets of cells and vesicles, which could be induced to differentiate *in vitro* by the addition of 20-HE. These primary cultures finally produced established cell lines from both wing and leg discs and were named with the prefix CME after their founders (Currie, Milner and Evans). At the same time as these cell lines were being developed another group of investigators had independently managed to succeed in producing disc cell lines. In this study (Ui *et al* 1987) disc cells were dissociated according to Wyss's earlier study of 1982, using protease and suspended in a medium (Cross and Sang 1978) supplemented with insulin, 10% FBS and usually with either larval haemolymph or supernatant from primary embryonic cultures.

Therefore established imaginal disc cell lines are now available in *Drosophila* cell biology. These represent a significant advance in the cell biology of *Drosophila* providing a useful defined epithelial cell line for a whole range of studies. However before these cell lines can be effectively used some of their properties need to be analysed. This thesis is about the cell lines that were established by Currie, Milner and Evans in 1988. The work has centred on trying to characterise the cells in culture and attempts to analyse their properties *in vitro*.

Figure 1

Figure 1 : Schematic representation of the imaginal discs in the *Drosophila* larva and the structures that they give rise to. Only one of the three leg discs is illustrated. (After Fristrom et al 1969).



Chapter 2

General Materials and Methods

General Materials and Methods

This chapter gives details of general methods that were used throughout the study. Each chapter has individual sections dealing with more specific methods that relate to that particular chapter. Many of these more general methods are mentioned in Currie, Milner and Evans (1988); Peel, Johnson and Milner (1990); and Cullen and Milner (1991). The following sections deal with routine cell culture methods, cryogenic preservation of cells, electron microscopy and protein determination techniques.

Cell culture

The cells were kept in Shields and Sang's MM3 medium (Shield and Sang 1977—see Table 1) which had been modified for imaginal disc cell growth by the addition of insulin, 20-hydroxy ecdysone and a fly extract (see Table 2).

Culture medium

The culture medium is made up as per table 1, the pH adjusted with Sodium Hydroxide and sterile filtered with a 0.22 μm filter.

Preparation of additives

Fly extract

Fly extract was prepared from axenically cultured flies. 200 well nourished and egg-laying flies were taken and homogenised in 1.5 mls of MM3 medium without additives. Originally the flies were sexed and a ratio of 150 females to

50 males were used, however this was found to be unnecessary for routine cell maintenance although this is still done for culture initiation. This extract is then centrifuged at 2600 r.p.m. (1500g) for 15 minutes at 4°C. The orange supernatant is taken, off together with as much of the oily film as possible, which primarily consists of fat body. This is then heat treated in a water bath for 5 minutes at 60°C, to inactivate the enzyme tyrosinase which otherwise will turn the extract black. After heat treatment the extract is centrifuged at 2600 r.p.m. (1500g) for 1.5 hours at 4°C. The supernatant is taken off, filtered through a 0.22 μ m Millipore filter and frozen at -20°C.

Insulin

Insulin was obtained from Sigma (25 I.U./mg), 10 mg dissolved in 500 μ l of 0.01 N Hydrochloric acid (HCl) and made up to a concentration of 12.5 I.U./ml in Phosphate Buffered Saline (PBS) without Calcium or Magnesium (D=).

Ecdysteroids

Ecdysteroids were obtained from Simes of Milan, stored at -20°C, weighed with a Mettler ME30 microbalance and dissolved in MM3 medium without additives.

Routine Passaging of cells

Cells were routinely kept in culture by weekly passaging. The old culture medium was removed and the cells washed with D=, this was done to remove the inhibitory effects

on trypsin action of serum components in the medium. Then a 0.1% solution of trypsin (Difco Bacto) in 2 mM Ethylenediaminetetra-acetic acid (EDTA) in D= was added to cover the cell layer and the cells exposed for 5 minutes at room temperature. After this period 2 mls of normal medium containing 2% Foetal Calf Serum (FCS) was added to the dish, the serum inactivating the trypsin. Then the cells were pipetted off the tissue culture plastic and centrifuged at 1500 r.p.m. (150 g) for 5 minutes. The supernatant was removed and the pellet resuspended in 1 ml of complete medium. An aliquot was taken and a cell count performed on a diluted cell sample in a Neubauer haemocytometer. Cells were plated out at a concentration of 3×10^6 cells/ml onto a 5 cm Petri dish (Nunc). The cells were then placed in a humidified incubator which is set at 25°C with a 5% CO₂ atmosphere.

Cryogenic preservation

Cells can be frozen and stored in liquid nitrogen. Cells are used that are in active growth, the cells are passaged as per normal and counted in a haemacytometer. The cell concentration is diluted in complete medium to 1×10^7 cells/ml and 10% sterile Dimethyl sulphoxide (DMSO) added, 1 ml of this cell suspension is then put in a cryogenic storage vial (Jencons). These vials are then immediately put in a polystyrene freezing container (see Fig. 2.1) as DMSO is toxic to the cells whilst the cell suspension is still unfrozen. This container consists of an Amersham polystyrene box with a sponge placed in the bottom and a foam partition placed over

the top and a foam rack placed on top into which liquid nitrogen has been poured until the sponge at the bottom is just covered. The vials are kept in this container for 2 hours and then transferred to a liquid nitrogen freezer.

Microscopy

Cells were routinely observed under phase contrast with a Leitz Diavert inverted photomicroscope. For fixed slide preparations of cells (e.g. immunocytochemistry) a Zeiss Universal microscope was used.

Routine scanning electron microscopy(SEM) and transmission electron microscopy(TEM)

See table 5 for composition of buffer solutions

SEM

Cells were prepared for microscopy in two main ways depending on how the cultures were grown. Cells were either grown on plastic coverslips (Lux-Thermanox) which could easily be manipulated and cut into pieces for preparation or were grown in normal culture dishes, the bottom of which was later punched out. The general procedure was as follows. Cells were washed firstly in D= and then in fixation buffer (50 mM phosphate buffer pH 7.57) without fixative and then fixed for 20 minutes in 2.5% glutaraldehyde in buffer. The cells were then rinsed 2-3 times in rinsing buffer and dehydrated in a graded series of alcohols (50,70,90,96,100%) and changed twice in absolute alcohol. Samples were critical point dried in a

Samdri 780 critical point drier and sputter coated in an Emscope Sc500. The material was examined in a Jeol 35CF scanning electron microscope.

TEM

For TEM preparation cells were either grown on Lux plastic coverslips and embedded directly or the cells removed from the culture surface embedded in agar. The general procedure for preparation was as follows. The culture medium was taken off and the cells washed 2-3 times in D= and then in fixing buffer. The cells were then fixed in 2.5% glutaraldehyde for 20 minutes at room temperature, washed twice in rinsing buffer for 10 minutes and then postfixed in 1% Osmium tetroxide for 20 minutes. The cells were then dehydrated in a graded series of alcohols and embedded in Araldite resin. Sections were cut on an LKB ultramicrotome, stained with uranyl acetate and lead citrate (Reynolds 1963) and viewed with a Philips 301 Electron microscope.

Protein determination

Bradford method

This protein assay relies upon the binding of a dye to any protein present producing a colour change which can be measured in the spectrophotometer. This is based on an assay developed by Bradford (1976). The results from unknown protein samples are compared to a set of samples of known protein concentration, a standard curve fitted and the results of the

unknowns read off.

Standard curve- Bovine serum albumin (BSA) was used as a standard at a concentration of 1 mg/ml. Using this stock concentration aliquots were pipetted out with a Hamilton syringe (Sigma), these were as follows: 0, 2, 4, 6, 8, 10, 15 and 20 μ l. Two replicates were taken of each standard.

Unknown sample- Two protein samples were taken from the samples generally of 5 and 10 μ l. Care was taken to ensure that all the protein was thoroughly dissolved, the samples were sometimes diluted in 0.1 M Sodium Hydroxide (NaOH) which was also used in dissolving the samples.

To the unknown samples, an equivalent amount of 0.2 M NaOH was added to dilute the samples. Then 200 μ l of the Bradford reagent (50 mg Coomassie Blue (Brilliant Blue G) in 25 mls 95% alcohol, 50 mls 85% Phosphoric acid and made up to 100 mls with water) and 800 μ l of distilled water was added, the reaction tubes thoroughly mixed and allowed to stand for 15 mins. The solution was then read at 595 nm in the spectrophotometer. Values for the Optical Density (O.D.) were read and a standard curve made (or regression analysis used) using the O.D. values obtained from the samples of known protein concentration. The protein concentration of the unknown samples can then be read off this calibration curve.

Table 1

Shields and Sang's MM3 medium (Shields and Sang 1977)

The pH of 6.7-6.8 is achieved by the addition of 1% NaOH.

<u>Reagent</u>	<u>mg/100 ml</u>	<u>Reagent</u>	<u>mg/100 ml</u>
Aspartic acid	30	Cysteine HCL	20
Threonine	50	Lysine HCL	85
Serine	35	Proline	40
Asparagine	34	Glycine	50
Glutamine	60	KCl	260
alpha-alanine	150	MgSO ₄ .7H ₂ O	400
Valine	40	Monosodium glutamate	786
Methionine	25	NaH ₂ PO ₄ .2H ₂ O	88
iso-leucine	25	CaCl ₂ .2H ₂ O	932
Leucine	40	Glucose	1000
Tyrosine	25	T.C. Yeastolate(Difco)	100
Phenylalanine	25	Choline chloride	5
beta-alanine	25	Oxaloacetic acid	25
Histidine	55	BIS-TRIS buffer	105
Tryptophan	10	Penicillin G.Na	3
Arginine	50	Streptomycin sulphate	10

Table 2: Complete medium used for imaginal disc cell growth

Shields and Sang's MM3 medium with the additions as per Currie, Milner and Evans (1988) and Cullen and Milner (1991)

2% Foetal Calf Serum (FCS) -added 2 days before
1 ng/ml 20-hydroxy ecdysone
0.125 I.U./ml Insulin
25 µl/ml Fly Extract

This was then sterile filtered using 0.22 µm Millipore filters.

Table 3: Saline solutions used in routine tissue culture

Phosphate buffered saline (PBS)

Dulbecco's formula, (Dulbecco and Vogt 1954) pH 7.2, filter sterilised.

NaCl	8000 mg/l
KCl	200 mg/l
Na ₂ HPO ₄	1150 mg/l
CaCl ₂ .2H ₂ O	132 mg/l
KH ₂ PO ₄	200 mg/l
MgCl ₂ .6H ₂ O	100 mg/l

D (PBS, Ca²⁺ and Mg²⁺ free)

Dulbecco's formula, (Dulbecco, and Vogt 1954) pH 7.2,
filter sterilised.

NaCl	8000 mg/l
KCl	200 mg/l
Na ₂ HPO ₄	2000 mg/l
KH ₂ PO ₄	400 mg/l

Table 4: Buffer solutions used for electron microscopy

Phosphate buffer (50 mM, pH 7.57)

KH ₂ PO ₄ (anhydrous)	0.41 g/500 ml
Na ₂ HPO ₄ (anhydrous)	3.12 g/500 ml

Glutaraldehyde fixative.

Buffer	90 ml
Sucrose	0.6 g
25% Glutaraldehyde	10 ml

Rinsing buffer.

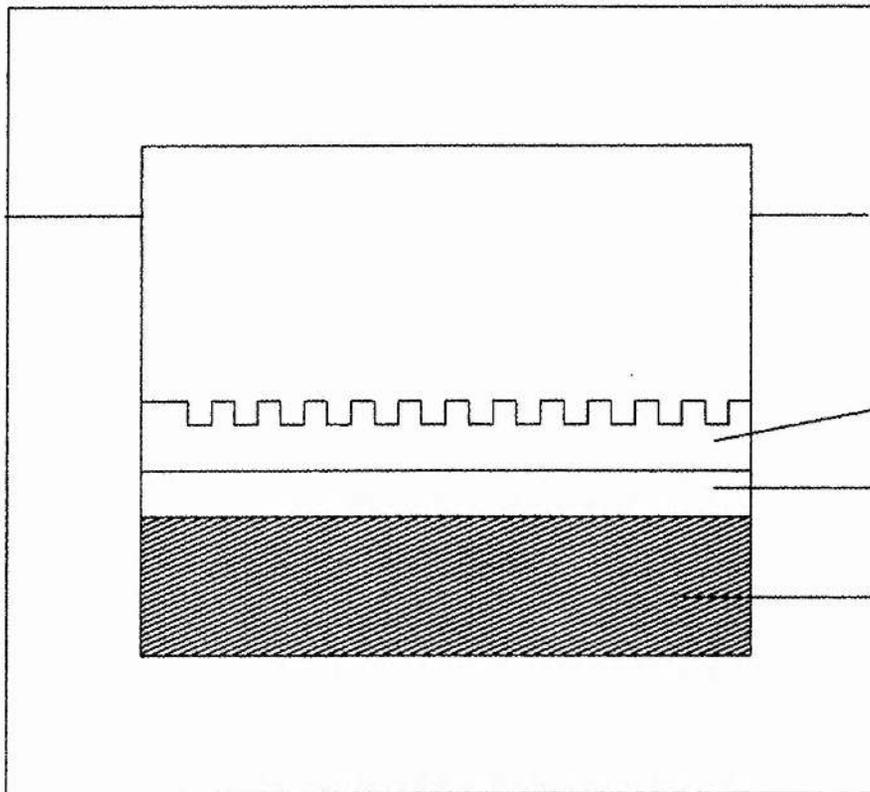
Buffer	100 ml
Sucrose	2.0 g

Osmium buffer.

Buffer	100 ml
Sucrose	4.0 g

Figure 2.1

Figure 2.1 : Diagram of Polystyrene box used to freeze cells before transferring to the liquid nitrogen freezer.



Vial rack

Partition

Sponge

Chapter 3

Characterisation of imaginal disc cells in culture

Characterisation of imaginal disc cells in culture

Introduction

Cells from both wing and leg imaginal discs have been growing in culture for three years and have reached passage numbers of over fifty. These cultures can therefore be truly described as established cell lines.

Imaginal discs consist of a polarised epithelium surrounding a lumen, the epithelium facing inwards and surrounded basally by a non-cellular basement lamina (Fig. 3.1). There are four different cell types that make up the disc, namely epithelial cells that make up the true epithelium and the peripodial membrane, aepithelial cells that are thought to be muscle precursor cells and nerve and tracheolar cells (Ursprung 1972). Although the great majority of imaginal disc cells are epithelial there are a number of different cell types which could conceivably give rise to a component of our established imaginal disc cell lines. It is also possible that once in culture disc cells could undergo dedifferentiation and lose some of their epithelial characteristics so it is important to analyse what the established cell lines actually consist of.

The ultrastructure of disc cells in culture was analysed and in an attempt to assess their stability and cellular origin in culture two different experiments were carried out. Firstly, a study of the chromosome complement of the cells was undertaken to see whether the cells showed any signs of karyotype instability in culture. Secondly in order to

investigate the cellular origin of the cells and analyse the stability of cell morphology in culture, the cell lines were cloned. This would directly test whether a single cell could give rise to several different morphologies in culture and determine whether or not the established cell lines are derived from a defined cell type.

Ultrastructural analysis

The vesicles in primary culture show many of the features of intact imaginal disc tissue. The vesicles consist of an epithelium surrounding a lumen which shows apical-basal polarity (Fig. 3.2) and possess septate desmosomes, zonula adherens, apical microvilli and gap junctions (Fig. 3.3, 3.4 ; Peel et al 1990) consistent with what is seen in mature imaginal disc tissue (Poodry 1980).

Established cell lines derived from these primary cultures show a different structure. Cells do not grow as an organised epithelium and lacked any apical-basal polarity (Fig. 3.5, 3.6). The cells do not show any signs of contact inhibition of growth and often grow up into large multicellular aggregates (Fig. 3.7). The fine ultrastructure of these cells shows that the cytoplasm exhibits numerous free ribosomes and shows very little endoplasmic reticulum, and this is very similar to the fine structure of the columnar epithelial cells of the imaginal disc (Ursprung 1972). An occasional piece of rough endoplasmic reticulum (RER) is seen often associated with a cell phagocytosing an adjoining dead cell fragment (Fig. 3.8). Cells seem to lack the junctional complexes that are characteristic of disc tissue.

There appear to be four different classes of cells in culture, epithelial and fibroblast-like cells which constitute the major component of the lines along with smaller numbers of lamellocyte-like and sickle-shaped cells (Figs. 3.9.). These four types of cells remain in culture over a long period of time with the exception of the sickle-shaped cells. These cells are very common in the early stages of culture but with time they become a smaller proportion of the established cell lines and after 10-15 passages are hardly ever seen in culture. Sickle-shaped cells are non-adherent to the tissue culture plastic and are often seen floating above the other cells, although they do occasionally attach.

Virus-like particles are often seen frequently in paracrystalline array both in the nucleus and cytoplasm of cells in culture (Figs. 3.10). Some of these are very similar to the arrays seen during the cytoplasmic morphogenesis of Picornaviruses (Brun and Plus 1980).

Karyotype analysis

Cells were plated out at a concentration of 3×10^6 cells in a 5cm Petri dish (Nunc) and left for a couple of days to ensure that they were in active exponential growth. The cells were incubated, harvested and fixed as in Schneider (1973) but chromosome spreads were carried out differently as below.

Cells were incubated with $0.06 \mu\text{g/ml}$ colcemid (Demecolcine) for 18 hours. After this incubation the cells were pipetted off the culture surface. A volume of distilled water (about 4 ml) was then added to the culture volume and

left for 20 minutes with another volume of distilled water being added after 10 mins and again after 15 mins. The fixative used was a mixture of glacial acetic acid:methanol (1:3). A few drops were added to the cell suspension now heavily diluted with distilled water, this dilution caused the cells to swell making the chromosomes more visible later on. The cells were centrifuged down for 10 minutes at 1000 r.p.m. Then a small amount of fixative (0.1 ml) was added to the pellet, which was then allowed to stand for 20 minutes at room temperature before a larger volume of fixative (2.9 ml) was added. The pellet was mixed gently by blowing air through the solution using a pipette, thus avoiding bursting the swollen cells and consequently losing the chromosome complement.

The cells were centrifuged, washed in fresh fixative, pelleted again and resuspended in a small amount of the supernatant fixative. Slides were pre-cooled on a block of dry ice for 5-10 mins so that a thin sheet of frost covered them. The slides were taken off and allowed to warm slightly until the frosting started to disappear. Then two drops of the cell suspension were dropped onto the slide, any excess blotted off and the slide gently warmed over a spirit burner. After drying the slides were stained for 10 mins in a 3% Giemsa solution in buffer at pH 7.2. Karyotypes were then scored. The karyotypes of the cells in culture showed that the vast majority have a diploid chromosome complement. The percentage of diploid cells in the wing cell line CME W2 was 92% , the remainder being made up of aneuploid cells. This culture was at passage number 20 and had been in culture for approximately 10 months. Most of the cells that showed a change in the chromosome complement

showed a doubling of the complement (4n). The leg line CME L2 which had been in culture for about 5 months and had a passage number of 15 showed a similar percentage of diploid cells (Fig. 3.11).

Cloning

The two conventional techniques for cloning *Drosophila* cells consist of either using a feeder layer of irradiated or Mitomycin-C treated cells and plating the single cell suspension in soft agar (Bernhard and Gehring 1975; Richard-Molard and Ohanessian 1977; Schneider and Blumenthal 1978) or using conditioned medium and cloning by progressively diluting the cells (Nakajima and Miyake 1975; Ashburner 1990; Schneider and Blumenthal 1978). These methods were attempted with imaginal disc cell lines. Cells were diluted in differing concentrations of conditioned medium: fresh medium in 96 well plates. Conditioned medium was taken from exponentially growing cultures and filtered, cells were diluted to a concentration where on average only 1-2 cells would be present in each well which contained 400 μ l of medium. Cells adhered to the tissue culture plastic and often threw out cell processes but even after 3-4 weeks showed no signs of division. Feeder layer methods were also tried, cells were plated out at 3×10^6 in a 5cm Petri dish and allowed to grow for 2-3 days to ensure that they were in active growth and producing conditioning factors. Cells were then irradiated or treated with mitomycin-C, to stop cell division and a layer of soft agar (0.3%) poured on top of this feeder layer, then

another layer of soft agar was added together with a dilute suspension of cells (200 cells/ml).

However these methods were found to be unsuccessful and so a more unusual method was used derived from mammalian tissue culture techniques. This consisted of the use of a special cloning dish which was manufactured from two different Petri dishes (Fig. 3.12). The plate was made by sticking an inverted 3cm Petri dish base to the underside of the lid of a 5cm Petri dish. This was achieved by dipping the rim of the smaller dish into propylene oxide, thus partially dissolving the plastic and allowing it to be stuck to the lid of the larger dish. This method was used rather than glue as it produced a direct binding between the two plastic surfaces without the use of any substance which might prove toxic and affect cell growth. A feeder layer of cells was plated out at a concentration of 3×10^6 cells/ml on to the bottom of the 5 cm dish and left to grow for 2-3 days to ensure that the cells were in active growth and conditioning the medium. Single cells were prepared using standard dissociation procedures, plated out on to the upturned bottom of the 3 cm dish and allowed to adhere overnight. Routinely a concentration of 100 cells/ml was used producing approximately 10-12 clones per dish. The medium was then pipetted off, the lid inverted and laid onto the 5 cm dish containing the feeder layer so that the surface of the 3 cm dish was immersed in medium. This arrangement allowed the diffusion of conditioning factors from the feeder layer to supplement the medium and enable the single cells to give rise to clones but prevented any contamination by the cells of the feeder layer which adhered

firmly to the plastic of the 5 cm dish. The feeder layer was changed at 4-5 day intervals to ensure that the cells were in active growth and adherent to the plastic. Due to the arrangement of the two dishes, the feeder layer cells and the cloning plate layer were in different planes of focus allowing regular visual checks to be made ensuring that there was no contamination of the cloned cells from the feeder layer. The positions of the single cells were mapped out and carefully followed during growth to ensure that the groups of cells were clonal (Fig. 3.13). Once the clones were growing and had reached a sufficient cell density to sustain growth the lid with the attached base was transferred to a fresh dish without a feeder layer for further growth. After a few weeks clones that were large enough were transferred to 96 well plates and then to progressively larger culture vessels and grown up as cloned cell lines.

These cloned lines on analysis showed the whole range of different morphologies seen in uncloned lines (Fig. 3.14) indicating that the cells were derived from a specified cell type rather than from several. Some cloned lines appear to be more uniform in appearance than others and at least one cell line seems to show a preponderance of epithelial-like cells rather than fibroblasts, even so these lines exhibit all the different classes of morphology. The cloned line C1.8+ shows a very uniform appearance especially in the early stages of culture (Fig. 3.15.1) whereas the cloned line C9 has a much more disorganised appearance and exhibits a large percentage of cells with an epithelial-like morphology (Fig. 3.15.4). Cloned cell lines have a higher growth rate than uncloned cell

lines and are more stable in culture than other uncloned cell lines.

Table 1: Cloned lines obtained

<u>Wing Cell lines</u>	<u>Morphology</u>	<u>Ecdysone</u>
8	F	++
7	F	++
13	F	++
C9	E	+
9	F	++

Leg cell lines

Astrew	F	+++
Prussacks	F	+++

E= Epidermal

F= Fibroblast-like

+ little response

++ dramatic response

+++ dramatic response at lower titre

Discussion

The ultrastructure of cells in established lines differs from that seen in mature imaginal disc tissue. In primary culture, vesicle cells appear to be very similar to disc tissue. Cells retain their apical-basal epithelial polarity,

possess numerous junctional complexes, and have microvilli which project inwards and appear to secrete extracellular cuticle-like material. Established cell lines grow in a less organised way, the cells do not show any apical-basal polarity and seem to lack the junctional complexes seen in primary culture.

In primary cultures there is a period of vesicle growth from disc fragments and finally cells appear on the plastic often close to a vesicle or fragment which give rise to clumps of actively growing cells and subsequently to cell lines (Cullen and Milner 1990). Vesicle cells have the ability to enlarge, often to quite large dimensions but do not give rise to cell lines. Only vesicle cells retain their apical-basal epithelial polarity.

Epithelial polarity is dependent on a number of different factors, two of the most important being extracellular matrix and polarity of nutrient uptake (Simons and Fuller 1985; Rodriguez-Boulan and Nelson 1989). In imaginal discs *in vivo* the basal surface of the columnar epithelial cells is nearest the basement membrane, the apical surface facing inwards to the lumen (Poodry 1980). Imaginal cells receive their nutrients in a unidirectional manner from the haemolymph which bathes the basement membrane on the exterior surface of the disc. In imaginal epithelial cells it is likely that the combination of the basement membrane with its constituent extracellular matrix molecules and the nutritive uptake from the basal surface generates and maintains their apical-basal polarity.

Cells in culture do not secrete the complicated basement membrane that is seen *in vivo*. *In vivo* the basement membrane consists of a whole range of different extra-cellular matrix molecules, notably laminin (Garzino et al 1989), collagen (Fessler and Fessler 1989), fibronectin (Gratecos et al 1988) and proteoglycan (Brower et al 1987). Haemocytes which are often found adhering to the basement membrane of third instar imaginal discs (Milner and Muir 1987) stain strongly for collagen IV and laminin (Lunstrum et al 1988, Le Parco et al 1986) major components of the *Drosophila* basement membrane. It may be that *in situ* basement membrane is secreted by both imaginal epithelial cells and haemocytes in a collaborative manner. This is the case in a vertebrate situation where fibroblasts that adhere to muscle cells cooperatively contribute to a basement membrane that is found between them (Sanderson et al 1986). In the developing wing of *Manduca sexta*, haemocytes have been found to contribute to the formation and degradation of the basal lamina (Nardi and Miklasz 1989), suggesting that haemocytes are important in the formation of the basal lamina and hence in maintaining apical-basal polarity. Therefore in an *in vitro* situation where the epithelial cells are isolated from any such interaction then basement membrane may be more difficult to produce and this may contribute to the lack of discernible extracellular material and loss of polarity.

In primary culture vesicle cells retain their apical-basal polarity. The cells receive their nutrients unidirectionally via their basal surfaces by virtue of their organisation. Such vesicles do not possess a discernible

basement membrane as seen in imaginal discs *in vivo*. However these vesicles derive from disc fragments which presumably contain basement membrane components and these may aid in the development of this type of growth. In established lines cells do not grow in a vesicle morphology and adhere directly to the tissue culture plastic. In this mode of growth the cells basal surface is adhering to the substrate and is isolated from the nutritive medium. So therefore it is not surprising that cells which adhere in this way do not exhibit any apical-basal polarity and lack the junctions that are associated with this mode of growth. Experiments growing established cell lines on nitrocellulose membranes allowing nutrient uptake from the cells basal surface suggest that this method of cell culture allows the cells to develop more intercellular junctions than is seen with ordinary culture methods (R.Wheater personal communication). This suggests that apical-basal polarity may be re-established under different culture conditions, perhaps with reconstituted basal membrane components.

Studies of virus prevalence in both natural and laboratory populations of *Drosophila melanogaster* reveal that both have a high level of endogeneous viruses. In data collected by Brun and Plus (1980) studying both natural and laboratory populations of *Drosophila*, 40% and 47% respectively were infected with viruses mostly of the Picornavirus type. It is not surprising given the high prevalence of endogeneous viruses in populations of *Drosophila melanogaster* that cell lines are infected. Indeed all of our lines that have been studied so far have been found to be infected. A study of the prevalence of endogeneous viruses in

permanent *Drosophila* cell lines revealed that 69% contained one or more types of virus (Plus 1978). In our cell lines virus particles are more common in aggregates of cells at later stages of passage, where the cells will probably be starved of nutrients. This is to be expected as in flies more virus particles are found in suboptimal conditions where the flies are old or malnourished (Schneider 1975). The greater prevalence of viral infection in *Drosophila* cell lines in general and in our cell lines in particular reflects a number of different factors. Cell lines have been in existence for a long period of time, 20 years for the K_C cell line (Echalier and Ohanessian 1970), 3 years for imaginal disc cell lines (Currie, Milner and Evans 1988), and over such a long period of time the opportunity for contamination is great. The high level of infectivity in our cell lines could possibly be a reflection of the inclusion of a fly extract in the medium components, meaning that the cells are continuously exposed to a potentially contaminating agent. In flies whilst vertical transmission between generations occurs not all *Drosophila* viruses are capable of such transmission, reducing the overall load as compared to cell lines where no such barrier to transmission exists. It appears that in *Drosophila* there is a naturally high level of virus infection and that virus infection does not appear to greatly harm the cells infected, so it should not be regarded as a great impediment to the use of imaginal disc cell lines.

Karyotype analysis of the cells in culture shows that they retain a diploid chromosome complement even in cell lines that have been in culture for a considerable length of time.

This shows that the chromosome complement of the cells remains fairly constant and that the culture conditions do not cause any karyotype instability.

Morphological analysis of established cell lines revealed that there are 4 different cell types seen in culture. These were classified as epithelial and fibroblastic-like which make up the bulk of the culture together with smaller numbers of lamellocyte-like and distinct sickle-shaped cells (Currie, Milner and Evans 1988). These cell types persisted in culture over a long period with the exception of the sickle-shaped cells which gradually became less of a component of the lines and after approximately 10-15 passages largely disappeared from culture. This appears to indicate that these cells do represent a distinct subpopulation of cells within the cell line and that they can not compete with the epithelial and fibroblastic-like cells which overgrow them.

Imaginal discs consist of a number of distinct cell types that could have given rise to the different morphological classes of cells seen in culture. In the mature imaginal disc the majority of cells are epithelial. These can be subdivided into the tall, columnar cells that make up the disc proper, and the more squamous cells that form the peripodial membrane which is involved in disc evagination (Milner, Bleasby and Kelly 1984). Other components of the disc include the adepithelial cells, thought to be muscle-precursors. These show no pronounced polarity and have a cuboidal or squamous morphology and are found between the true epithelial layer and the basement lamina (Poodry and

Schneiderman 1970). Between these two layers is also found a small proportion of nerve cells, which go to form the neuronal component of the bristle cell complex and some tracheolar cells. Besides the actual cellular components of 3rd instar imaginal discs there is also a possibility of contamination with blood cells, as these have been found to adhere to the basement lamina of the disc on dissection (Milner and Muir 1987) and could conceivably have been included in primary cultures of dissociated disc tissue. In established cell lines some cells are seen that have a very similar morphology to a class of haemocytes termed lamellocytes (Rizki 1978) and have themselves been designated lamellocyte-like cells (Currie, Milner and Evans 1988). This raises the possibility that a small proportion of an established cell line is derived from these haemocytes. The sickle-shaped cells that appear early on in the evolution of an established cell line do seem to represent a distinct sub-population of cells, and are gradually lost from culture with increasing passage number suggesting that they are overgrown by the more proliferative epithelial and fibroblastic-like cells. So it seems likely that these cells originate from a distinct subset of imaginal disc cells.

Morphological analysis of cloned colonies and established cloned lines indicated that the progeny of a single cell could contain cells with epithelial-like, fibroblast-like and lamellocyte-like morphologies (Fig. 3.14). If the established cell lines originated from a number of different cell types then cloned cell lines should exhibit a limited range of morphologies compared with uncloned lines.

However this is found not to be the case. Differences in individual cell morphology found in both established and cloned cell lines must therefore be the result of localised differences in cellular environment rather than differences in cellular origin. This indicates that the established cell lines do come from a defined cell type and not from a range of different cell types.

Figure 3.1

Figure 3.1: Diagrammatic representation of a saggital section of a leg imaginal disc, showing the general structure of an individual imaginal disc. The disc is an infolding of the larval epidermis consisting of a sac of epithelial cells. One side of this sac consists of thickened columnar epithelial cells which show typical apical-basal epithelial polarity with apical microvilli projecting into the lumen. The other side of the sac consists of thin squamous epithelial cells known as the peripodial membrane. Basement membrane material covers the entire disc, between this material and the disc epithelium lies the adeptithelial cells and the nerve and tracheolar cells (After Poodry and Schneiderman 1970).

Labelled structures include:-

- ad** Adeptithelial cells
- bm** Basement membrane
- CE** Columnar epithelium
- L** Lumen of disc
- N** Nerve stalk (connecting to the ventral ganglion)
- Pm** Peripodial membrane
- S** Stalk (connecting the disc to the larval hypodermis)

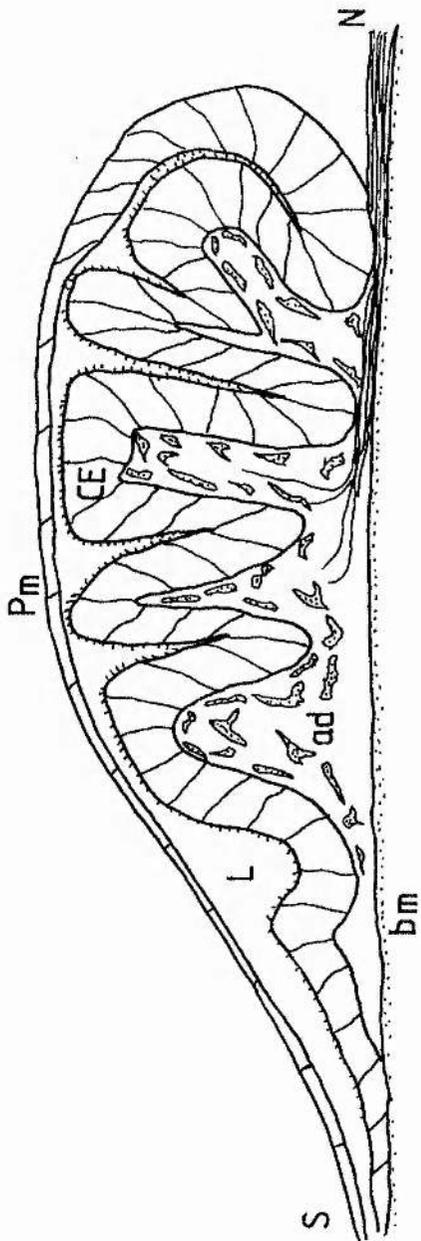


Figure 3.2

Figure 3.2 : Ultrastructure of vesicles in wing primary cultures. The vesicles show apical-basal polarity with apical microvilli (mv) secreting ECM material (E) facing the interior of the lumen (L). Scale bar = 3 μ m



Figure 3.3

Figure 3.3 : Ultrastructure of vesicles in primary culture.

Cells show apical-basal polarity with apical microvilli with plaques (ap) secreting extracellular material (E) into the lumen of the vesicle. Scale bar = 0.5 μm .

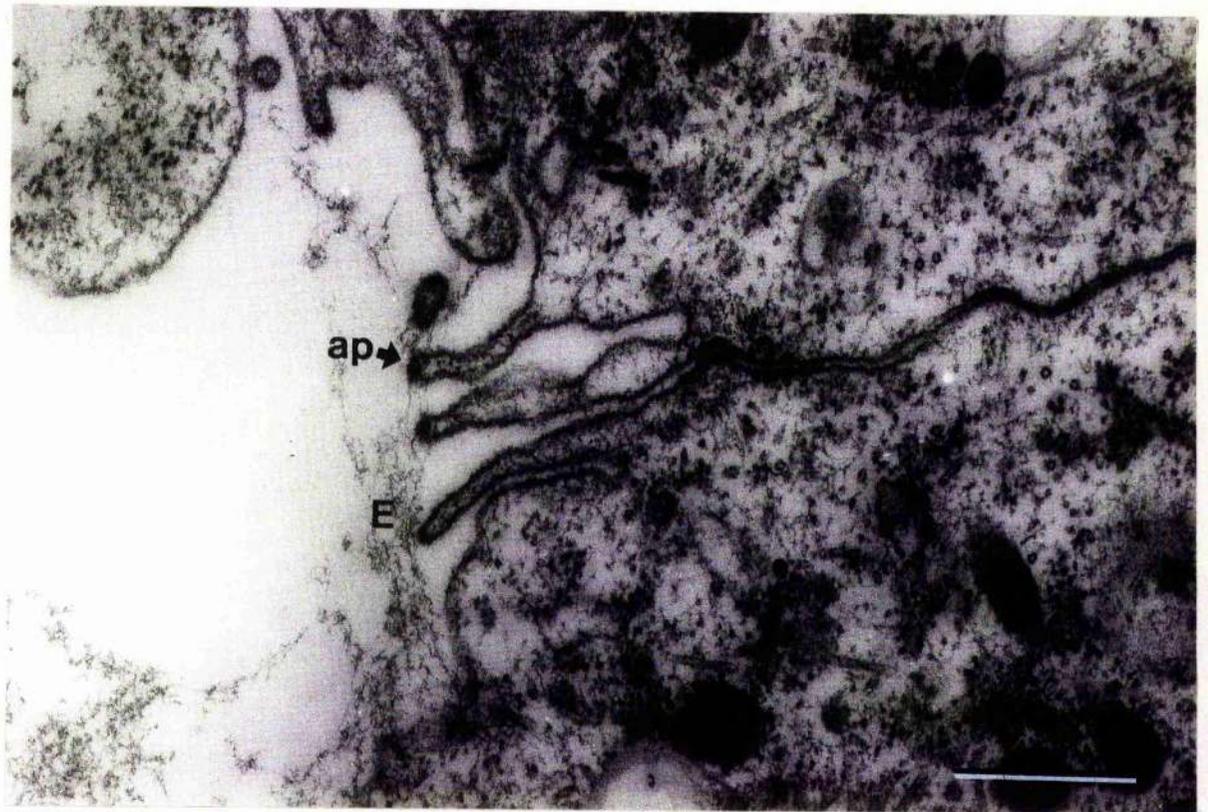


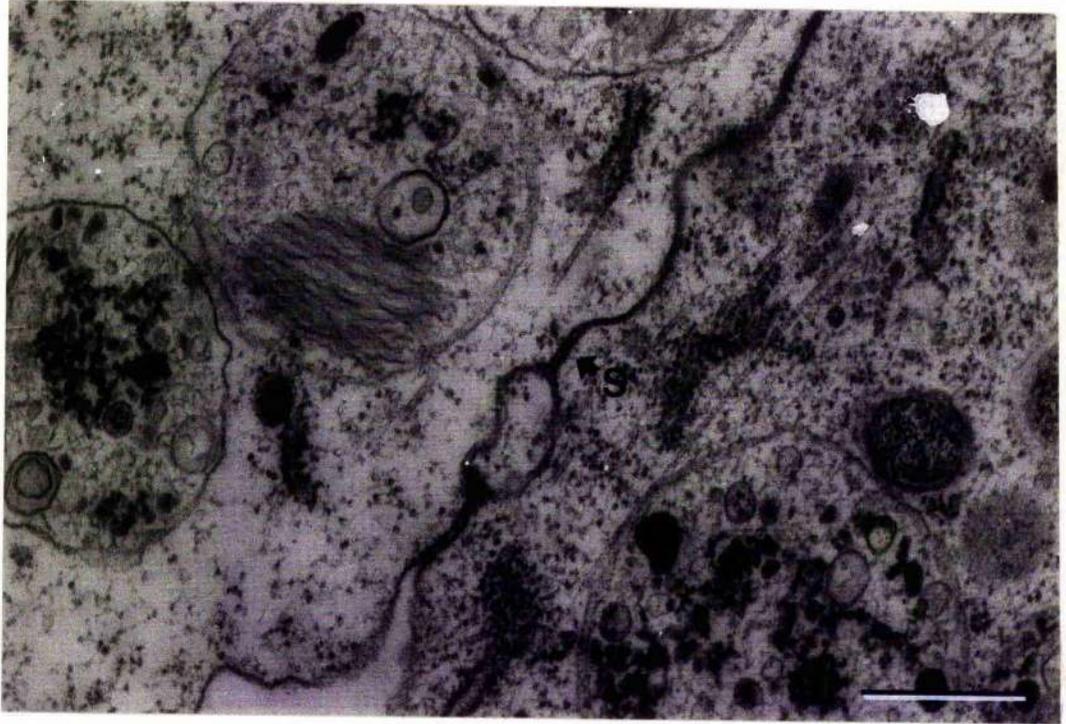
Figure 3.4

Figure 3.4 : Ultrastructure of vesicles from primary wing cultures. Intercellular junctions occur between cells such as septate and gap junctions.

Fig. 3.4.1 : Septate junction (S). Scale bar = 0.5 μm .

Fig. 3.4.2 : Gap junction (G). Scale bar = 0.4 μm .

1



2

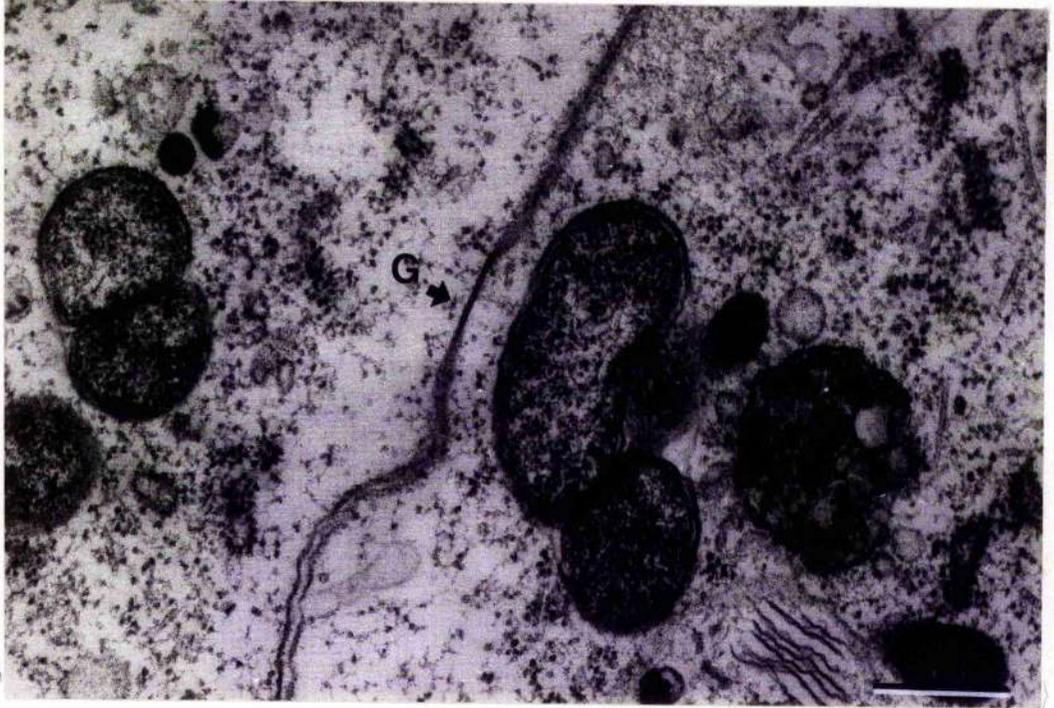


Figure 3.5

Figure 3.5 : Ultrastructure of cell lines. TEM of aggregate of cells from the wing cell line W2. Note the lack of any apical-basal polarity and the presence of numerous cell processes (cp) packed with microtubules. Scale bar = 2 μ m.

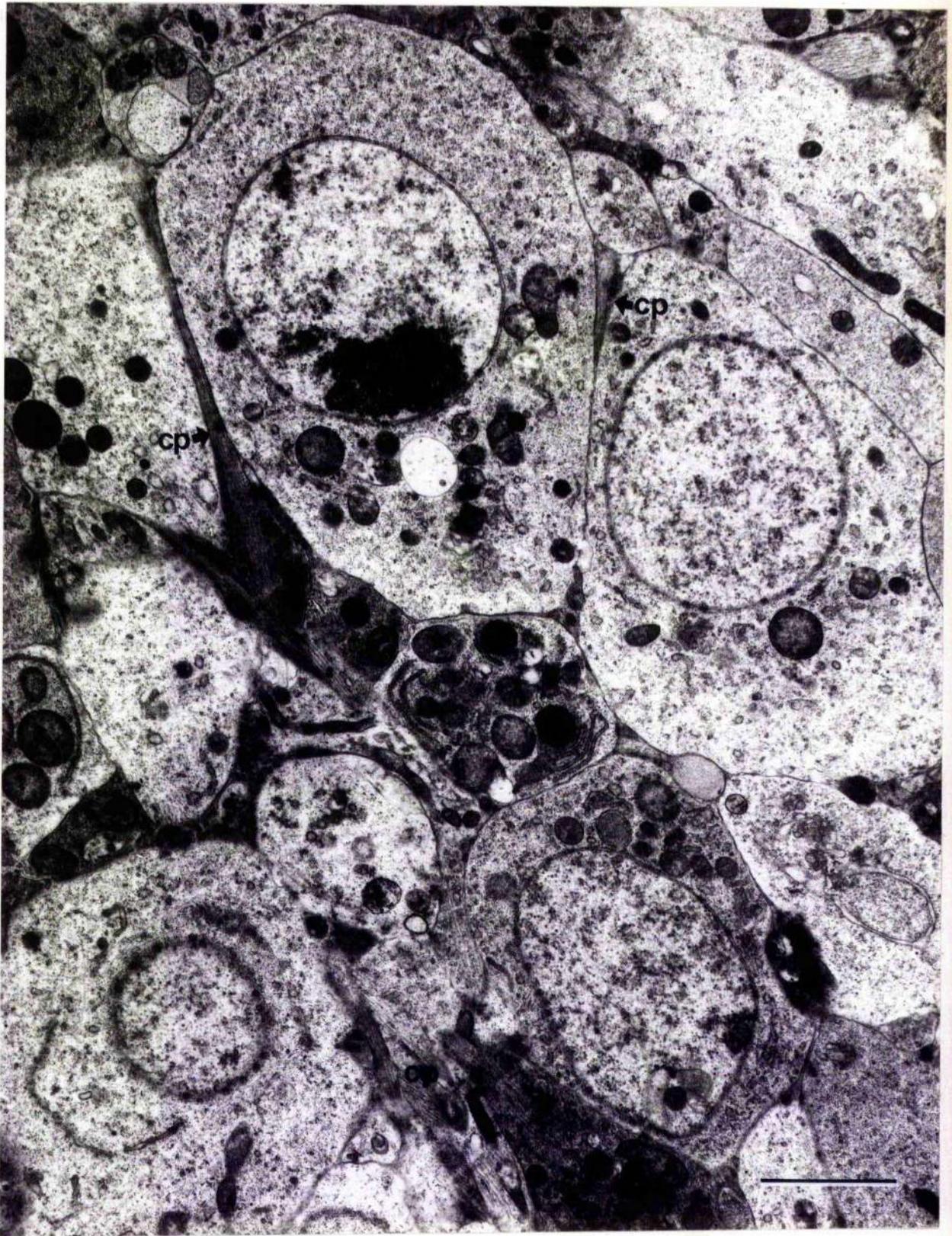


Figure 3.6

Figure 3.6 : Ultrastructure of disc cell lines. SEM of wing cell line W1, 2 days after passaging showing the lack of any organised epithelial structure. Scale bar = 10 μm .

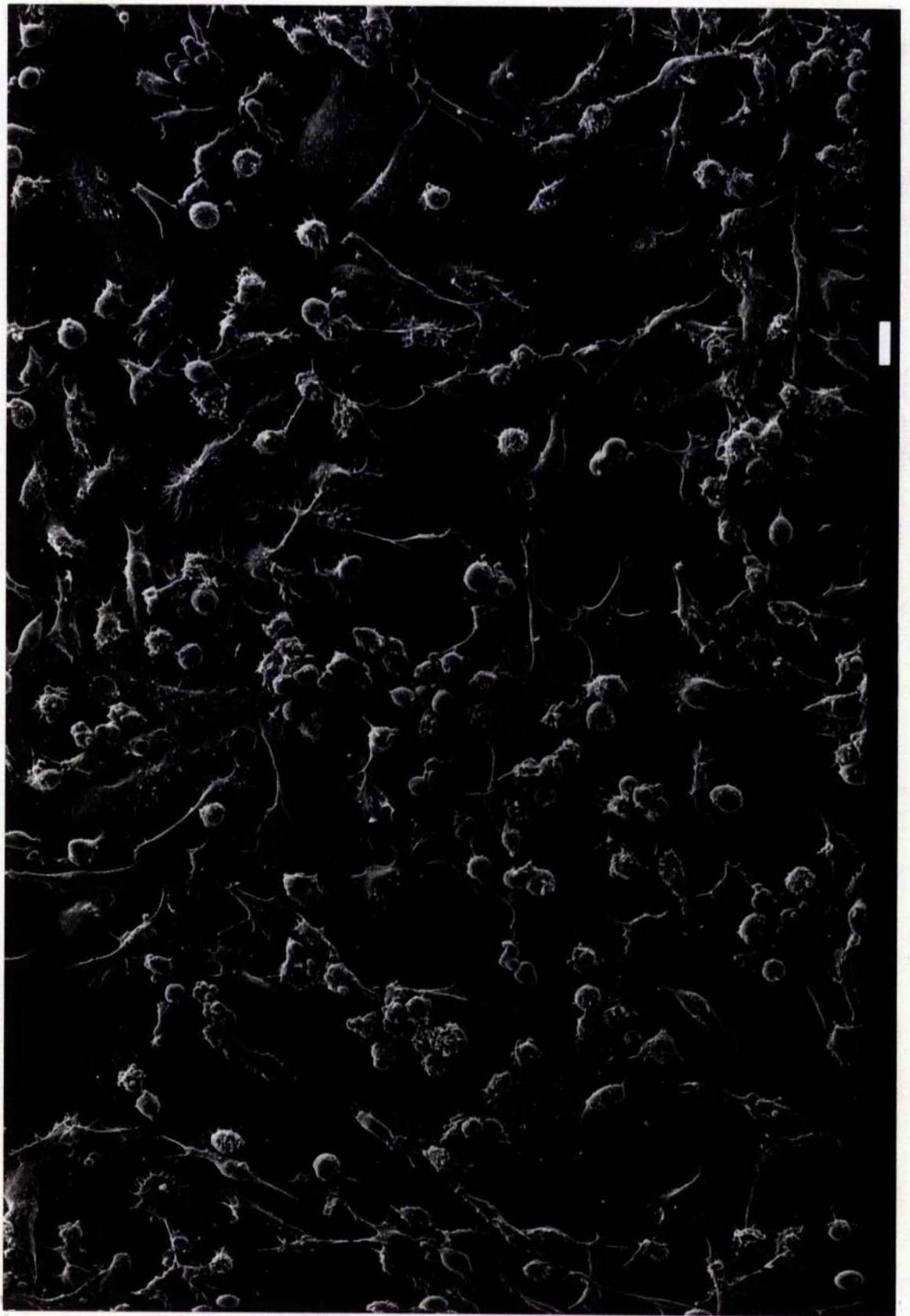


Figure 3.7

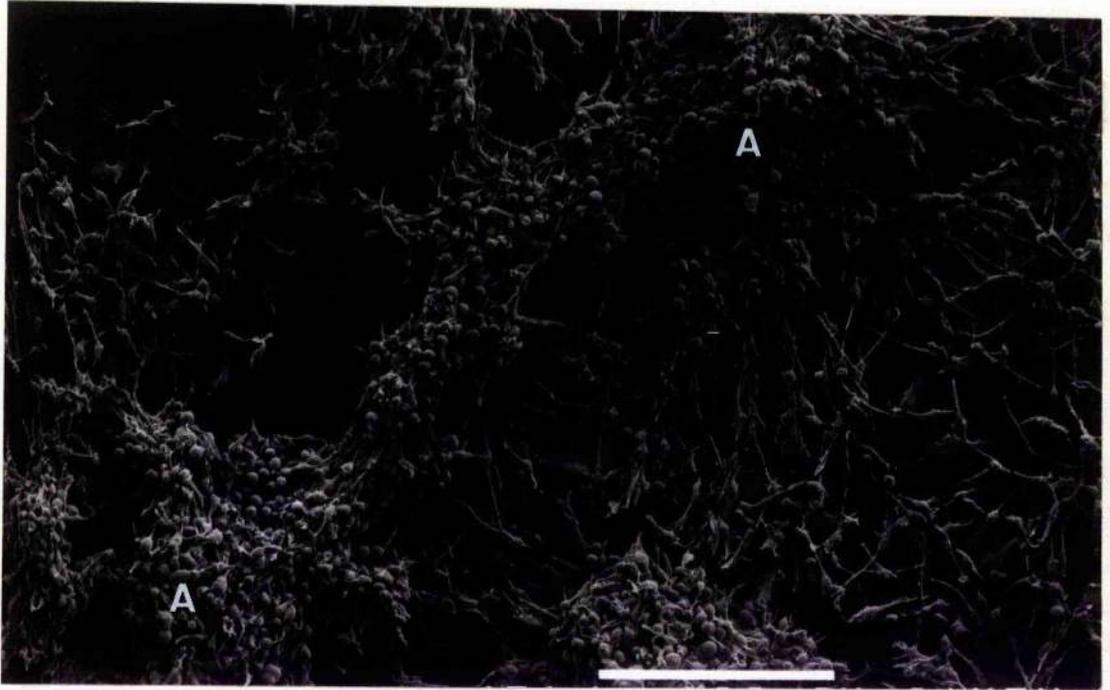
and

Figure 3.8

Figure 3.7 : Ultrastructure of disc cell lines. SEM of wing cells from the line W1 7 days after passaging. Note that the cells do not show any sign of contact inhibition and continue to grow beyond confluency into multicellular aggregates (A). Scale bar = 100 μm .

Figure 3.8 : Ultrastructure of disc cell lines. TEM of leg cell from the line LI phagocytosing a neighbouring dead cell fragment. Note the presence of Rough Endoplasmic Reticulum (R) in the cell, which is very uncommon in ordinary cells where ribosomes are free in the cytoplasm but seems to be a feature of cells that are acting as macrophages. Scale bar = 5 μm .

7



8

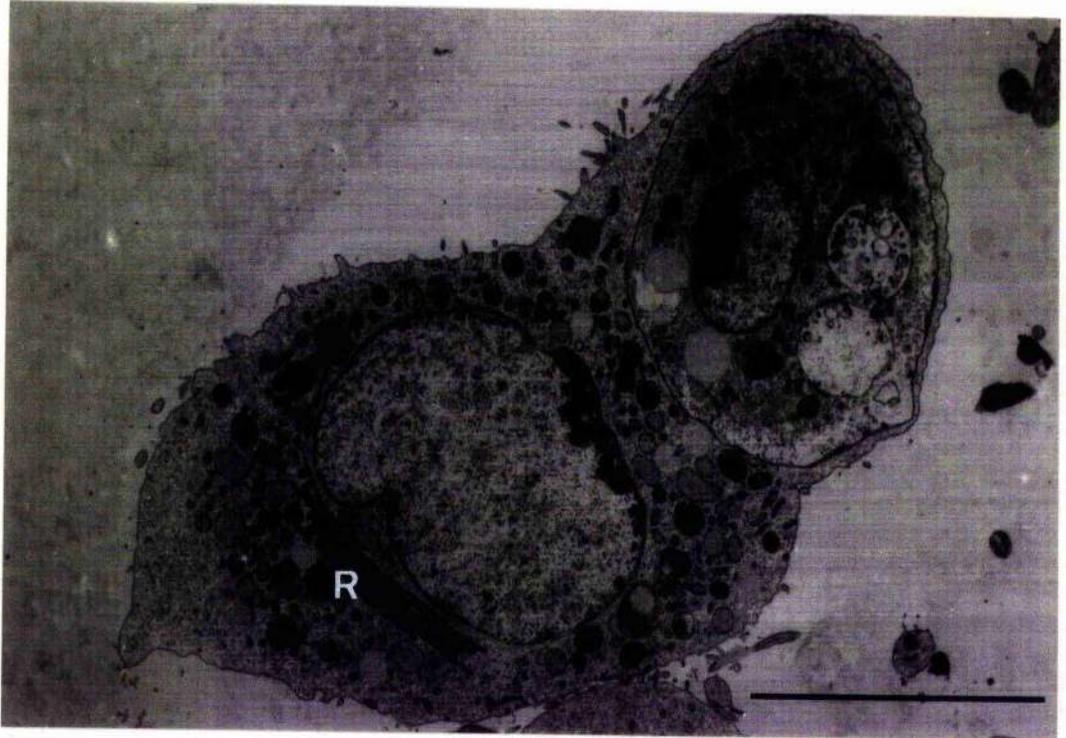


Figure 3.9

Figure 3.9 : Different morphological types of cells found in culture.

Fig. 3.9.1 : SEM of a leg cell line (LI) showing epithelial-like (E) cells. Scale bar = 10 μ m.

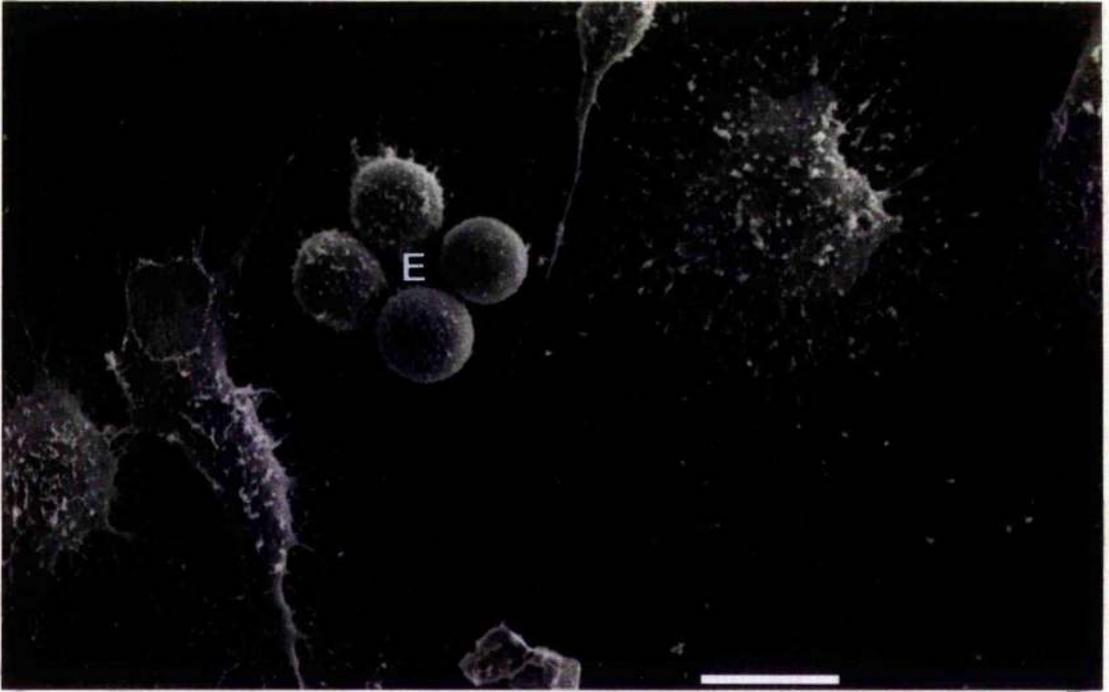
Fig. 3.9.2 : SEM of a wing cell line (W1) showing characteristic fibroblastic-like (F) cells. Scale bar = 10 μ m.

Fig. 3.9.3 : Light micrograph of a very large lamellocyte-like (L) cell (from wing cell line, P2) occasionally found in culture. Scale bar = 30 μ m.

Fig. 3.9.4 : SEM of a smaller lamellocyte-like (L) cell (from leg cell line, LI) found in culture. Scale bar = 10 μ m.

Fig. 3.9.5 : Sickle shaped cells (S) from the wing line W2. Scale bar = 80 μ m.

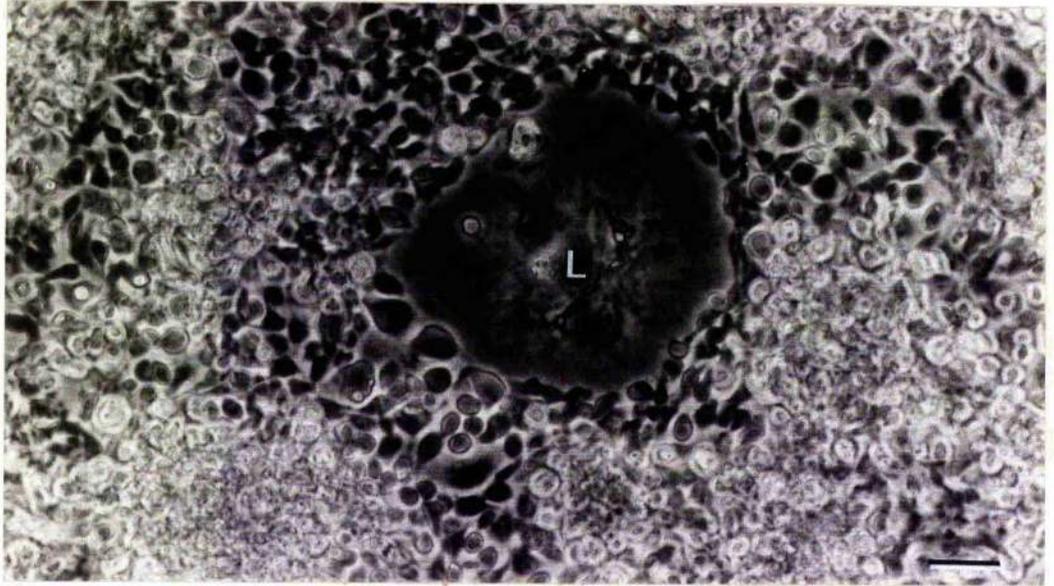
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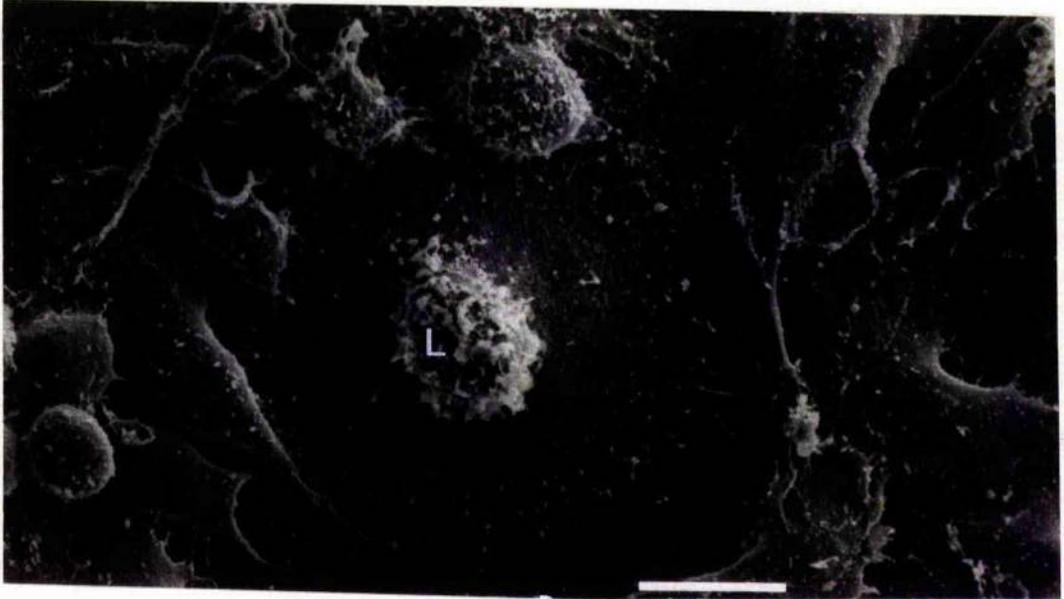
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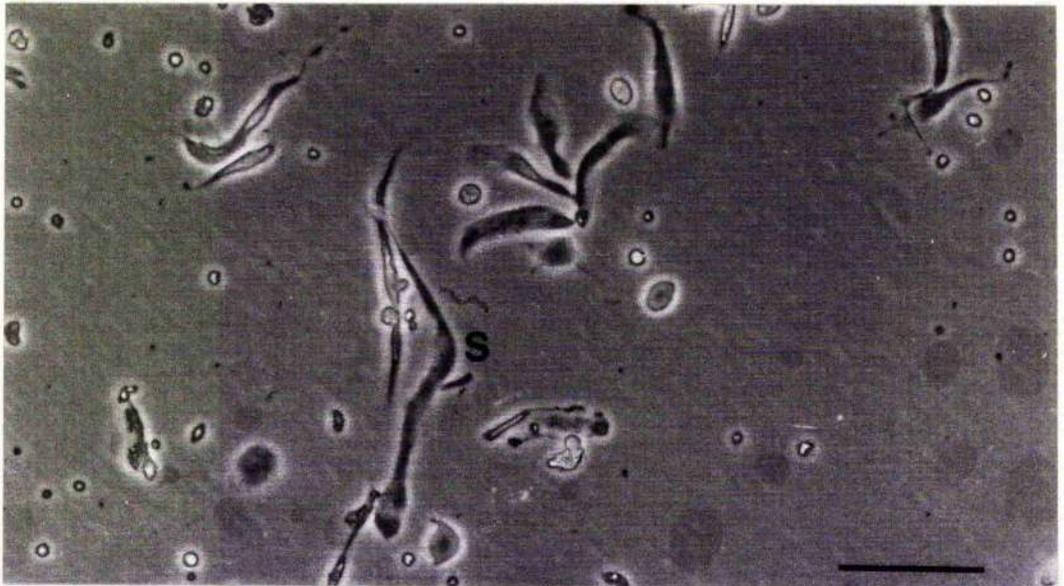


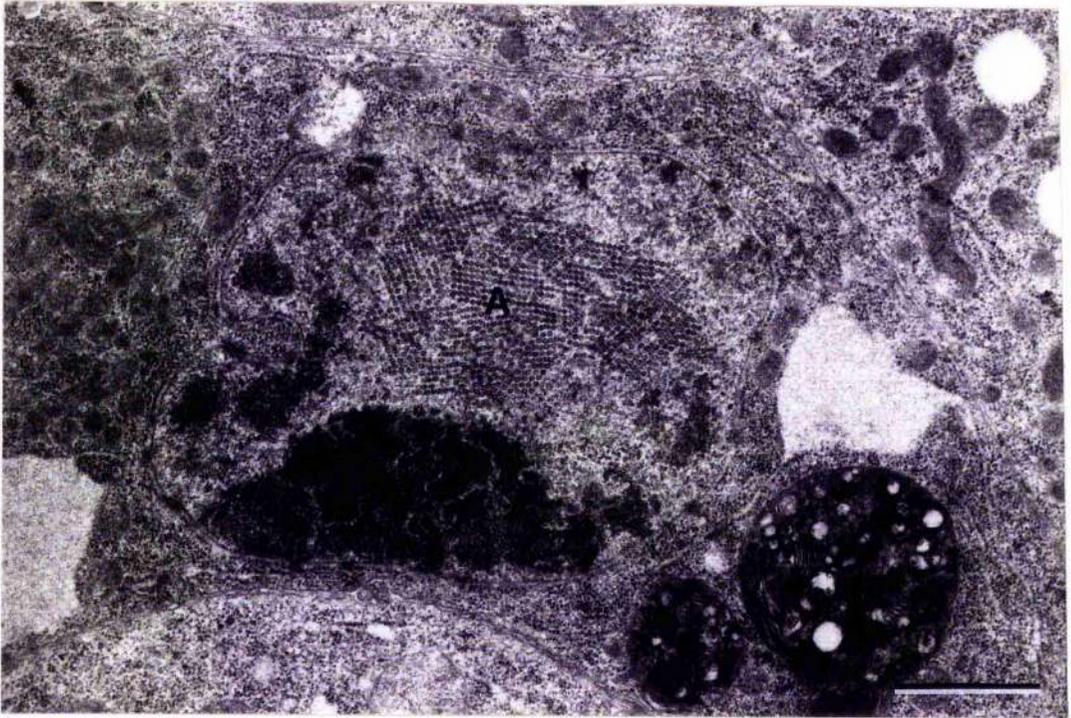
Figure 3.10

Figure 3.10 : Ultrastructure of cells in culture. TEMs of cells showing viral particles.

Fig. 3.10.1 : TEM showing viral particles in a paracrystalline array (A) in the nucleus. Scale bar = 1 μm .

Fig. 3.10.2 : TEM showing different sorts of viral inclusions (V) in the nucleus and cytoplasm of the cell. Viral particles are randomly arranged in the nucleus and in a cytoplasmic inclusion in a crystalline array (A). Scale bar = 0.5 μm .

1



2

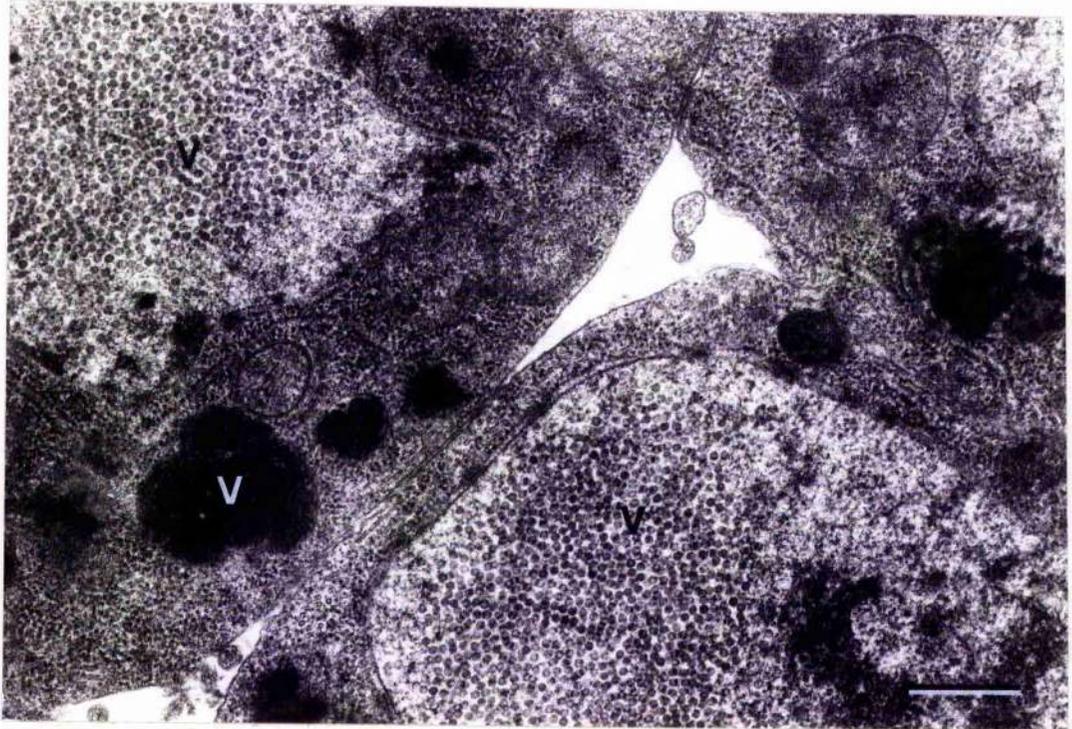


Figure 3.11

Figure 3.11 : Karyotype of cells in culture. Cell of the L2
leg line showing a diploid karyotype. Scale bar = 5
 μm .



Figure 3.12

Figure 3.12 : Cloning dish devised for cloning imaginal disc cell lines. The dish consists of a 5 cm Petri dish (5), the lid of which has been removed and the bottom of a 3 cm Petri dish (3) cemented to the underneath of it. This lid is then placed back on the 5 cm Petri dish, the surface of the attached bottom of the 3 cm dish being immersed in complete medium. This allows a surface for plating out the cells to form clones (C) which is suspended above a feeder layer (F) of cells grown in the 5 cm dish. The cloning experiment can easily be followed without the disturbing the dish by focussing either on the bottom of the 5 cm dish to look at the feeder layer and also focussing on the bottom of the 3 cm dish to closely follow the cloning experiment.

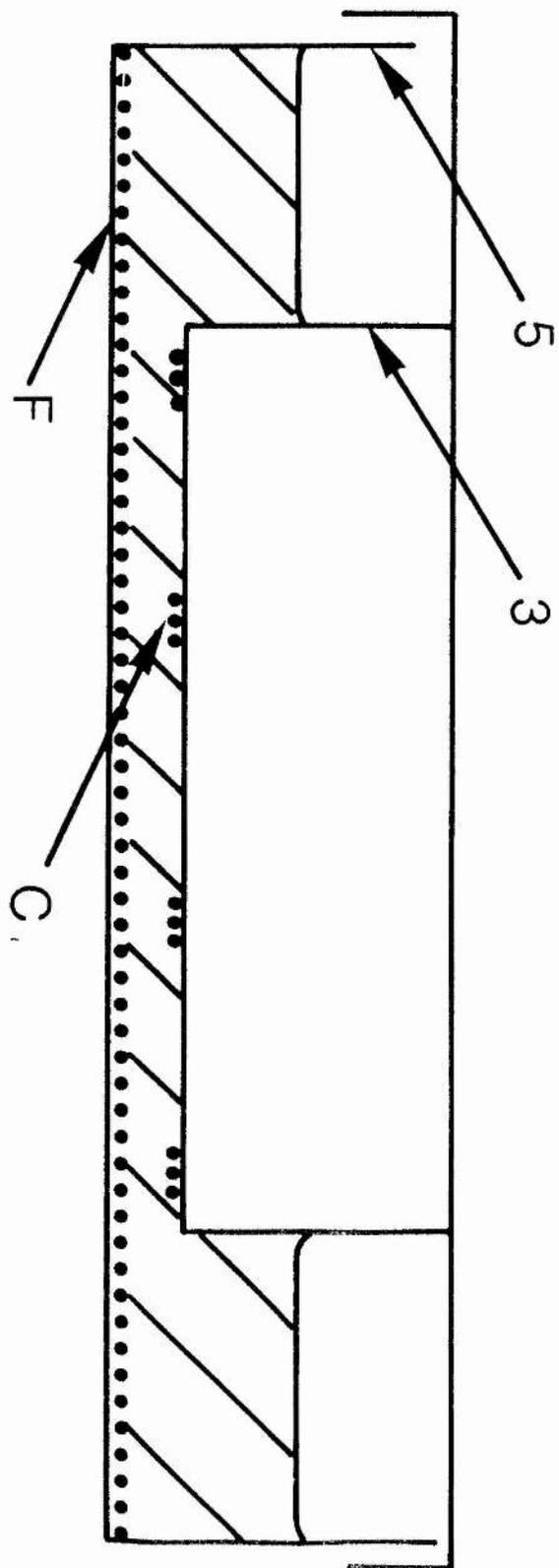


Figure 3.13

**Figure 3.13 a-e : The establishment of cloned cell lines
from the wing line W1.**

**a) Clone at the two cell stage 13 days after the
cloning experiment was started.**

**b) Another clone from the same dish at a more advanced
stage of growth at 13 days after initiation.**

c) The same clone as in (b) after 18 days.

d) The same clone at 22 days after initiation.

**e) A clone just before subculture. The cells in the
centre of the clone are beginning to form aggregates
(A).**

**Scale bars represent 50 μm in a and b, and 100 μm in
c,d and e.**

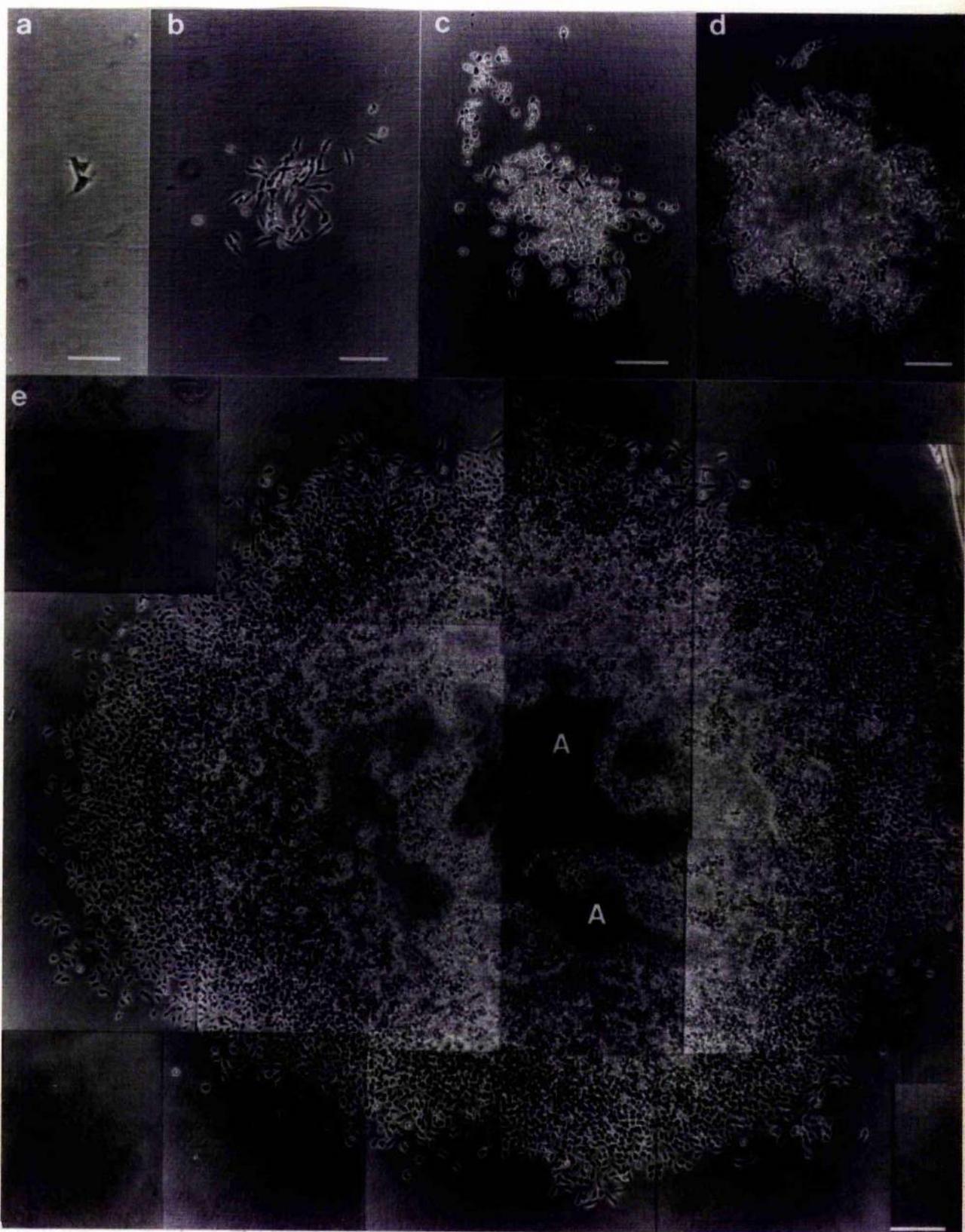


Figure 3.14

Figure 3.14 a-d : Cell morphology in cloned lines.

a) The edge of a leg clone 35 days after clone initiation, which includes epithelial-like (E), bipolar fibroblast-like (F) and flattened lamellocyte-like (L) cells.

b),(c),(d) Cells from wing clone 13, 11 passages after cloning showing the three types of cells mentioned above. Cells were fixed in 4% paraformaldehyde for 10 min, and stained in Mayers haematoxylin. Scale bars represent 50 μm in (a) and 20 μm in (b),(c) and (d).

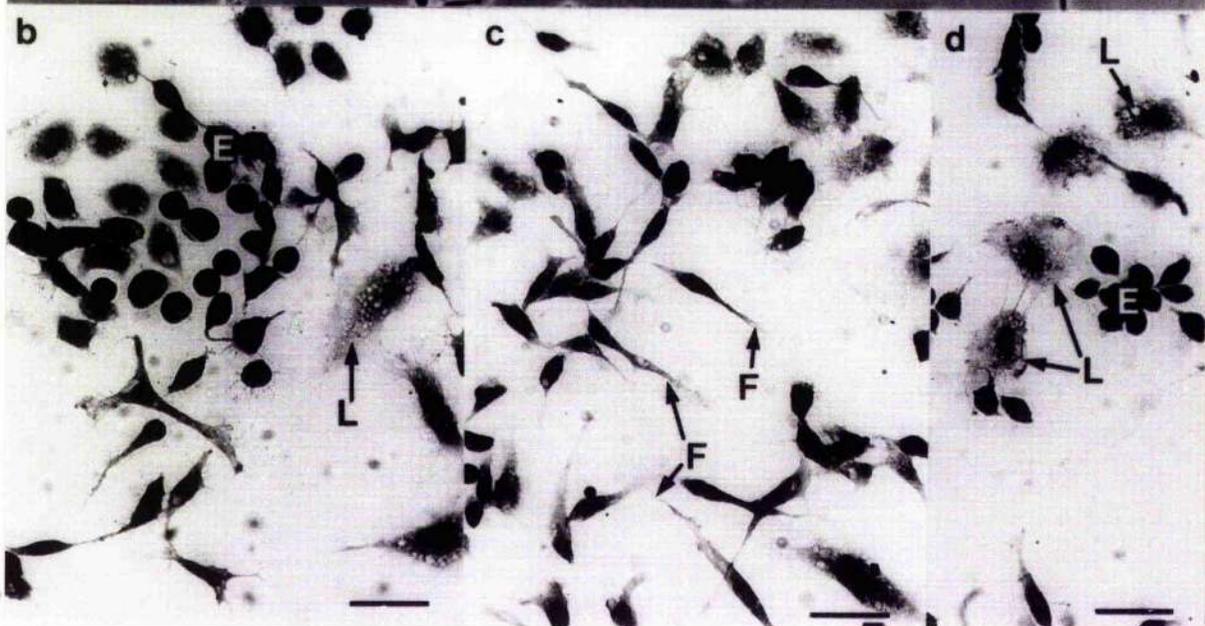


Figure 3.15

Figure 3.15 : Light micrographs showing the diversity of different cloned Wing cell lines. All scale bars = 30 μm . Figs. 3.15.2-4 : All cell lines are 5 days post passage and were seeded at the same density (3×10^6).

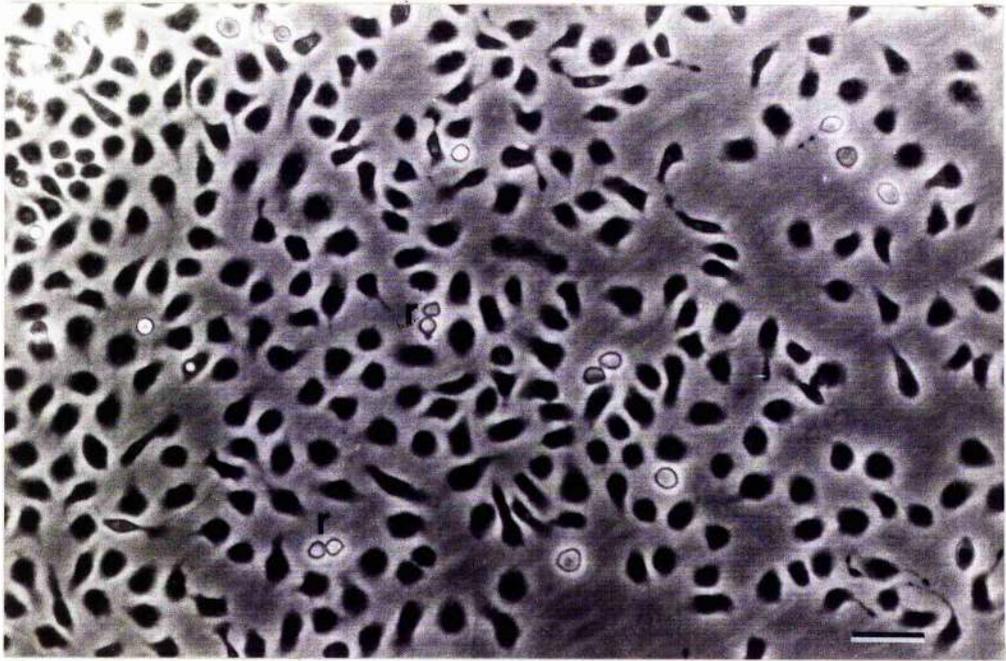
Fig. 3.15.1 : Cl.8+ at low density showing overall uniformity of the cells. Note the rounded cells (r), some of which have just divided.

Fig. 3.15.2 : Cl.8+, cells are in the process of aggregating.

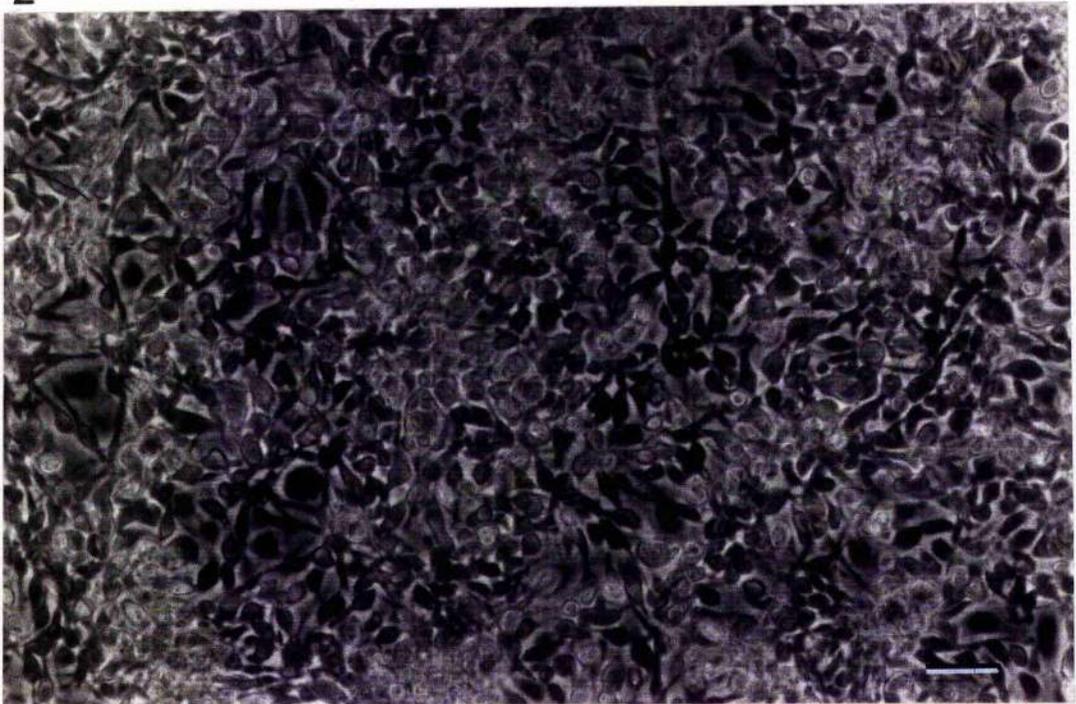
Fig. 3.15.3 : Cl.7, cells have clearly begun to aggregate (A).

Fig. 3.15.4 : C9, cells show a different overall morphology than the other cloned cell lines containing many more rounded epithelial-like (E) cells than other cloned lines.

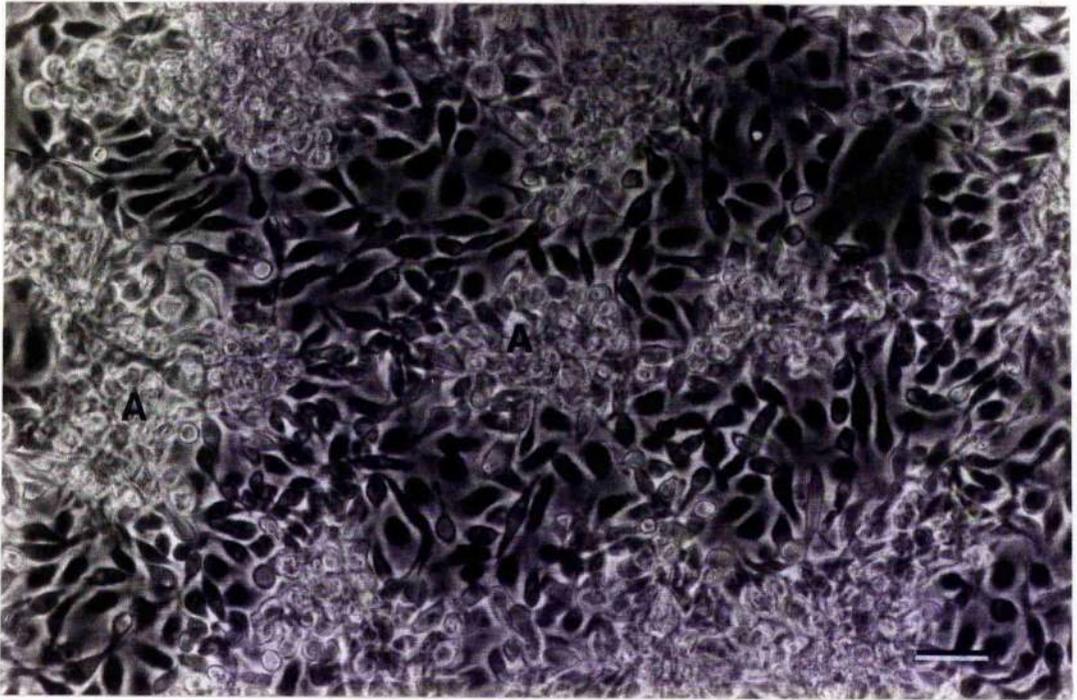
1



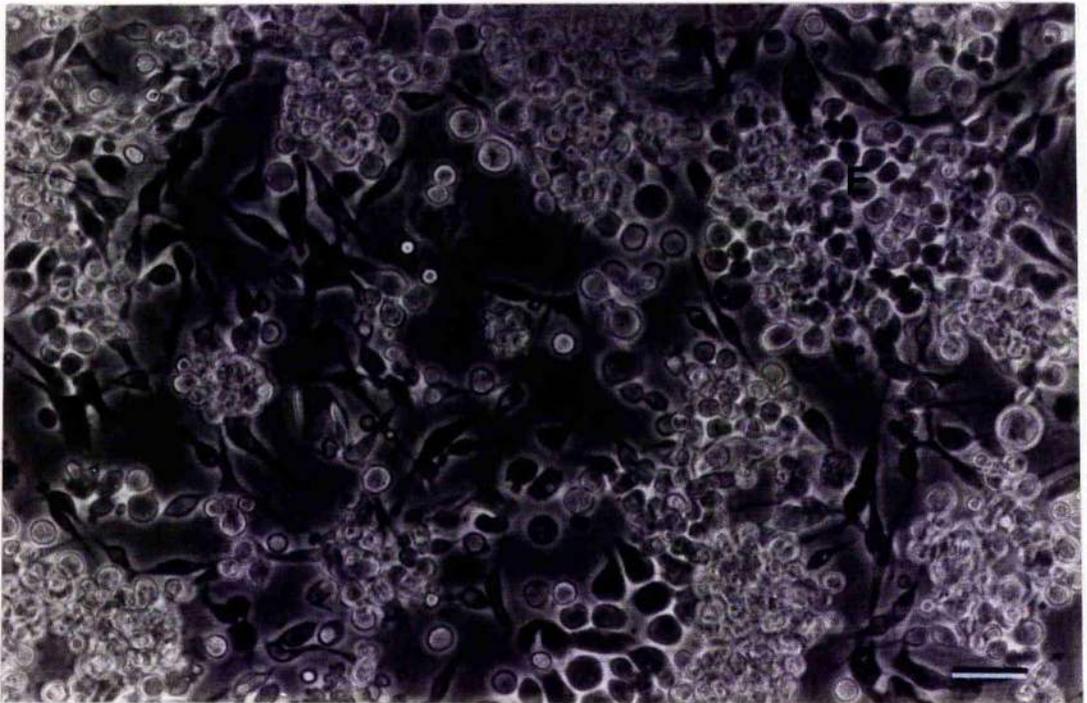
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3



4



Chapter 4

Morphological responses of cells to ecdysteroids

Morphological responses of cells to ecdysteroids

Introduction

Ultrastructural studies on the effect of 20-HE on imaginal discs dissected out and placed in organ culture reveal that the process of evagination occurs in two stages (see Figure 4.1). Firstly there is a process of elongation of the appendage within the disc sac and then eversion of the disc to form the adult structure. These studies have helped to reveal the way 20-HE acts to bring about these transformations. The lack of a defined imaginal disc cell line has prevented a more cellular analysis of the way that 20-HE acts on individual cells within that disc epithelium. In the absence of such a line embryonic cell lines have been used extensively.

Already established *Drosophila* embryonic cell lines show a variety of responses to 20-HE. One of the first reactions to 20-HE is a rapid cessation of cell division (Wyss 1976; Rosset 1978), followed by a change in morphology of the cells and aggregation (Courgeon 1972; Berger 1978; Woods and Poodry 1983). Thus it seems that even in cell lines derived from embryonic tissue one of the major responses of the cells to 20-HE is a change in cell morphology. Therefore it was thought to be important to test the responses of imaginal disc cell lines to 20-HE, enabling a comparison to be made between the effects of 20-HE on disc cells *in vitro* with disc cell *in vivo* and also with embryonic cell lines. Imaginal disc cell lines were subjected to different regimes of 20-HE application and the morphological response of the cells studied.

Results

Imaginal disc cell lines exhibit various responses to 20-HE. Firstly cells stop dividing and there is some cell death. The cells then elongate and aggregate and throw out long processes which often form connections between different aggregates. The optimal concentration of 20-HE found to elicit these responses is 10 ng/ml (2×10^{-8} M) 20-HE. Higher concentrations of 20-HE tend to elicit a greater degree of cell death without any appreciable differences in the morphological response.

20-HE has a very dramatic effect on the cell division rates in imaginal disc cell lines as can be seen from the graphs in Figure 4.2, showing cell yield against concentration of both ecdysone and 20-HE over 6 days in culture. Two primary uncloned wing cell lines were chosen, W1 and W2, the difference being that one had been in culture for approximately one year and was at passage number 40 (W1) and the other (W2) had only been passaged 6 times. As can be seen both ecdysone and 20-HE have a dramatic effect on cell yield. Both reduce the numbers of cells in culture, indicating that cell death is a major feature of the response of the cells to ecdysteroids. Comparing the effects of ecdysone and 20-HE on an individual cell line it can be seen that to achieve the same effect with ecdysone that is seen with 20-HE approximately a 100 fold greater concentration of ecdysone is needed. Thus 20-HE seems to be much more active (100x) in reducing cell yield and causing cell death than ecdysone.

Comparing the two lines and bearing in mind that both are uncloned primary wing lines but at different passage numbers it can be seen that they have different sensitivities to ecdysone and 20-HE. The W2 cell line seems to be much more sensitive to the effects of 20-HE and ecdysone than the W1 line. For instance in the W2 line, it takes only 3 days for the cell yield to decrease to below 1×10^5 cells, even at the lowest concentration of 20-HE (10 ng/ml). However with the W1 line this level is never reached and the cell yield remains above this threshold even after 6 days at the highest concentration of 20-HE (100 ng/ml). For both ecdysone and 20-HE treatments it can be seen that the W2 cell line is much more sensitive to the effects of ecdysteroids than W1. This is probably related to the length of time that W1 has been in culture as compared to W2. For the control values it can also be seen that with length of time in culture the growth rate of the cell lines becomes higher. After 6 days in culture where both lines were seeded at the same density, cell yield in W1 is double that in W2.

Morphological transformation

A concentration of 10 ng/ml of 20-HE was routinely used, as this appeared to give the maximum amount of morphological transformation without causing the greater degree of cell death associated with higher concentrations. The range of morphological transformations that occur on 20-HE application can be seen in the set of pictures from Figure 4.3 where an optimal concentration (10 ng/ml) of 20-HE was added to a

cloned wing line Cl.8+ and followed at 1 day intervals over a week.

Day 1 (Figure 4.3.1.)

Cells were plated out at a concentration of 3×10^6 cells/5 cm Petri dish. As can be seen from the control (Fig. 4.3.1 a) this produces a fairly even distribution of cells over the tissue culture plastic. Even after just 1 day the effect of addition of 10 ng/ml 20-HE can be seen (Fig. 4.3.1 b): Although the same cell concentration was used for plating out, the cells appear much less dense and there are a number of bare areas free of any attached cells. Along with this it can be seen that often in these areas there are a number of particles which probably consist of dead cell fragments. This indicates along with the decreased cell density that even after 1 day the application of 20-HE has resulted in some cell death. Several of the cells that are remaining have already undergone a process of cell elongation and process extension. Thus even after only 1 day in culture the effects of 10 ng/ml 20-HE can be seen. There has been a decrease in cell density and the appearance of dead cell fragments indicating that cell death has occurred. Some of the cells have undergone a morphological transformation, elongating and throwing out processes.

Day 2 (Figure 4.3.2)

After 2 days it can be seen that the control culture is much more dense and is approaching confluency. The experimental culture (Fig. 4.3.2 b) shows some noticeable

differences. Again several of the cells appear to be elongated and to have processes, also there seems to be the first signs of aggregate formation.

Day 3 (Figure 4.3.3)

The control culture has reached confluency whereas the experimental culture after 3 days with 20-HE shows a reduction in the numbers of cells present along with a number of dead cell fragments in culture. The cells possess long processes and filopodia.

Day 4 (Figure 4.3.4)

The control cells are now quite dense in culture and have passed the point of confluency and have begun to aggregate. The treated cells again show an elongated morphology and also the process of aggregation into small discrete tight aggregates is continuing.

Day 5 (Figure 4.3.5)

The control culture is now very dense, the aggregates are now multilayered. The 20-HE treated cells have continued to aggregate and now there are a number of small discrete aggregates. These aggregates often have processes radiating out from them indicating that cells are either throwing out processes once they have aggregated or that process extension is one of the reasons for aggregate formation, pulling cells together.

Day 6 (Figure 4.3.6)

Cells in the control (Fig. 4.3.6 a) have continued to aggregate and it can now be seen that there are some areas of the tissue culture plastic where few cells are present next to often very large multicellular aggregates. The hormonally treated culture continues to show the presence of a number of small aggregates often with cells and cell processes radiating out from them. Also it can be seen that some cells have taken up a very flattened like morphology.

Day 7 (Figure 4.3.7 a,b,c,d)

Again the control culture shows that the cells are now very dense and are arranged into large multicellular aggregates. The experimental culture can again be seen to be composed of small discrete aggregates of cells often with very long processes extending from them and often connecting different aggregates (Fig. 4.3.7 d). From Fig. 4.3.7 b it appears as though a cell has extended a process towards an aggregate suggesting that it is by this formation of cell extensions that aggregation occurs. A close up of the aggregate from Fig. 4.3.7 b in Fig. 4.3.7 c shows that the aggregate has an outer bilayer surrounding the cells. This can also be seen in Fig. 4.5. This perhaps suggests that there is some process of cuticle production in culture. Indeed often when cells are 20-HE treated, material can be seen floating in the culture medium which resembles pupal cuticle (Fig. 4.4). In Fig. 4.3.7.b it can be seen that some of the processes of 20-HE treated cells have begun to arborise. Fig. 4.5 shows a small discrete aggregate after 7 days at 10 ng/ml of 20-HE

with a bilayer surrounding the aggregated cells and a number of cell processes extending from the base of this aggregate. Some of these processes also have inclusions.

Some of the effects of 20-HE on the process of aggregate formation and process extension can be more clearly demonstrated by SEM's of cells exposed to 20-HE. Fig. 4.6.1 shows a control culture of Cl.8+ cells after 1 week in culture. Figs. 4.6.2, 4.6.3 and 4.6.4 show the effect of 10 ng/ml of 20-HE on those cells. Figs. 4.6.2 and 4.6.3 show close ups of small aggregates showing the rounded nature of many of these aggregates with basal extensions which maintain their adherence to the substrate. Fig. 4.6.3 shows a cytoplasmic fringe to such an aggregate with numerous filopodia that probably would not be seen with phase contrast microscopy. Fig. 4.6.4 shows a lower power picture of cells exposed to 20-HE where some small aggregates with processes and cells radiating out can be seen. At this stage of the culture (7 days) these aggregates are only loosely attached to the substrate and are easily dislodged and often some of these aggregates can be seen floating freely in the culture medium.

These effects were achieved with 10 ng/ml of 20-HE continuously present in the medium. The effects of this level of 20-HE could be seen even after 1 day (Fig. 4.3.1 a,b), suggesting that 20-HE is very quick acting. To test whether or not 20-HE was required to be continuously present in order to elicit the range of responses seen, cells were treated with 20-HE and the medium changed after 1 or 2 days. The effects of this treatment was compared with cells that had been cultured

in the continuous presence of 20-HE for a week. The Figs. 4.7.1, 4.7.2, 4.7.3 and 4.7.4 show the results from such an experiment. Fig. 4.7.1 shows the control cells after 1 week, the culture appears quite dense with some large aggregates. Fig. 4.7.2 shows the same culture where 10 ng/ml of 20-HE was present for only 1 day and then replaced with fresh medium without 20-HE. It can be seen that the culture is much less dense than the control indicating that a 1 day pulse of 20-HE has slowed down the rate of cell division in these cultures. Fig. 4.7.3 shows a culture that had 20-HE present for 2 days before the medium was changed. If you compare this with Fig. 4.7.4 where 20-HE was present for the entire 7 day period you can see that there was not much difference between the two. This indicates that 20-HE is indeed quick acting and quickly commits the cells to a reduction or cessation in cell division after 1 day. It appears to require 2 days before 20-HE induces the aggregation and cell elongation that is typical of cells exposed to 20-HE continuously.

Thus it appears that at 10 ng/ml 20-HE produces a range of responses, firstly causing the cells to stop dividing and causing some cell death. The cells then undergo a process of morphological transformation, cells elongate, throw out processes and aggregate.

At 10 ng/ml 20-HE produces a dramatic transformation to the cells, therefore what effects do lower levels of 20-HE have on the cells? Experiments were carried out to determine whether lower concentrations of 20-HE would induce the whole range of responses at a lower level or just particular parts of the response. Cells were grown at varying levels of 20-HE

below 10 ng/ml to see what effects these lower levels of 20-HE had on the cells. Fig. 4.8.1 shows the control culture after 1 week, the cells are very dense and have aggregated. It should be borne in mind that these cells have been cultured in the standard imaginal disc cell line culture medium which contains 1 ng/ml (2×10^{-9} M) 20-HE. Fig. 4.8.2 shows a culture that was grown with 2 ng/ml (4×10^{-9} M) 20-HE, the cells appear less dense than in the control (Fig. 4.8.1) and although they have aggregated do not form the large conglomerated aggregates seen in Fig. 4.8.1. A culture grown with 4 ng/ml (8×10^{-9} M) 20-HE is shown in Fig. 4.8.3. The cells are much less dense but aggregates of cells have formed. Fig. 4.8.4 shows a culture that has been incubated with 20-HE at a level of 6 ng/ml (1.2×10^{-8} M), again the cells appear to be less dense and the aggregates are bigger and more definite with cell processes radiating outwards. At 8 ng/ml (1.6×10^{-8} M) in Fig. 4.8.5 this process can be seen to have gone further. There are now large areas of bare tissue culture plastic, the aggregates are more defined and the cell processes extending from these aggregates are longer. Fig. 4.8.6 shows a culture incubated with 10 ng/ml (2×10^{-8} M) 20-HE for a week.

Therefore it seems that even at much lower concentrations of 20-HE down to 2 ng/ml the cells respond. It seems that the reduction in cell division rate is the most sensitive part of the cells response to 20-HE occurring even at 2 ng/ml. Aggregation also seems to be one of more sensitive responses of the cells, being commonplace at 4 ng/ml. Cell processes radiating from aggregates seem to be more prevalent

at higher levels of 20-HE although cell elongation seems to occur at lower 20-HE titres (4 ng/ml).

Therefore it seems that at lower levels of 20-HE the response of the cells is progressively reduced. Again the cessation or reduction in cell division is characteristic of both low levels of 20-HE and also in cultures briefly exposed to an optimal concentration. This indicates that this cessation of cell division appears to be a very early and primary action of 20-HE, followed later by cell elongation, aggregation and process extension.

This response to 20-HE is apparent in both wing and leg imaginal disc cell lines and also in nearly all of the clones so far isolated from these primary lines. However there are a few exceptions to this. For instance the leg lines so far tested (3) whilst showing all the features that have been mentioned previously do appear to be somewhat more sensitive to 20-HE than wing lines. This can be seen in Fig. 4.9, which shows the effect of administering a 5 ng/ml (1×10^{-8} M) concentration of 20-HE to two cloned cell lines, one wing and one leg. Figs. 4.9.1 and 4.9.2 show the control and experimental respectively for the wing line Cl.8+. This produces a response which has been seen before in the low titre experiments, the cells are much less dense, and there are several aggregates. Although individually some of the cells have elongated there does not appear to be large numbers of processes radiating from aggregates as seen for the 10 ng/ml treatment.

In contrast Figs. 4.9.3 and 4.9.4 show the control and experimental for the cloned leg line L1 Astrew (L1A). The

difference between the control and 5 ng/ml treatment is more stark than for the cloned wing line Cl.8+, 5 ng/ml induces a similar response in the line as 10 ng/ml of 20-HE in the Cl.8+ cell line. The cells have aggregated in places, there are some areas of bare plastic and there are numbers of cell processes extending across the plastic. Note the flattened out cells (arrowed), these are often seen in 20-HE treated cultures.

One cell line produces a response which is significantly different from that which has been described above. The cloned wing cell line C9 shows an apparent insensitivity to the effects of 20-HE. Fig. 4.10 shows a culture of C9 treated with 100 ng/ml (2×10^{-7} M) of 20-HE for a week. At this level other cloned and uncloned cell lines would be very severely affected, most of the cells would have died and there would not be the extensive aggregation and cell process extension that is seen at lower 20-HE titres. However this cell line seems to be relatively unaffected by this extremely high level of 20-HE. There does appear to be some 20-HE this cell line, albeit very much reduced, the cells appear somewhat less dense than in the control and there appears to have been some aggregation.

Apart from this particular cell line all the lines so far tested have proved positive for the action of 20-HE. Even this cell line C9 does show some effect and is not completely insensitive to 20-HE. Thus besides the above mentioned differences the effect of 20-HE on all the cell lines tested was quite similar. Occasionally in culture patches of tanned cuticle-like material would be produced. This phenomenon was

most common in the cloned leg cell line L1A but was also apparent in other cell lines (Fig 4.11).

When conducting these experiments on the effects of ecdysteroids, the cultures were carried on beyond 7 days and any different effects observed. The 20-HE response did not change appreciably beyond this period and therefore 7 days was taken as a convenient incubation period for 20-HE to take effect. However occasionally after a while some cells began to divide again in the 20-HE treated cultures. Fig. 4.12 shows an aggregate where cells have begun to divide again. These cells were still growing in a medium containing 20-HE so it appeared that they had overcome the growth inhibiting effects of 20-HE. When a small group of these cells were observed, the cultures were allowed to continue growth and the medium changed to one without any 20-HE. This allowed any cells that had survived 20-HE treatment such as the small group in Fig. 4.12 to grow up and form a culture. This was repeated with alternating periods of no 20-HE and medium with 20-HE for a period of 2-3 months. During this period the 20-HE concentration was gradually increased during periods of 20-HE exposure.

This regime specifically selected for cells that were resistant to the effects of 20-HE based on their ability to continue dividing in medium containing 20-HE and without showing any morphological alterations. Eventually cells could be grown in medium containing 1.5 ug/ml of 20-HE, with no effect. Thus cells had been selected for 20-HE resistance. The effects of such selection can be seen in Fig. 4.13.(1-4). This shows the wing line Cl.8+ subjected to increasing levels of 20-HE (10-150 ng/ml (2×10^{-8} M - 3×10^{-7} M)) after 7 days.

This line was also used to select cells that were resistant to 20-HE and a line was grown up termed Cl.8R which was resistant to 20-HE. In Fig. 4.13.(5-8) increasing levels of 20-HE to 150 ng/ml (3×10^{-7} M) can be seen to have no effect on the cells whereas in Fig. 4.13.(1-4) increasing levels of 20-HE can be seen to have a dramatic effect on the parent line Cl.8+.

Thus by the use of an appropriate selective pressure, in this case increasing 20-HE levels, cells have been selected for resistance providing a useful control for a number of 20-HE experiments, especially those associated with biochemical aspects of 20-HE action.

Discussion

Imaginal disc cell lines exhibit a defined morphological response to 20-HE. The cells cease dividing, elongate, aggregate and throw out processes. This occurs at an optimal concentration of 10 ng/ml (2×10^{-8} M) 20-HE.

Embryonic cell lines also respond to the 20-HE by undergoing a morphological transformation. The most widely studied *Drosophila* embryonic cell line is K_C , which was first established by Echaliier and Ohanessian (1970). This cell line and subclones derived from it react to 20-HE with a similar response to that seen for imaginal disc cell lines, the cells undergo a cessation of division and major changes in morphology (Courgeon 1972, Cherbas et al 1980, Wyss 1976). Other *Drosophila* embryonic cell lines also undergo similar ecdysteroid responses (Berger et al 1978). For the K_C cell line Cherbas et al (1980) found that the optimal concentration

of 20-HE to induce a morphological response was 50 ng/ml (1×10^{-7} M) a half maximal response being achieved with 5 ng/ml (1×10^{-8} M). Rosset (1978) found a concentration of 10 ng/ml (2×10^{-8} M) to be optimal. Thus the response appears to be achieved with a very similar level of 20-HE in both embryonic and imaginal disc cell lines.

Cell division

One of the first responses of the cells to 20-HE is a cessation in cell division. This can be seen even after only a short exposure to 20-HE. In imaginal discs *in vivo* cell division occurs throughout the larval period, in contrast to other larval tissues, and ceases at the onset of metamorphosis (Nöthiger 1972). This would seem to suggest that imaginal differentiation involves a suspension of cell division and that this is under hormonal control. This would accord well with the finding that 20-HE causes a cessation in disc cell division *in vitro*. However *in vivo* imaginal discs are also exposed to periodic increases in ecdysteroid titre during the larval moult cycles. Cell lineage analysis of cell division rates through imaginal disc development shows that division rates are reduced during the larval moults (García-Bellido and Merriam 1971). This evidence tends to support the contention that 20-HE does have a controlling effect on cell division rates.

However studies on mass-isolated imaginal discs suggest that 20-HE increases DNA synthesis and therefore presumably division rates (Siegel and Fristrom 1978). These experiments were carried out in short term organ culture in Robb's medium,

in this regime DNA synthesis as measured by incorporation of tritiated thymidine which in this system decreases to a low basal level after 5 hours in culture (Logan et al 1975). The observed stimulation of DNA synthesis by 20-HE is an amelioration of this decline rather than a straightforward stimulation. The dramatic decline in DNA synthesis may well be a product of the inadequate culture conditions, especially in simple media such as Robb's. The effect of 20-HE in such situations may be due to the effects that steroid hormones have on cellular membranes and their ability to alter the transport of molecules into cells. Steroid hormones increase the uptake of molecules such as glucose, thymidine and uridine through the cell membrane (Jensen and De Sombre 1972). This has also been found to be true for disc cells, 20-HE dramatically increasing the uptake of uridine (Raikow and Fristrom 1971) and glucose (Siegel and Fristrom 1978). Therefore the observed stimulation of DNA synthesis by 20-HE in these situations may have more to do with the effect of 20-HE on the cell membrane increasing utilisation of the available media components rather than a direct receptor mediated stimulation by 20-HE.

Embryonic *Drosophila* cell lines also show a cessation of division following 20-HE addition (Wyss 1976; Rosset 1978; Cherbas et al 1980). Treatment of K_C cells with 100 ng/ml (2×10^{-7} M) 20-HE results in a cessation of division and arrest of the cells in the G2 phase of the cell cycle (Stevens et al 1980). In this study, treatment of the cells with this concentration of 20-HE did not result in any cell death. In other studies using the K_C cell line and sub-clones from it,

cell death does appear to be a factor in the cells response to 20-HE. Wyss (1976) found, using a clonal subline from K_C , that very low levels of 20-HE (3 ng/ml, 6×10^{-9} M) actually stimulated cell proliferation whereas higher concentrations (10 ng/ml- 2×10^{-8} M) caused almost a 50% reduction in cell numbers after 3 days. In another study (Cherbas et al 1980), 5 ng/ml (1×10^{-8} M) of 20-HE caused a transient increase in cell proliferation and then a reduction in division rates, whereas higher concentrations, 50 ng/ml (1×10^{-7} M) of 20-HE caused an arrest of division and a certain amount of cell death after 3 days in culture. Despite the differences that there appears to be even using the same cell line (K_C), a general feature of the response to 20-HE in all embryonic cell lines seems to be a cessation of cell division (Berger et al 1980).

Imaginal disc cell lines show a dramatic response to even quite low levels of 20-HE and just 24 hours of exposure (10 ng/ml, 2×10^{-8} M) is enough to induce a dramatic reduction in division (Figs. 4.7.1 and 4.7.2). Comparing the published results for embryonic cell lines with those achieved using disc cell lines it can be seen that disc cell lines show many similar features in their response to 20-HE. However it does appear that disc cell lines are much more sensitive to these effects. For example exposure to even 2 ng/ml (4×10^{-9} M) of 20-HE appears to produce a culture that is less dense than the control (Figs. 4.8.1 and 4.8.2). Wyss (1976) found that a similar level of 20-HE (3 ng/ml, 6×10^{-9} M) in the K_C cell line actually increased cell proliferation and that only higher concentrations inhibited division. Disc cell lines also seem to exhibit a greater degree of cell death in response to

20-HE than embryonic cell lines, after 7 days at 10 ng/ml (2×10^{-8} M) 20-HE, in the W2 cell line there was a 15 fold reduction in cell numbers.

The response of the cells to ecdysone appears to be very similar qualitatively to that of 20-HE but different quantitatively. The 20-HE is about 100x more effective at inducing a reduction in cell division rates than ecdysone. Organ culture studies of the relative effectiveness of ecdysone as opposed to 20-HE in inducing evagination in isolated imaginal discs agree with the figure arrived at with disc cell lines (Milner and Sang 1974). This indicates that with regard to this part of their ecdysteroid response the disc cell lines have retained the same characteristics as intact imaginal disc tissue.

Comparing the two different cell lines W1 and W2 and looking at their ecdysteroid response to both ecdysone and 20-HE it can be seen that there is quite a difference between the two even though they are both uncloned wing cell lines. The W1 cell line had been passaged for over a year and reached passage number of over 40 whereas the W2 cell line was only recently established and had been passaged only 6 times when the experiment was carried out. The W2 cell line is much more sensitive to 20-HE than the W1 line. However the two lines both show a similar magnitude of difference in their response to ecdysone and 20-HE. This indicates that although one line is more insensitive to hormone than the other, they both show qualitatively the same response. Obviously an extended period in culture results in some diminution in the ability of the cells to respond to 20-HE so that they become more

insensitive. Looking at the control values for W1 and W2 there appears to be quite a difference in that W1 has a much higher growth rate than W2, reflecting that these cells have adapted themselves to the prevailing culture conditions. This adaptation has resulted in higher growth rates and a growing insensitivity to 20-HE. Interestingly the cloned cell lines isolated, which have been passaged in some cases over 50 times since culture initiation show no such diminution in their response to 20-HE although they show a greatly increased growth rate as compared to uncloned cell lines.

One of the most notable features of the response of the cells to 20-HE is an initiation of cell death, as already mentioned one cell line showed a 15-fold reduction in cell numbers over 7 days at 10 ng/ml (2×10^{-8} M). Cell death is an important feature of metamorphosis in higher *Diptera*, however this has been generally thought to be restricted to the degeneration of the larval tissues (Locke 1981). Cell death plays a role in the production of some mutant phenotypes of *Drosophila* (Fristrom 1969), and also plays a part in normal imaginal disc development (Spreij 1971). Cell death has also been seen in some embryonic cell lines when they have been exposed to 20-HE (Cherbas et al 1980) although not to the same extent as that seen in disc cell lines. The extent of cell death that is seen in 20-HE treated disc cells *in vitro* may well be an exaggeration of a normal response.

Morphological transformation

After the cessation of cell division the next response of the cells to 20-HE is a dramatic morphological transformation. The cells elongate, throw out long processes and aggregate. Cells can be seen to elongate even after 1 day at 10 ng/ml (2×10^{-8} M) of 20-HE. At this stage the cells appear somewhat spindle shaped, later the cells throw out processes and begin to aggregate. At later stages of culture distinct aggregates of cells have formed and extremely long processes can often be seen extending from them. Although it appears that these cells are radiating processes out from these small aggregates, it may be that this extension of cell processes is one of the mechanisms bringing about aggregation. Cells may produce long processes which on contacting other cells could draw them together resulting in the formation of distinct regularly spaced aggregates.

Imaginal discs *in vivo* at metamorphosis undergo a process of evagination and differentiation in response to moulting hormones. This results in the transformation of a single layered epithelial sac into its relevant adult structure. This occurs in two stages, the disc epithelium firstly elongating within the sac and then everting through the stalk of the disc, in effect being turned inside out, to form the adult appendage (see Fig. 4.1). Evagination occurs by cell rearrangement (Fristrom and Fristrom 1975; Fristrom 1976) and cell shape changes (Milner *et al* 1984; Condic *et al* 1991). Eversion of the disc proper is mediated by cell shape changes in the overlying peripodial membrane, the cells of which change from a thin squamous epithelium to a more cuboidal

form, the resulting contraction pulling the elongated appendage through the stalk (Milner et al 1984). Evagination is reversibly inhibited by cytochalasin-B which disrupts the microfilament contractile system but not by colcemid which disrupts the microtubule system (Mandaron 1974; Fekete et al 1975; Fristrom and Fristrom 1975). The microfilament contractile system is thought to be important in mediating cell shape changes in morphogenesis (Wessells et al 1971), and this result suggests that cell shape changes are very important for the process of imaginal disc morphogenesis.

At the onset of metamorphosis in *Calpodes*, a Lepidopteran, the rise in ecdysteroid titre leads to the production of cell processes which can span several cell diameters and have been termed epidermal feet (Locke and Huie 1981; Delhanty and Locke 1989). These epidermal feet are thought to be important in allowing cell rearrangements at metamorphosis (Locke 1981; Nardi and Magee-Adams 1986). Studies on the cell biology of imaginal disc evagination *in vitro* (Milner and Muir 1987) reveal that complex cell interdigitation is common in the disc epithelium and that at later stages cell process extension is a feature of dorsal-ventral wing epithelial apposition. This shows that extension of cell processes within the disc epithelium as in epithelial foot extension in *Calpodes* may also be common in *Drosophila*.

Imaginal disc eversion is accelerated by treatment with the serine protease trypsin (Poodry and Schneiderman 1971). This was thought to be due to a loss of adhesion between cells allowing more rapid cell rearrangement and cell shape changes (Siegel and Fristrom 1978). Studies of endogeneous proteases

in imaginal discs reveals that protease inhibitors inhibit eversion and that disc cells synthesise and secrete several proteases one of which was found to be 20-HE dependent (Pino-Heiss and Schubiger 1989). An apical surface glycoprotein *gp125* is produced during imaginal disc morphogenesis by 20-HE dependent proteolysis (Birrer et al 1990), and this molecule has been localised in imaginal disc cell lines (A. Wallace pers. comm.). So proteases seem to have a role in imaginal disc morphogenesis. Pino-Heiss and Schubiger (1989) have shown that trypsin treatment of discs which accelerates disc eversion causes activation of endogenous protease activity by breaking up of protease-inhibitor and zymogen complexes. Disc protease activity is controlled by inhibition and activation systems (Pino-Heiss and Schubiger 1989), and these may be responsible for the localised proteolysis which would be necessary to allow rearrangement and cell shape changes in the disc epithelium to occur. Cell surface proteases have been found to be localised to pseudopodial extensions (Chen and Chen 1987). Therefore cell process extension may involve localised proteolysis under the control of inhibition and activation systems, so allowing cell shape changes and cell rearrangements within the disc epithelium.

An important aspect of evagination of imaginal discs *in vivo* may well be the 20-HE induced changes in cell morphology that bring about cell process extension allowing cell rearrangement and movement possibly with the aid of cell process localised proteases. The observation that *gp 125*, a cell surface glycoprotein produced by proteolysis is present in imaginal disc cell lines suggests that proteases are active

in this system. Embryonic cell lines also respond to 20-HE by cell elongation and cell process extension. The cells show increased motility and an increase in the content of actins and tubulin (Berger et al 1980, 1981; Sobrier et al 1989) in response to 20-HE.

Imaginal disc cells respond to 20-HE morphologically by cell elongation and the extension of cell processes. Imaginal disc eversion *in vivo* is dependent on cell shape changes and cell rearrangement both induced by 20-HE. Cell shape changes involving the actin network are important aspects of disc eversion (Condic et al 1990). It is perhaps not surprising that imaginal disc cells *in vitro* respond to 20-HE by initiating a change in their cell morphology and throwing out processes. Cell process extension in 20-HE treated disc cells *in vitro* and their joining up with different cells may be an exaggerated form of what occurs in intact epithelia bringing about cell rearrangement and shape changes. This may be similar to the response to 20-HE of epithelial cells in *Calpodes* epidermis and the production of so called epithelial feet. Another mechanism by which cell process extension may be an important mechanism in remodelling epithelia at metamorphosis is by proteolytic activity, localised to cell processes (Pino-Heiss and Schubiger 1989).

Aggregation

At the same time that cells are undergoing a morphological transformation and extending processes, cell aggregation occurs. Cells appear to become more adhesive to each other and less so to the tissue culture plastic and with

the aid of their cell processes, appear to draw themselves into an aggregation. This process of aggregation results in the formation of small discrete aggregates often with processes radiating out from one aggregate joining up with another.

Studies looking at the effects of 20-HE on protein synthesis in mass isolated imaginal discs *in vitro* reveal that 20% of cell membrane proteins are 20-HE dependent whereas only 1% of other cellular fraction proteins are (Rickoll and Fristrom 1983). This suggests that alteration of cell membrane proteins is one of the major targets for 20-HE action. It has been suggested that these proteins are involved in the cell rearrangements, cell movement and cell shape changes that occur in the disc epithelium during evagination (Rickoll and Galewsky 1987; Woods *et al* 1987). Analysis of some of these cell surface proteins reveals that they are related to similar proteins that are induced by 20-HE in embryonic cell lines (Woods *et al* 1987). A number of cell surface proteins in embryonic cell lines appear to be correlated with the 20-HE induced aggregation that is seen in these lines (Johnson *et al* 1983; Rickoll *et al* 1986). Further studies isolating these proteins in cell lines and producing antibodies revealed that increases in their protein levels were associated with 20-HE treatment in intact imaginal disc tissue as well (Rickoll and Galewsky 1987). Fab antibody fragments (monovalent antibodies which interfere with a molecules function by binding but not causing capping) of anti-P110, one of these 20-HE-dependent proteins, inhibited ecdysteroid induced aggregation of S3 embryonic cells (Galewsky and Rickoll 1989). Galewsky *et al* (1988) showed that this glycoprotein termed P110 is a cell

adhesion molecule in a cell binding assay where embryonic cells preferentially adhered to an isolated *P110* band on a nitrocellulose blot. Immunofluorescent studies of *P110* distribution in intact imaginal discs have also revealed staining in regions of cell-cell contact (P. Bryant pers. comm.).

All these data suggests that changes occur in cell surface proteins in response to 20-HE and that many of these proteins are involved in promoting cell-cell interactions via enhanced cell adhesion. This observation would agree with the finding that 20-HE causes aggregation in imaginal disc cells *in vitro* resulting in greater cell-cell adhesion in culture. Therefore it appears that disc cells *in vitro* respond in a similar way to intact disc tissue in response to 20-HE, there is a change in the adhesive interactions of the disc cells bringing about cell rearrangement in intact disc epithelia and aggregation in disc cells *in vitro*.

The result of these processes occurring *in vitro* is the production of small discrete aggregates with cell extensions protruding from them and often connecting up with other aggregates. On closer examination many of these aggregates can be seen to be surrounded by a thick bilayered membrane structure which can be seen clearly under phase contrast (see Figs. 4.3.6 (b), 4.3.7 (b,c) and 4.5). This may well be some sort of attempt at producing cuticle. EM studies were attempted but found to be very difficult as such small aggregates were only lightly adherent to the tissue culture plastic, and so were frequently lost during preparation. Pupal cuticle-like material can be sometimes seen floating around in

culture, (Fig. 4.4), so it does appear that some sort of cuticular production is being carried out in the cells. The appearance of this is very variable and does not always seem to correlate with 20-HE treatment and occasionally is seen in untreated cultures. Some lines, notably the leg cloned line LIA produces putative tanned cuticle in culture. This does not appear to be 20-HE dependent, there being no notable increase in response to 20-HE treatment. Occasionally other cultures also produce areas of tanned cuticle appearing as brown spots in an aggregate of cells (Fig. 4.11). If cells are maltreated either through being left in culture for too long without fresh medium or treated with a toxic chemical (Mitomycin C- used during cloning attempts) then sometimes the response is to produce large quantities of melanised (sclerotic) cells as a prelude to death.

Therefore it seems that these cells are able to produce cuticle-like material and to undergo melanisation *in vitro*, both of which do not occur in embryonic cell lines but which are an inherent part of imaginal disc differentiation *in vivo*. However the extent of this imaginal differentiation is poor and variable, perhaps better culture methods could produce better differentiation *in vitro*.

Resistance to 20-HE

Apart from the few exceptions noted, the response to ecdysteroids of different cell lines is fairly consistent. Only one cloned cell line C9 does not respond in a normal way to 20-HE, the reasons for which will be discussed further in Chapter 5.

As already mentioned, one of the first responses to 20-HE is a cessation in cell proliferation. Therefore any cell that can overcome this effect of 20-HE will have an enormous selective advantage over the other cells. This is what has happened with one of the cell lines where cells were specially grown to allow selection of cells that were insensitive to 20-HE. This produced a line 8R which was completely insensitive to 20-HE, as well as its complementary 8+ parent line that was 20-HE sensitive. This resulted in two cell lines being produced with exactly the same origins (cloned wing cells), the only difference being sensitivity to 20-HE.

Ecdysteroid resistant *Drosophila* cells have already been selected for using embryonic cell lines. Courgeon (1972) first reported that K_C cells maintained continuously in medium containing 20-HE eventually overcame the growth inhibiting properties of 20-HE. These cells appeared to arise spontaneously in culture at a frequency of about 1 in every 3×10^6 cells. The basis of this ecdysteroid resistance appeared to be an absence of ecdysteroid receptors although in this study 20-HE binding was low and there was high unspecific binding (Best-Belpomme and Courgeon 1975; Schneider and Blumenthal 1978). Cherbas (1980) also reported the occurrence of ecdysteroid resistant cell clones and that approximately 1 in 2×10^5 cells can form such a clone. Therefore it appears as though the selection of such lines is relatively easy due to the effect that 20-HE has on cell proliferation.

Provided an appropriate selective pressure can be applied to the cells this production of selective mutant cells could be carried out with a whole range of studies in mind.

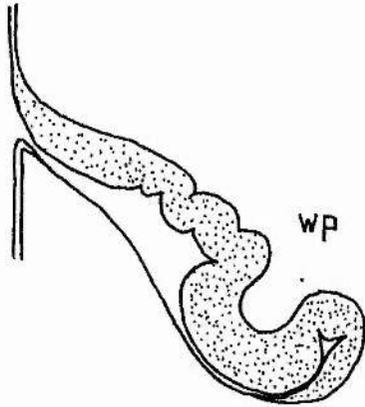
Transfection experiments could be carried out whereby the transfected gene also carried a bacterial antibiotic resistance gene with it enabling successful transformants to be recovered by applying the appropriate selective pressure (antibiotic in this case) to the cells.

Imaginal disc cell lines exhibit a profound and rapid morphological response to the 20-HE that in many ways mimics the response of intact imaginal disc tissue to 20-HE. The peculiarities of the culture environment mean that the response of the cells appears to be somewhat different from that of intact discs, and some aspects do appear to be abnormal (e.g. the excessive cell death seen in culture). However these may have more to do with the position the cells find themselves at 20-HE exposure (dissociated and in tissue culture) than any dilution of the cells imaginal characteristics. Disc cell lines exhibit a somewhat similar set of responses to embryonic cell lines with the exception that they appear to be more sensitive to 20-HE effects producing a greater response and also exhibit some sort of cuticular differentiation *in vitro* which embryonic cells never do.

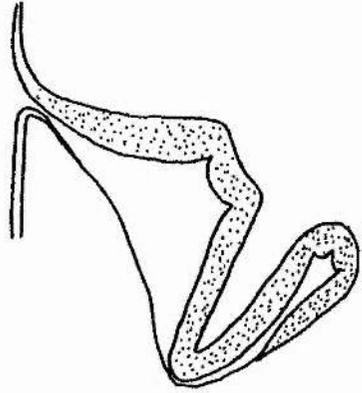
Figure 4.1

Figure 4.1 : Schematic representation of wing disc morphogenesis. The epithelium of the disc in the region of the wing pouch (**wp**) forms an expanded bilayer (**A-D**) which is everted (**C-D**) by contraction of the peripodial membrane (**solid**). After Fristrom (1988).

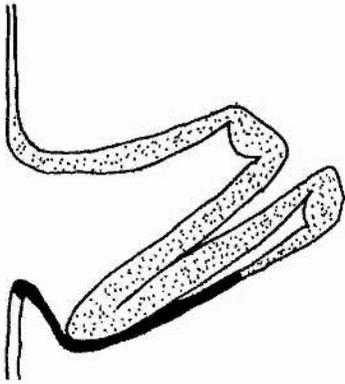
A



B



C



D

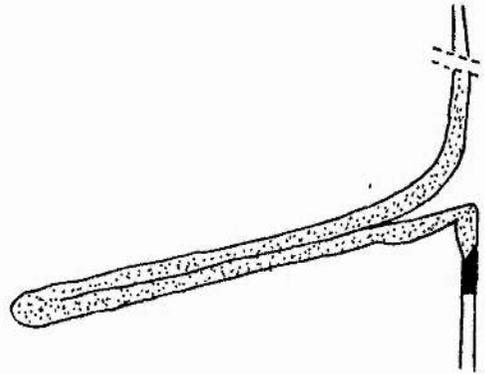


Figure 4.2

Figure 4.2 (a-d) : Graphs of the effects on cell division rates of 20-HE and ecdysone on two wing cell lines, W1 (p.40) and W2 (p.6). The graphs show cell yield measured daily over 6 days in cultures containing three different concentrations of hormone (10 ng/ml, 50 ng/ml and 100 ng/ml for 20-HE and 200 ng/ml, 500 ng/ml and 1 μ g/ml for ecdysone).

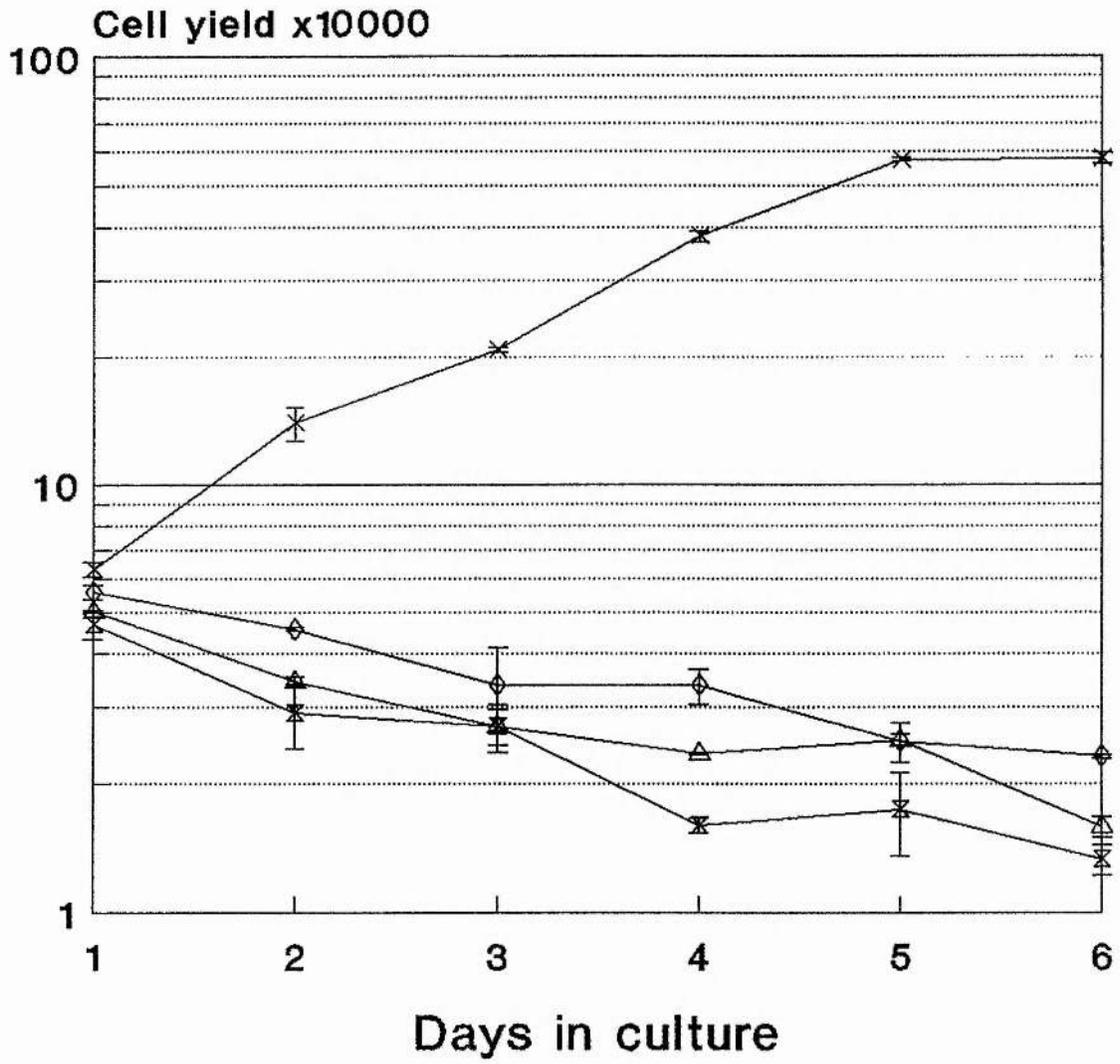
a.) W1 tested with 20-HE.

b.) W1 tested with Ecdysone.

c.) W2 tested with 20-HE.

d.) W2 tested with Ecdysone.

a



Level of 20-HE

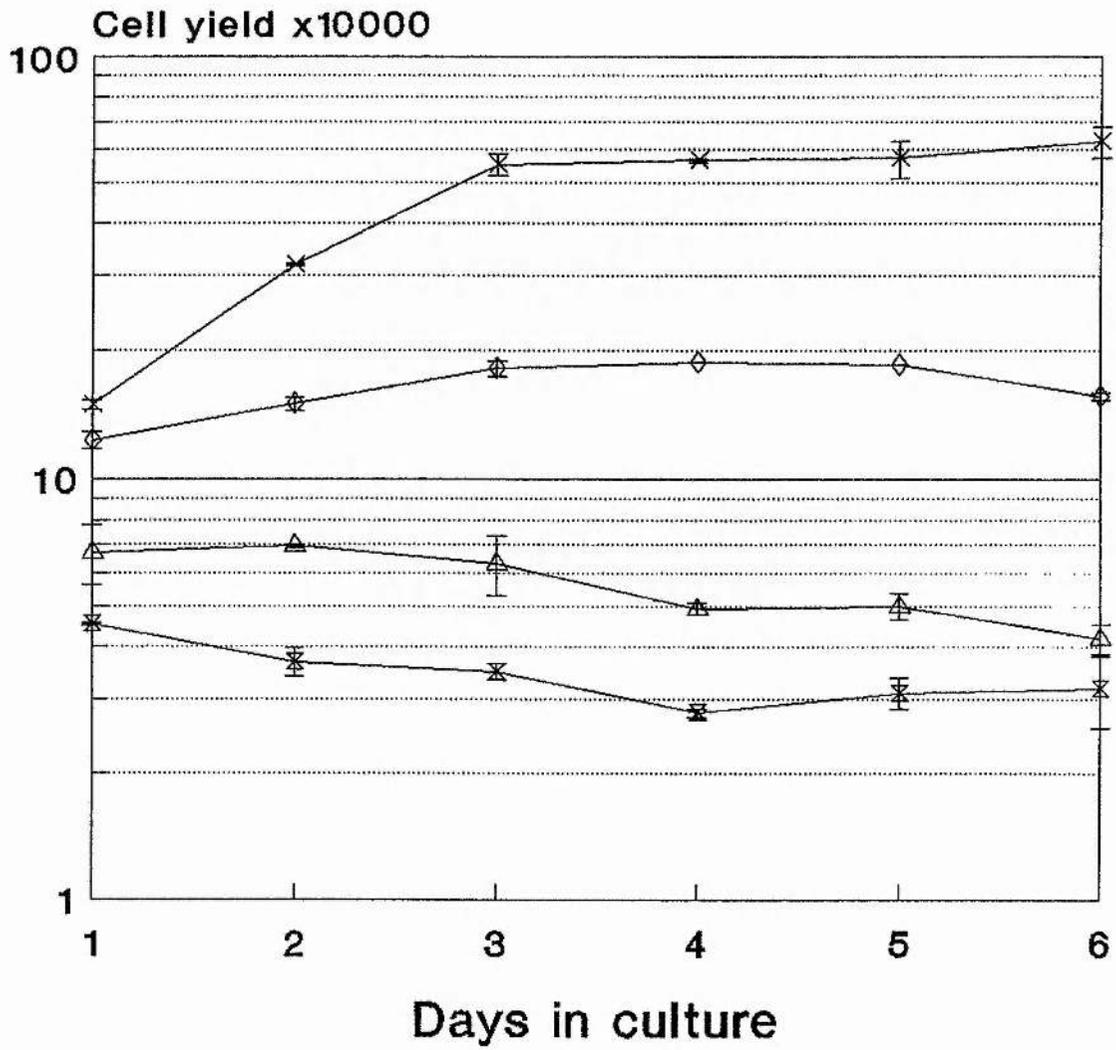
—x— Control

—◇— 10 ng/ml

—△— 50 ng/ml

—⊠— 100 ng/ml

b



Level of Ecdysone

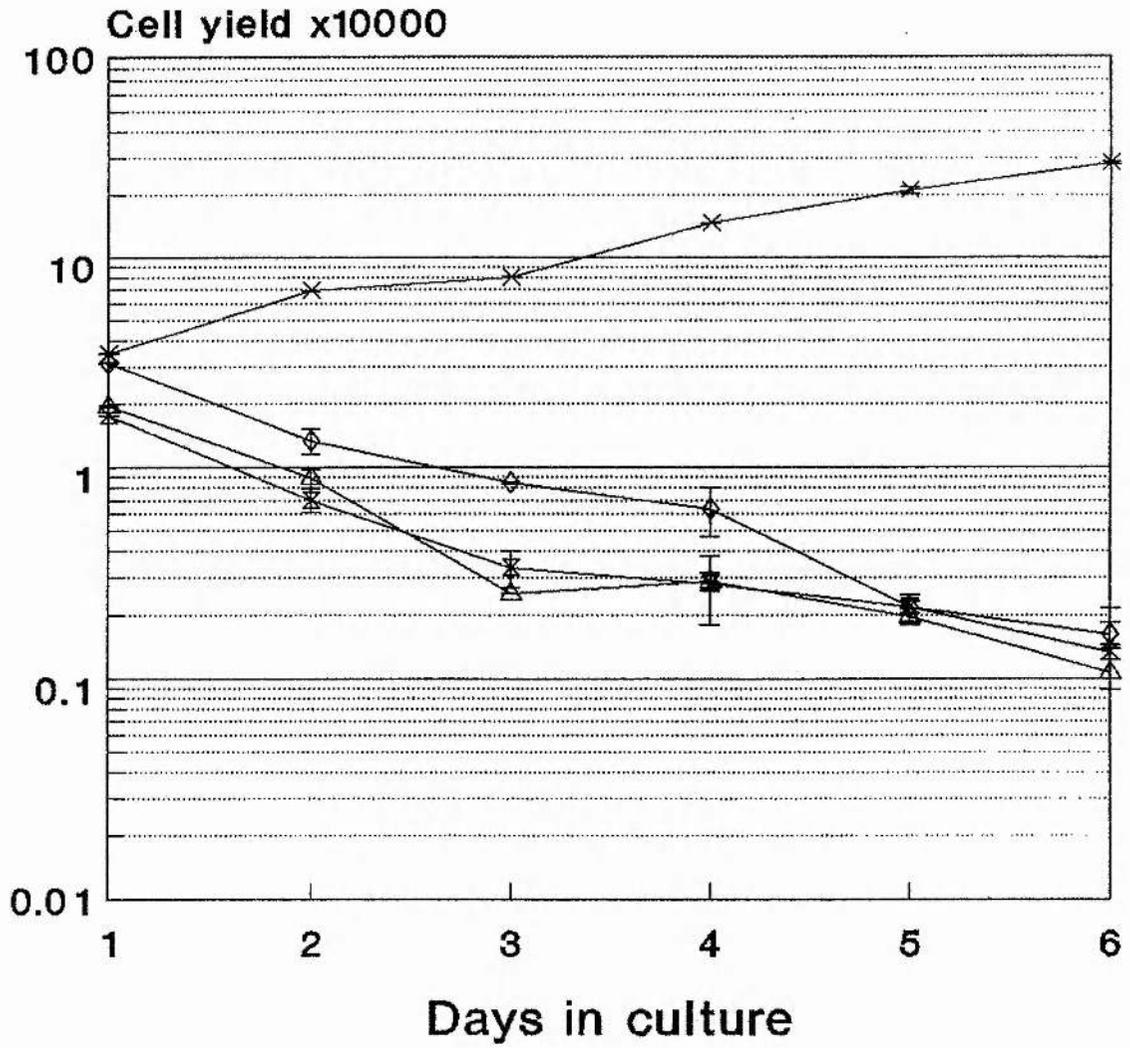
—x— Control

—◇— 200 ng/ml

—△— 500 ng/ml

—⊠— 1 ug/ml

c



Level of 20-HE

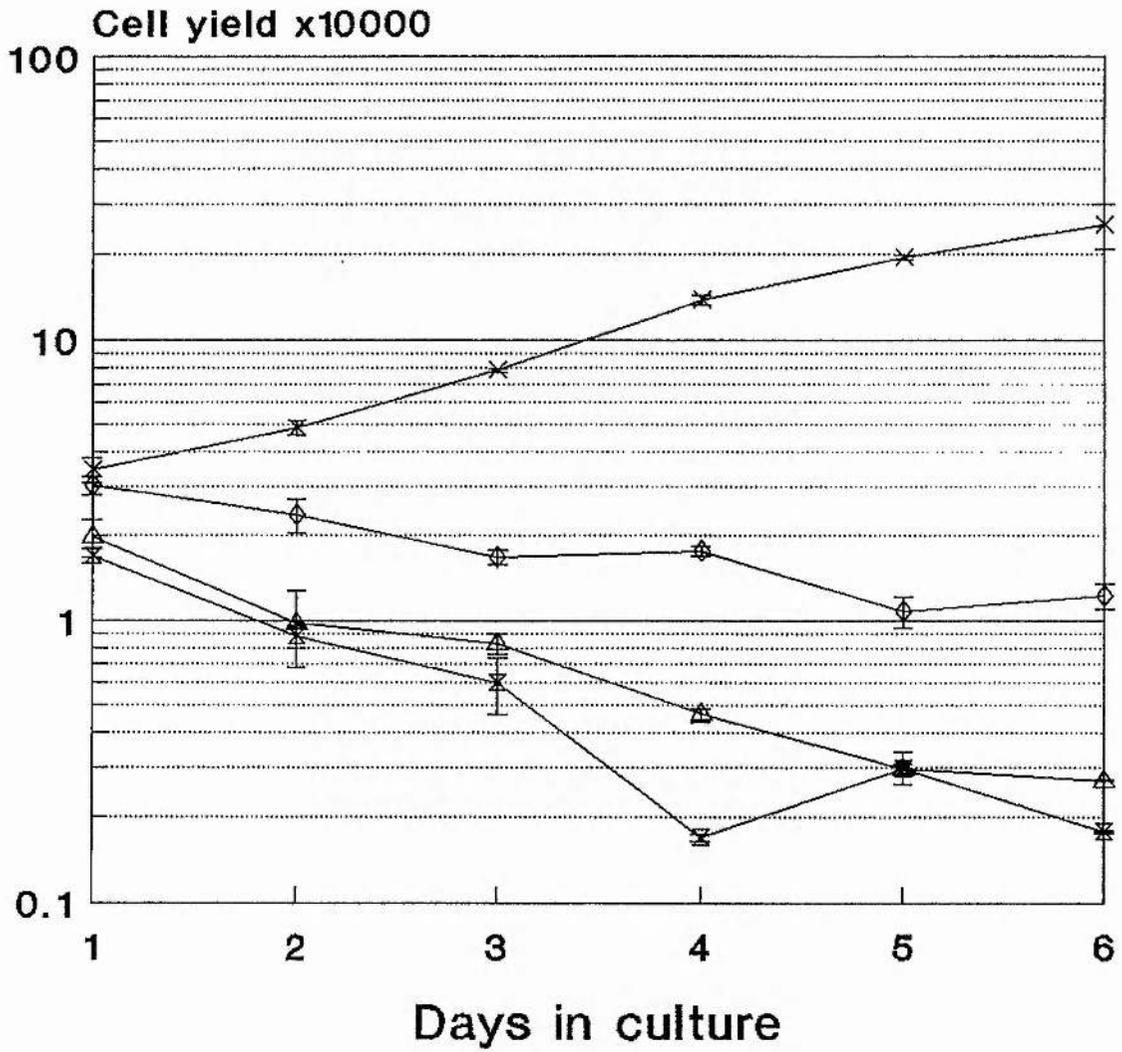
—x— Control

—◇— 10 ng/ml

—△— 50 ng/ml

—⊠— 100 ng/ml

d



Level of Ecdysone

—x— Control

—◇— 200 ng/ml

—△— 500 ng/ml

—⊠— 1 ug/ml

Figure 4.3

Figure 4.3 : Time course of exposure to 20-HE over a one week period using the cloned wing cell line Cl.8+ seeded at 3×10^6 cells per 5 cm Petri dish. Cultures were exposed to 10 ng/ml of 20-HE (2×10^{-8} M) in the (b) series. Control cultures are shown in (a)

Fig. 4.3.1 : Day 1 Note the elongated cells (arrowed) and the numbers of dead cell particles (P) in (b). Scale bars = 30 μm in (a) and 50 μm in (b).

Fig. 4.3.2 : Day 2 Scale bars = 50 μm . In (b) elongated cells (arrowed) the start of aggregates (A) can be seen.

Fig. 4.3.3 : Day 3, Scale bars = 50 μm . Note the elongated cells with processes in (b).

Fig. 4.3.4 : Day 4, Scale bars = 50 μm in (a) and 100 μm in (b). Aggregates (A) can be seen to be forming in both (a) and (b).

Fig. 4.3.5 : Day 5, Scale bars = 50 μm in (a) and 100 μm in (b). Aggregates (A) can be seen to be forming in (a) and aggregates (A) with cell processes(cp) in (b).

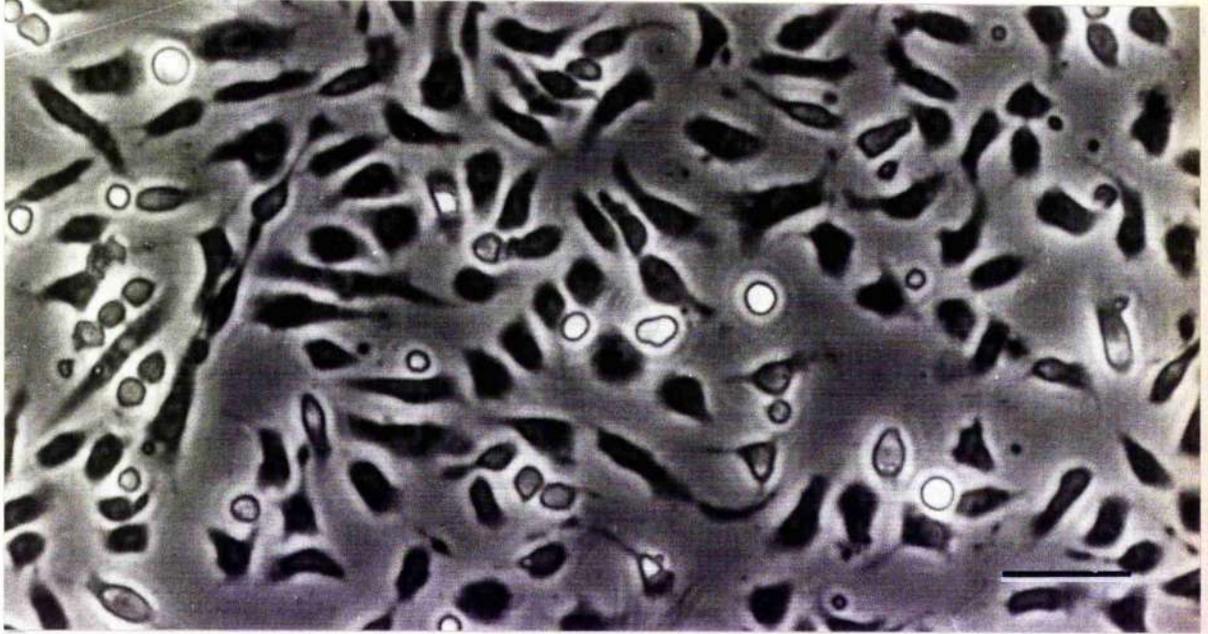
Fig. 4.3.6 : Day 6, Scale bars = 50 μm in (a) and (b). The aggregates (A) are now much larger in (a). Distinct aggregates (A) with cells and cell processes (C) extending around the periphery. Note the presence

of cells with flattened (F) morphology.

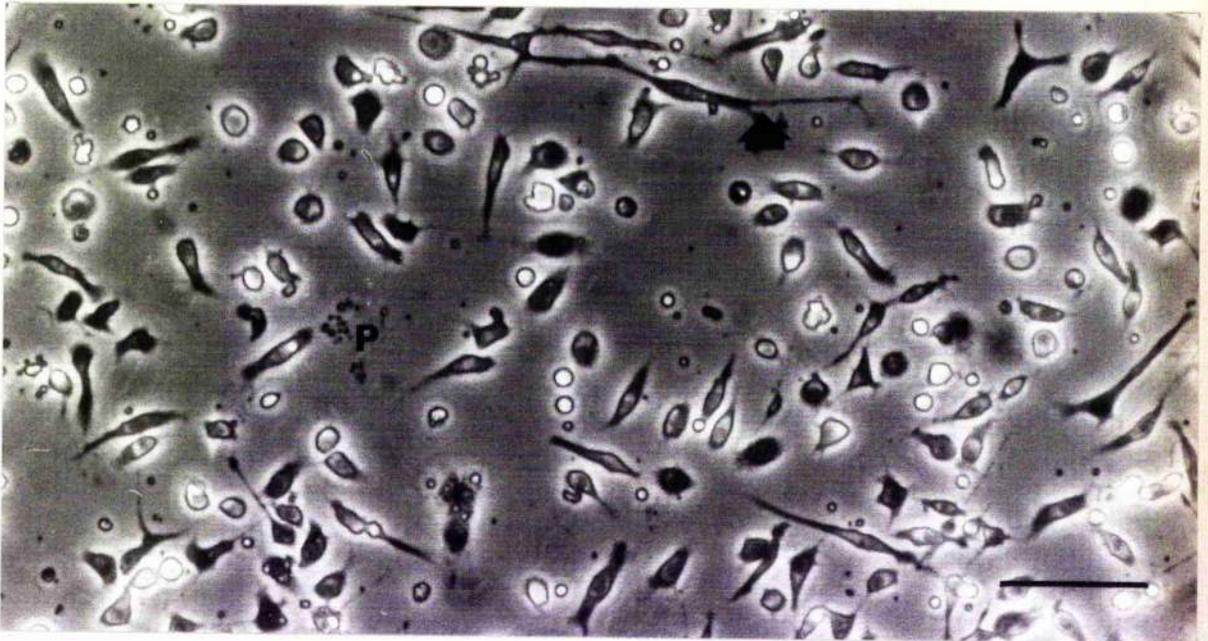
Fig. 4.3.7 : Day 7 Control and 10 ng/ml in (a) and (b) respectively. The cells have aggregated into large multicellular aggregates (A) in the control culture whilst in (b) the culture now consists of small distinct aggregates with very long cell processes, in this picture a cell (arrowed) can be seen to be extending a process towards an aggregate suggesting that this may be one mechanism for bringing about aggregate formation. (c) shows a blowup of the aggregate in (b) showing the bilayered structure surrounding the cells (arrowed) and (d) shows a different view illustrating how long cell processes (arrowed) can extend between aggregates. Scale bars = 50 μm in (a) and (b), 15 μm in (c) and 100 μm in (d).

1

a

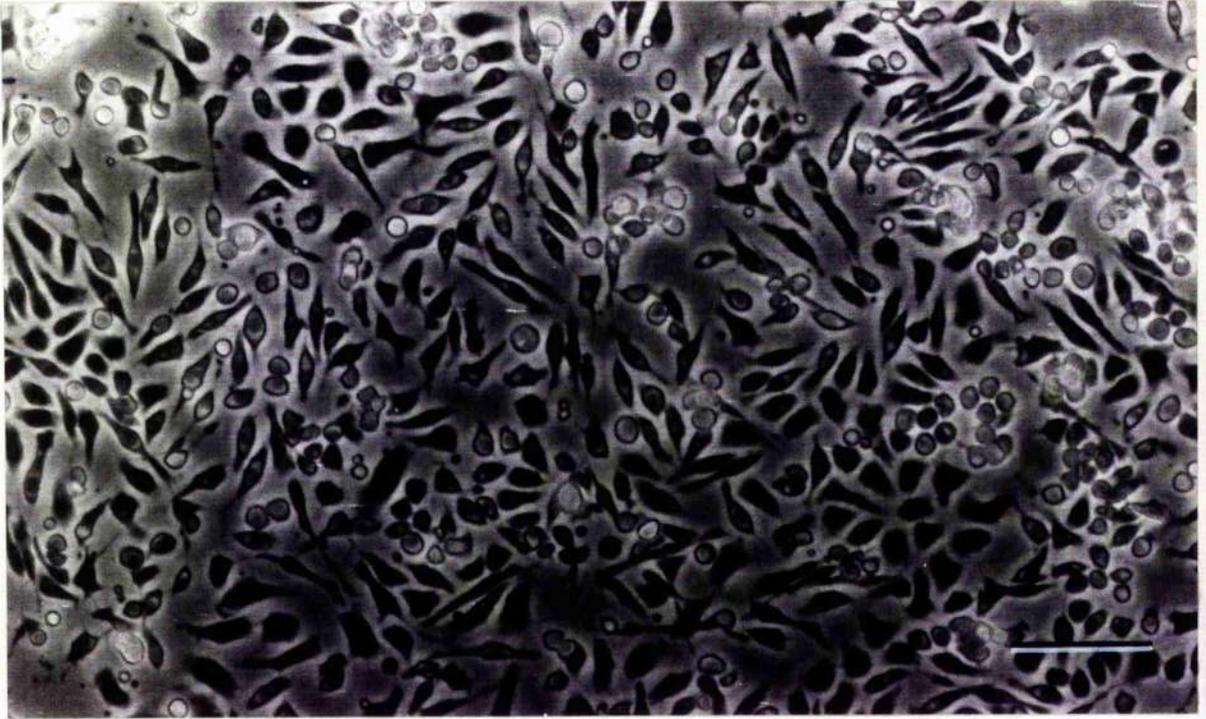


b

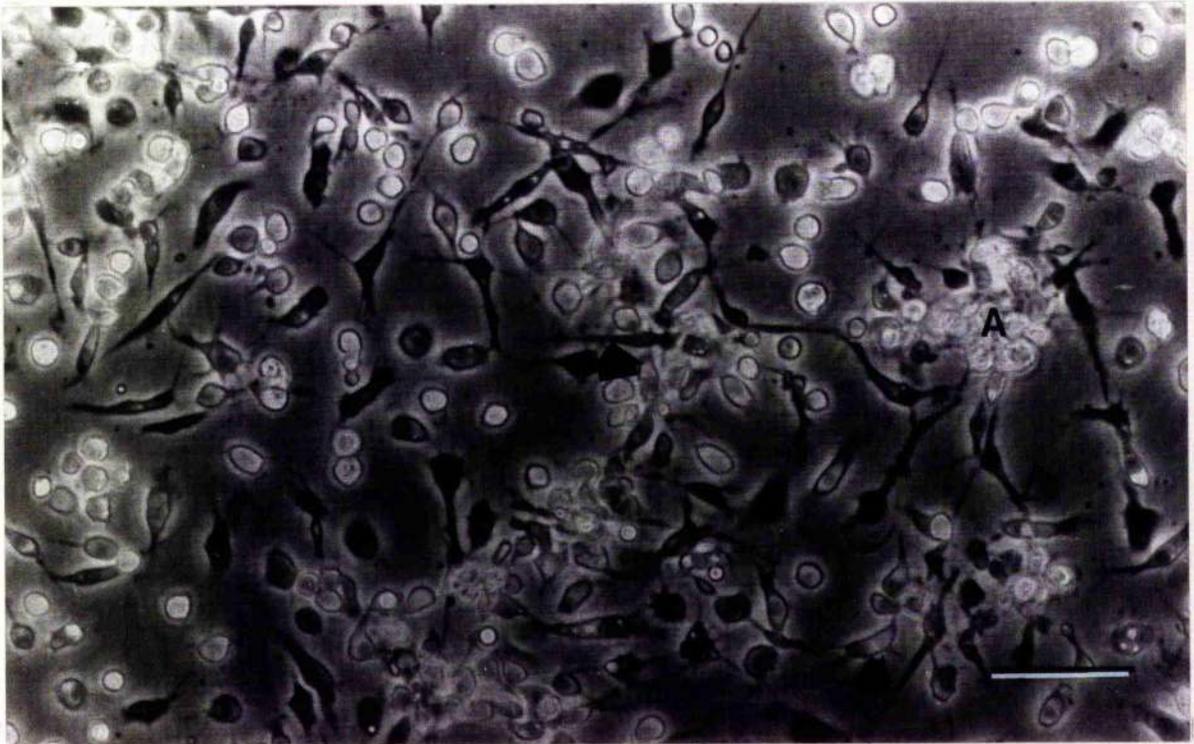


2

a

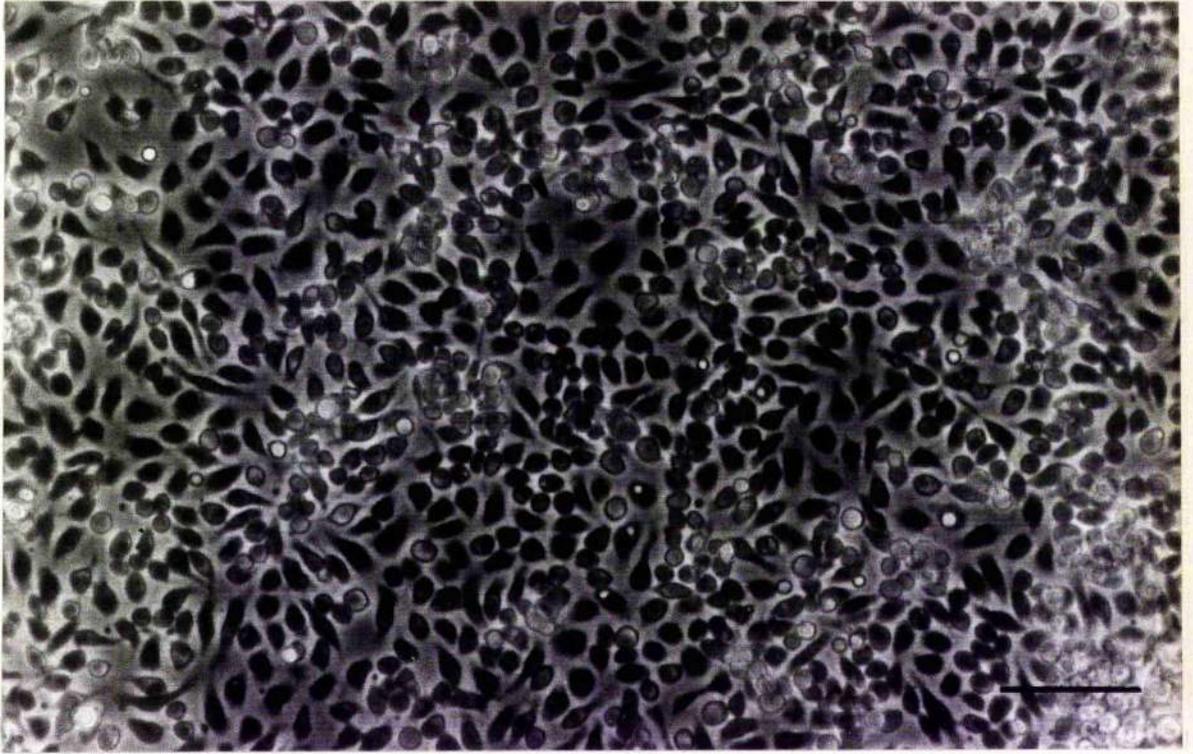


b



3

a

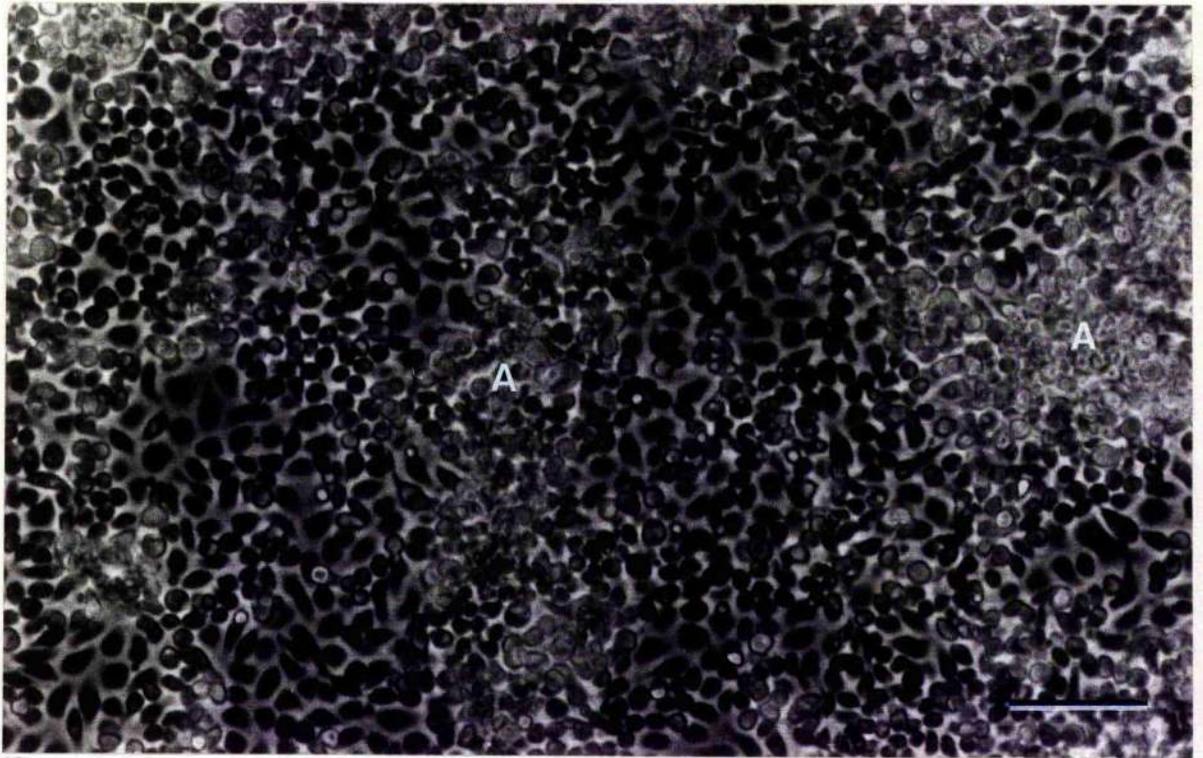


b



4

a

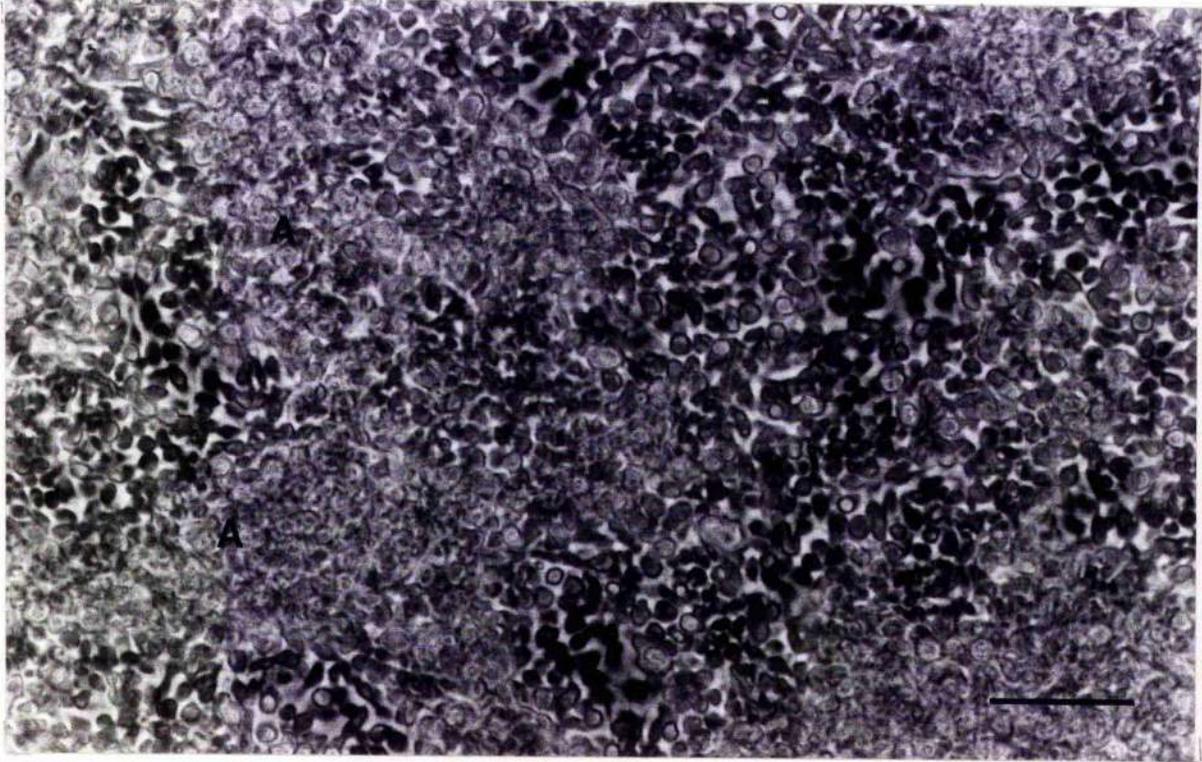


b

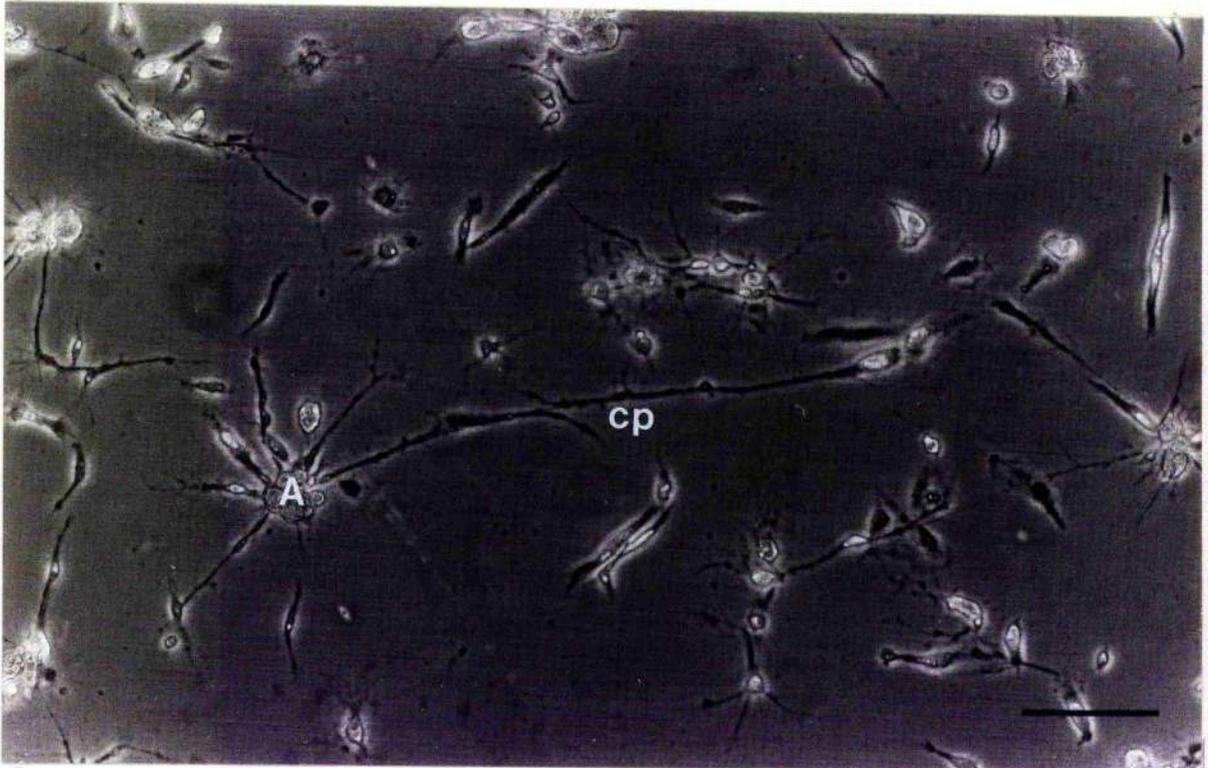


5

a

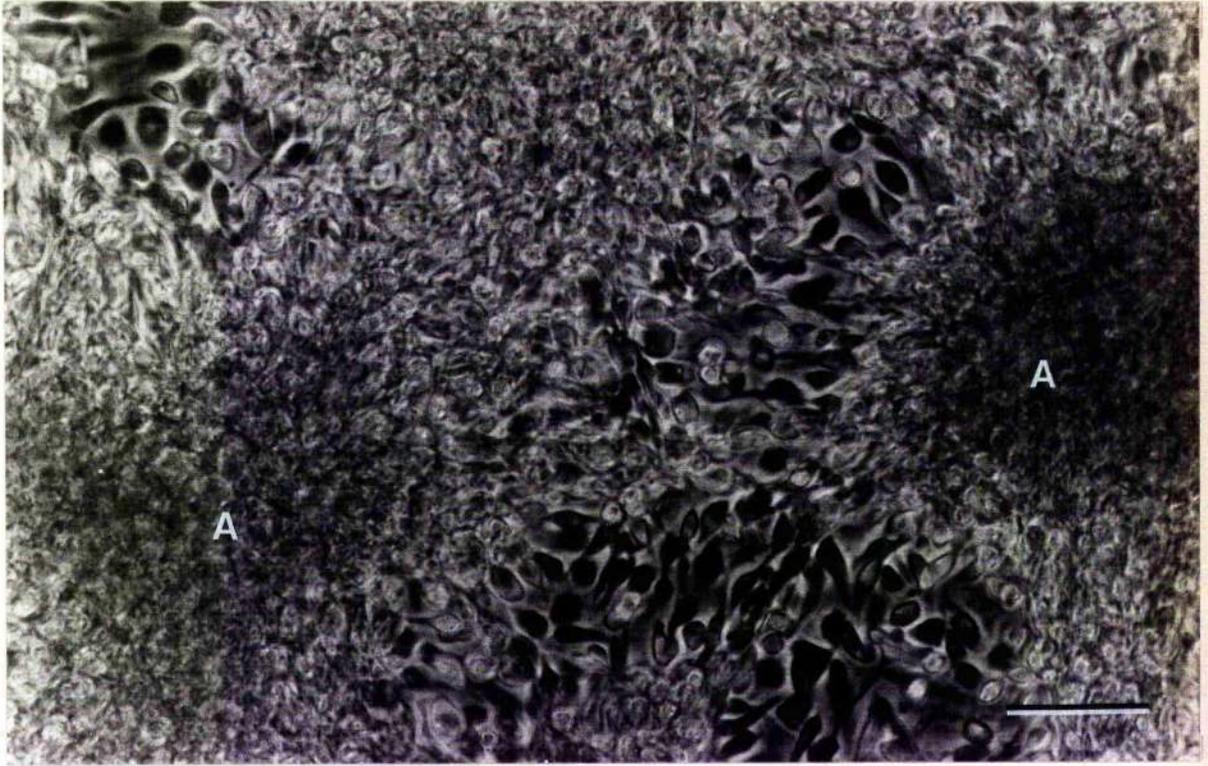


b

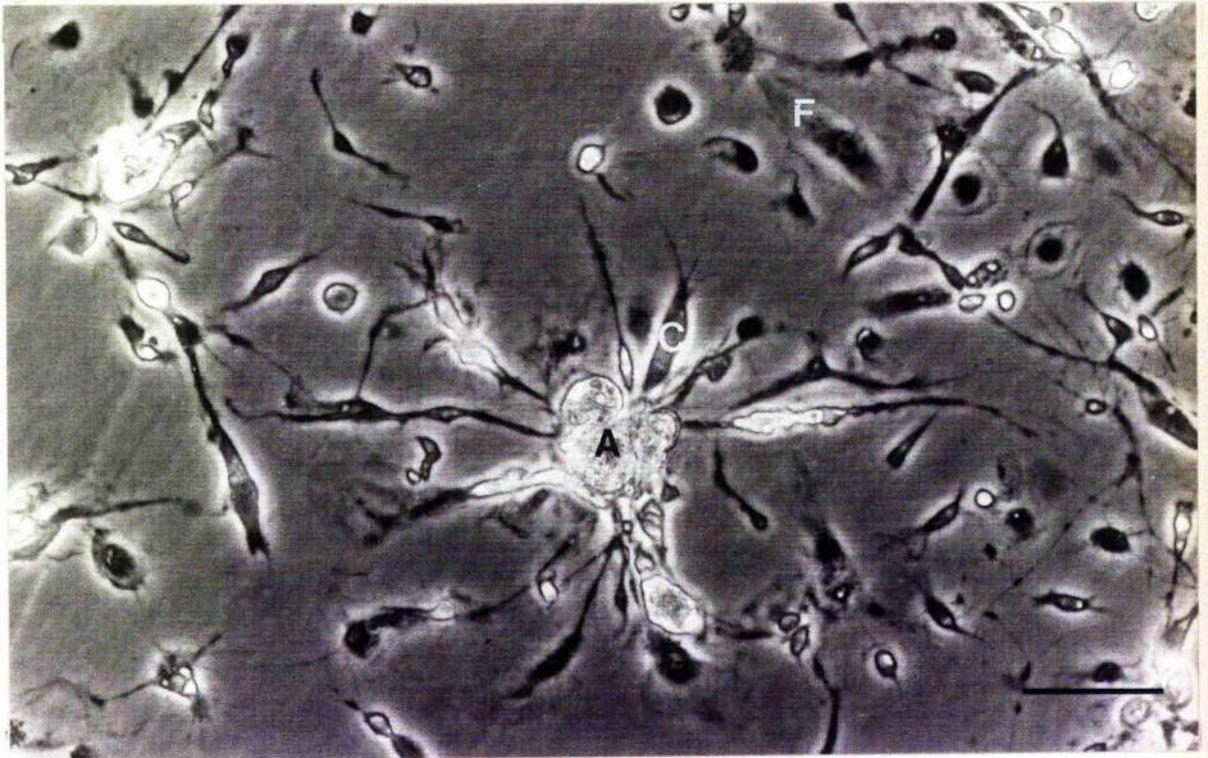


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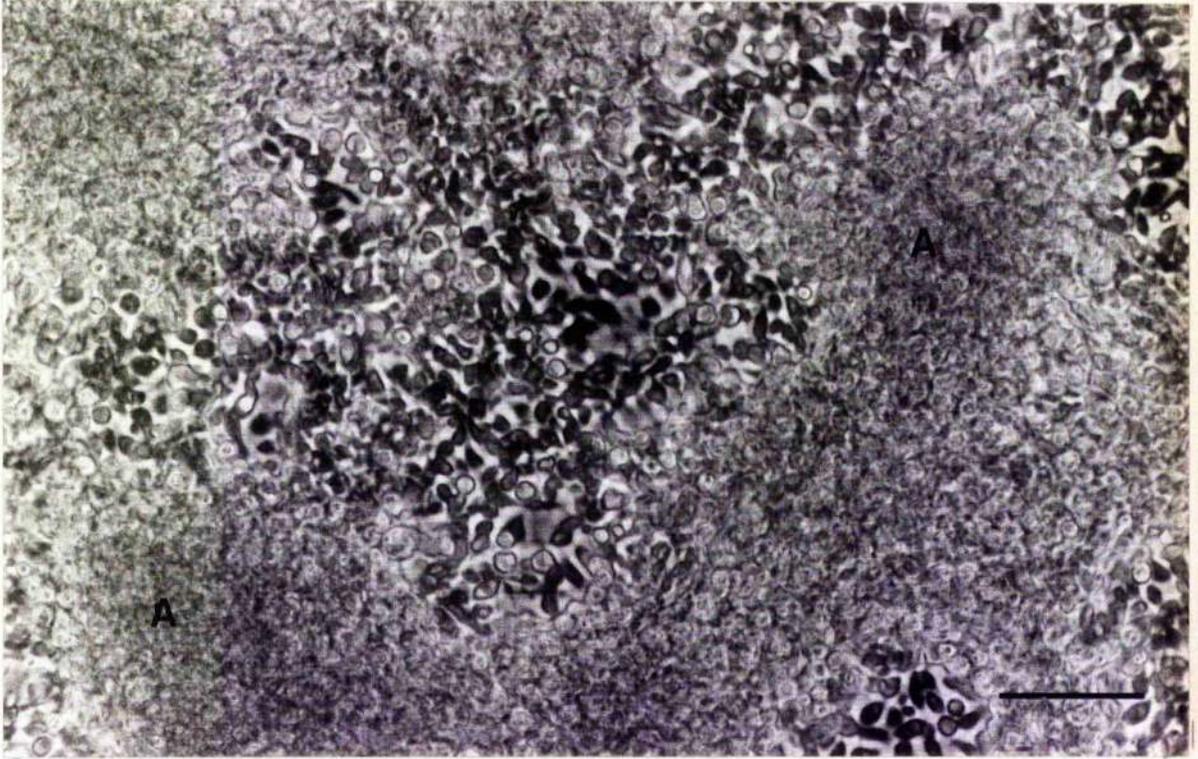
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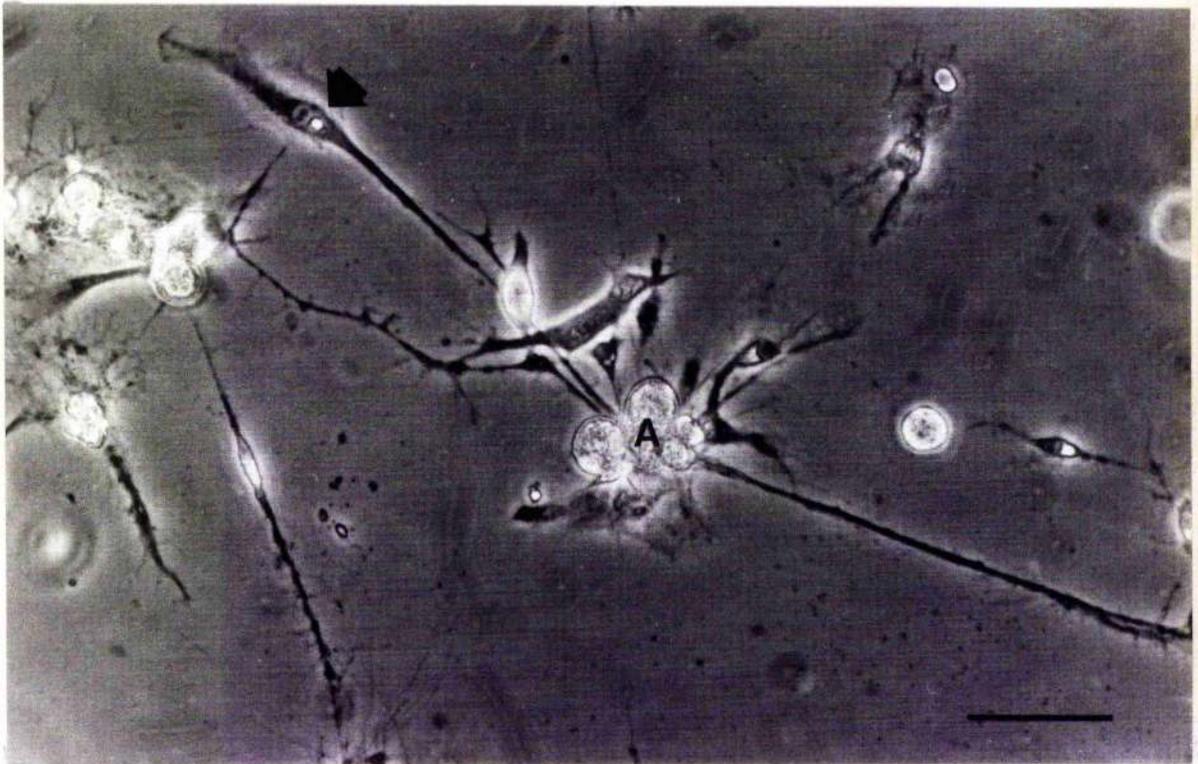
b



a

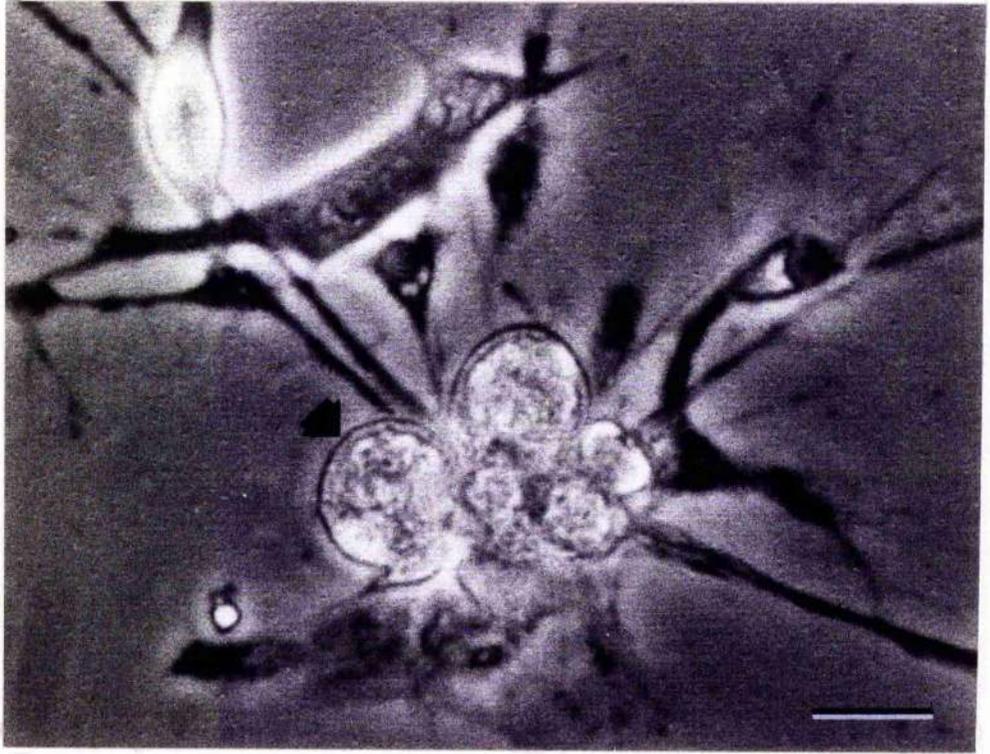


b



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c



d

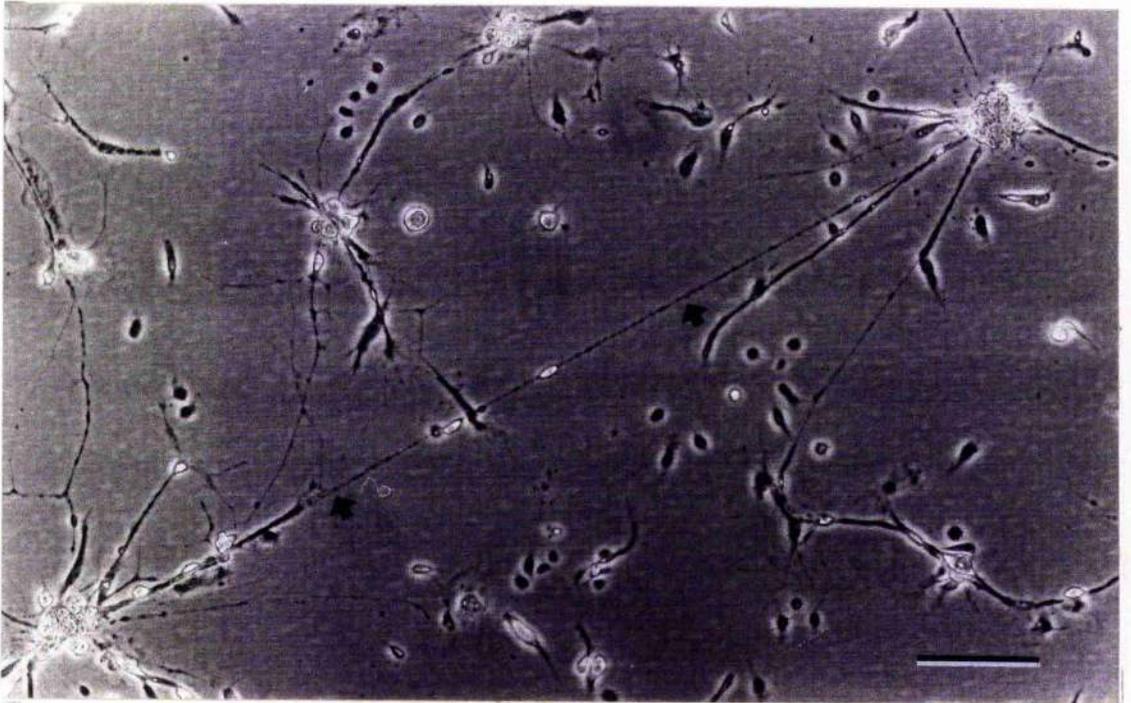


Figure 4.4

Figure 4.4 : Putative pupal cuticle-like material found floating in culture medium of a culture of cloned wing cells (Cl. 13) treated with 50 ng/ml of 20-HE for one week. Scale bar = 50 μ m.



Figure 4.5

Figure 4.5 : Aggregate of cells from a culture of cloned wing cells (Cl. 8+) treated with 10 ng/ml of 20-HE for 7 days. Note the bilayered structure surrounding the cells in the aggregate (arrowed). Scale bar = 30 μ m.

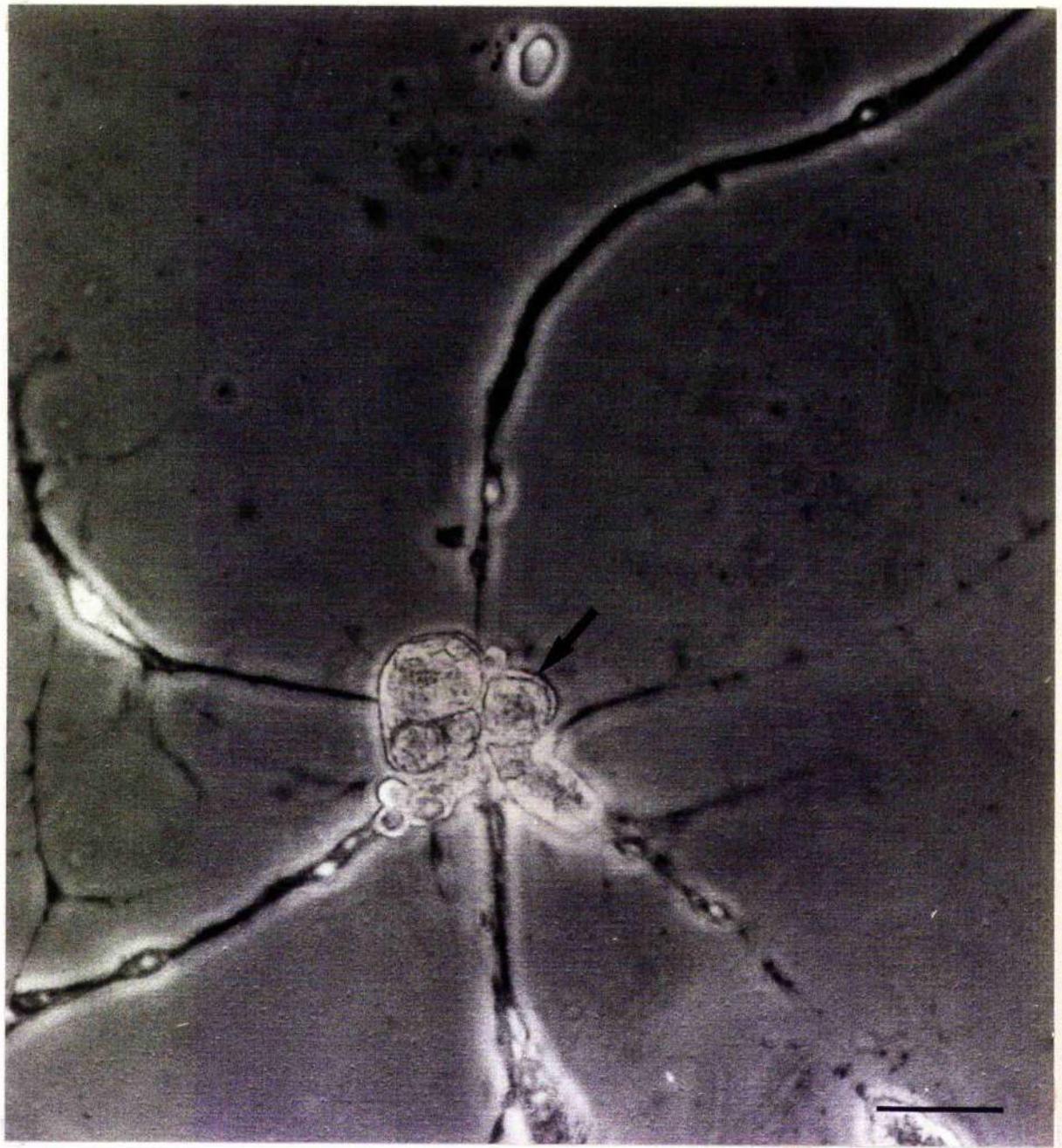


Figure 4.6

Figure 4.6 : Morphological responses of the cloned wing cell line Cl.8+ to 20-HE at 10 ng/ml (2×10^{-8} M) after 7 days in culture.

Fig. 4.6.1 : Control, cells grown in complete medium without 20-HE. Scale bar = 10 μ m.

Fig. 4.6.2 : Small clump of cells in culture treated with 20-HE. Scale bar = 10 μ m.

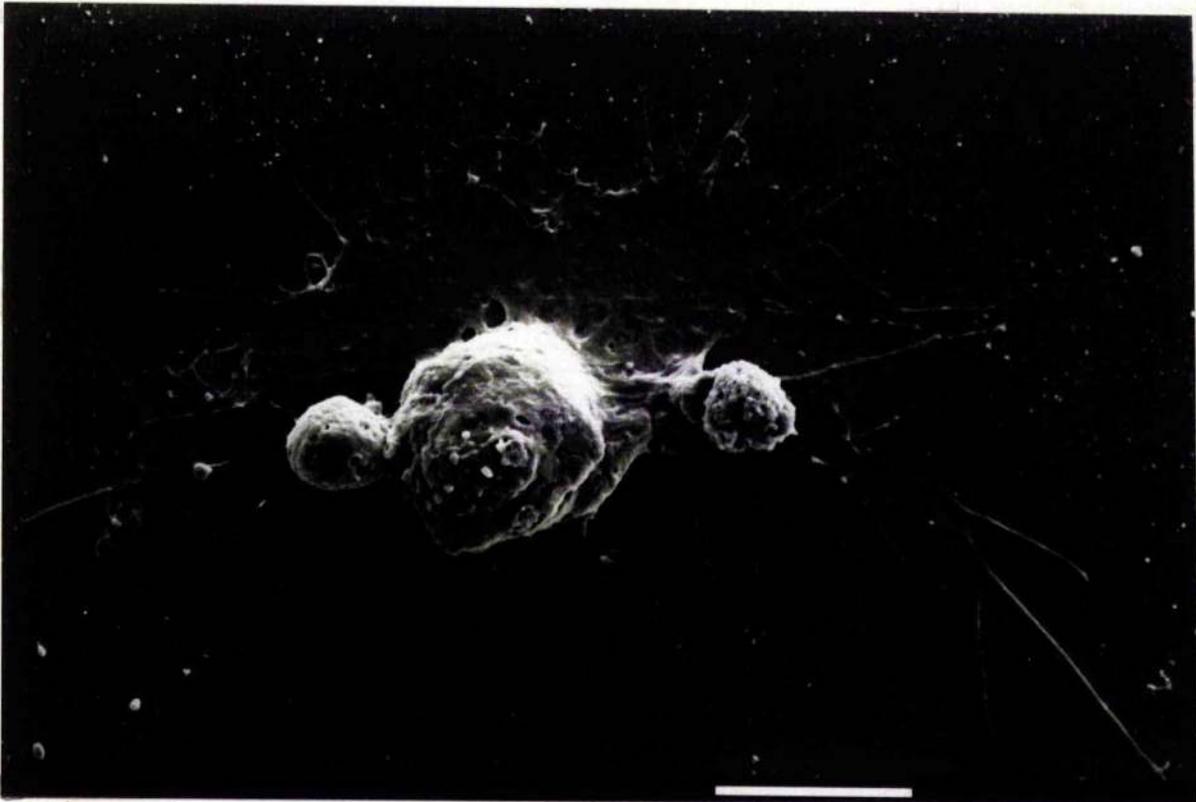
Fig. 4.6.3 : 20-HE treatment, small clump of cells with cytoplasmic sheet (S) extending from the base of the aggregate. Scale bar = 10 μ m.

Fig. 4.6.4 : 20-HE treatment, overview of culture showing a number of small aggregates with extensive cell processes (cp). Scale bar = 10 μ m.

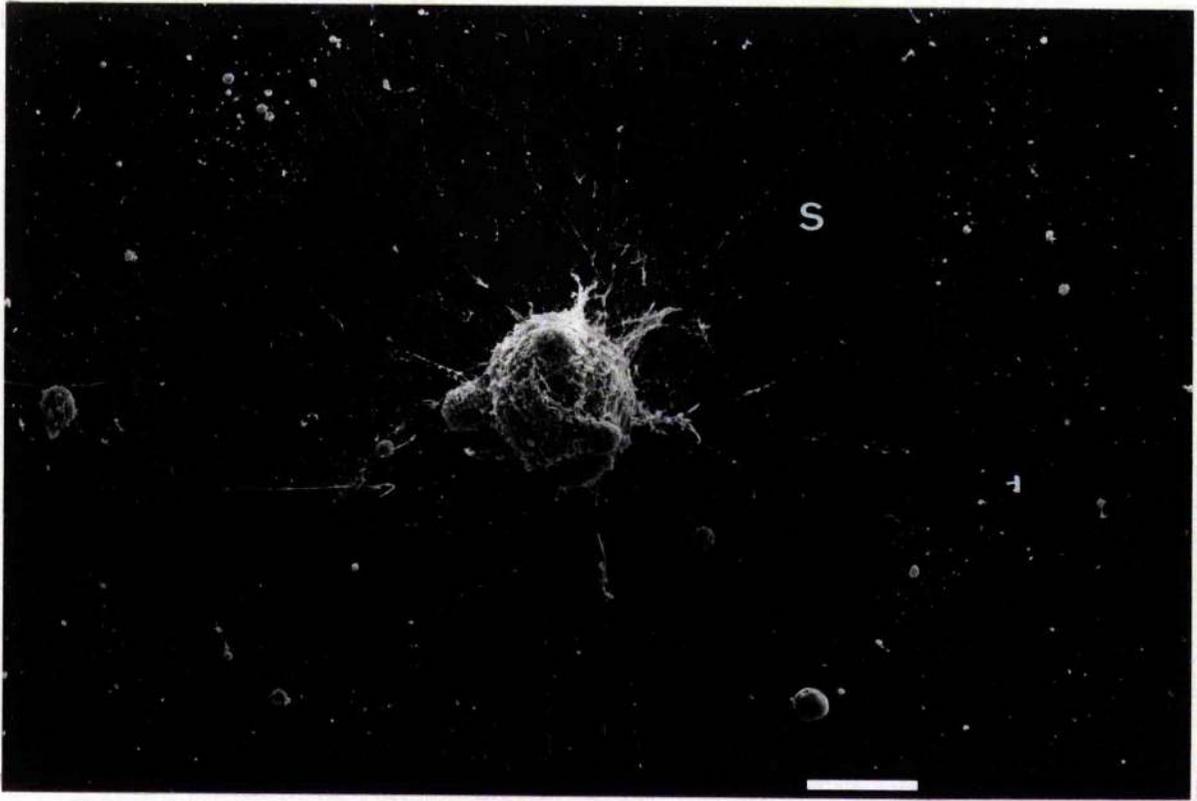
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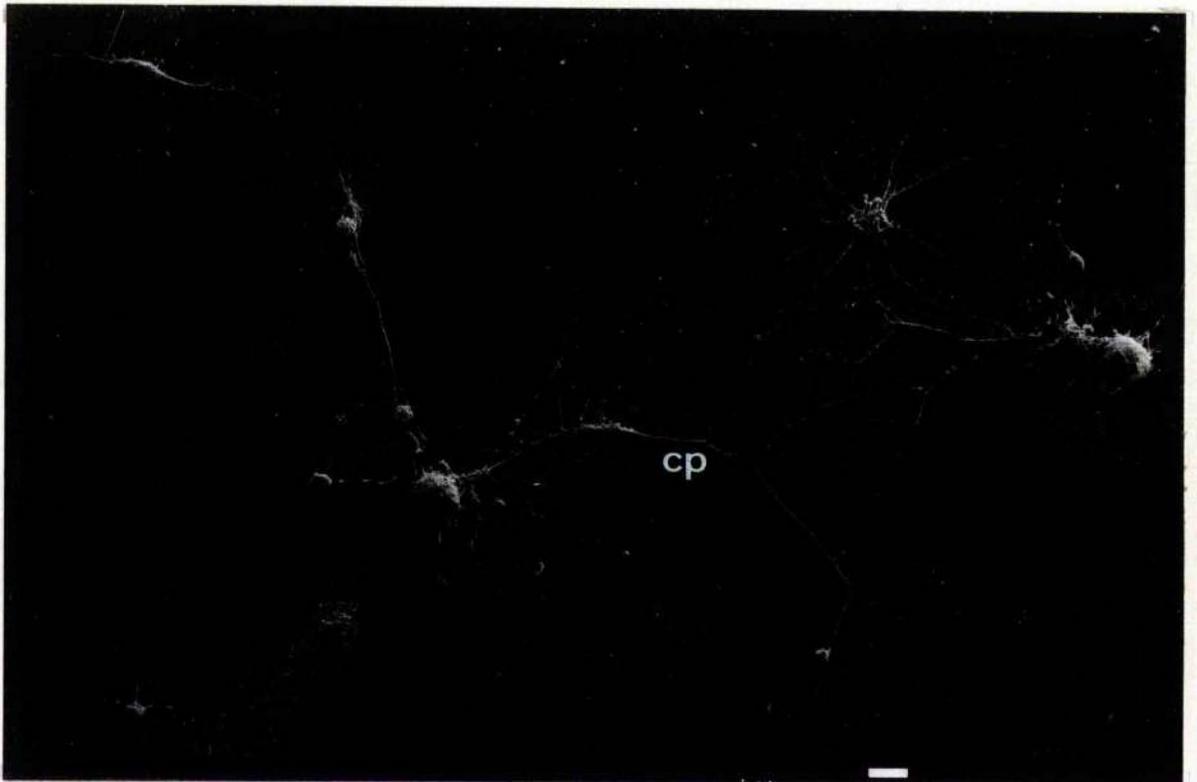


Figure 4.7

Figure 4.7 : Exposure time needed for 20-HE action. Cells from the wing line W1 were incubated with 10 ng/ml 20-HE for 1, 2 and 7 days with medium changes in the 1 and 2 day cultures. Scale bars = 100 μ m.

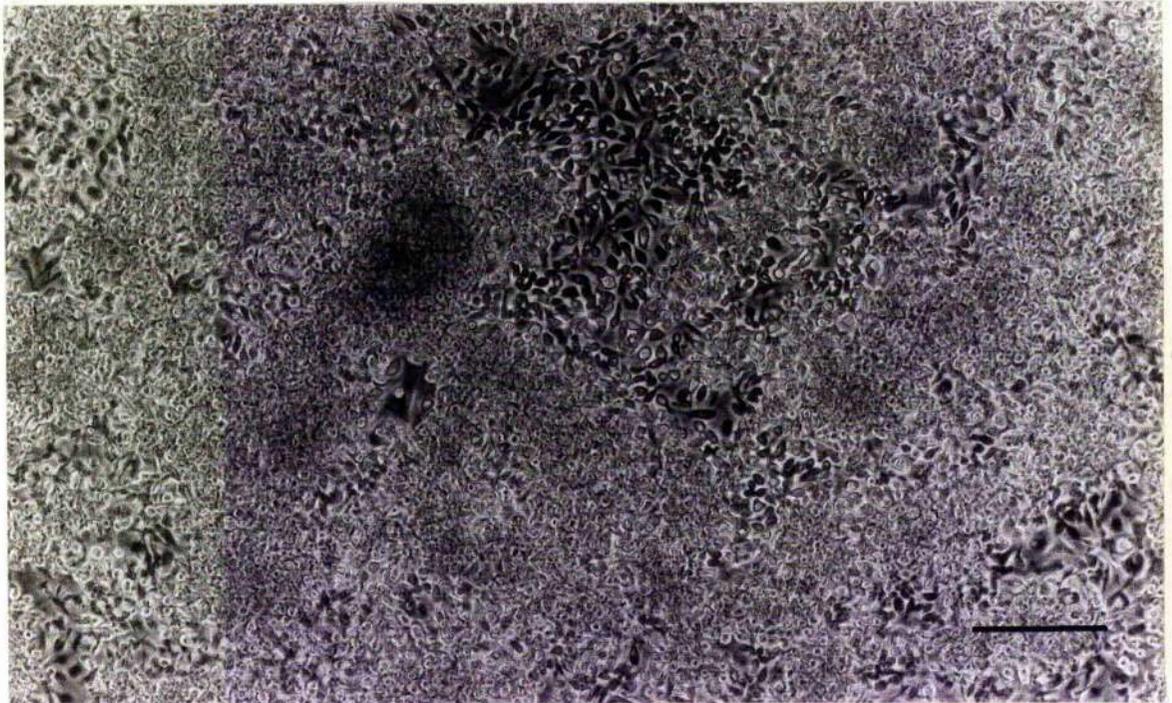
Fig. 4.7.1 : Control culture in complete medium for 7 days.

Fig. 4.7.2 : 20-HE treatment for 1 day with a medium change.

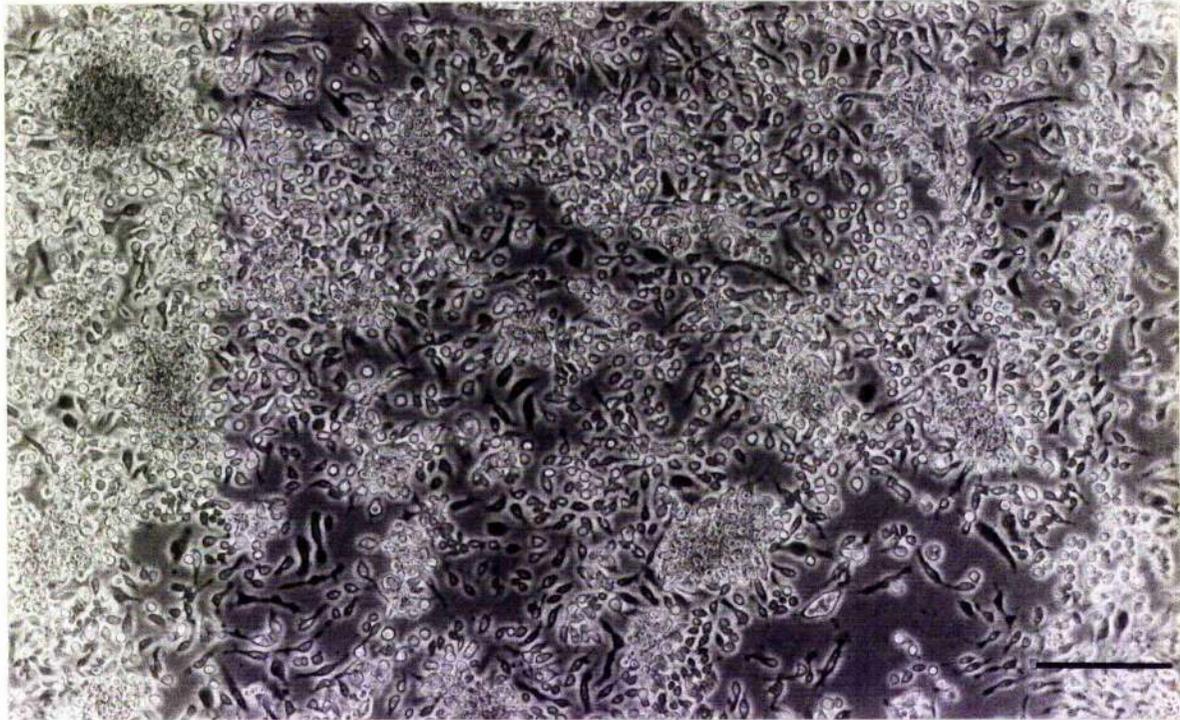
Fig. 4.7.3 : 20-HE treatment for 2 days with a medium change.

Fig. 4.7.4 : 20-HE treatment for 7 days without any medium change.

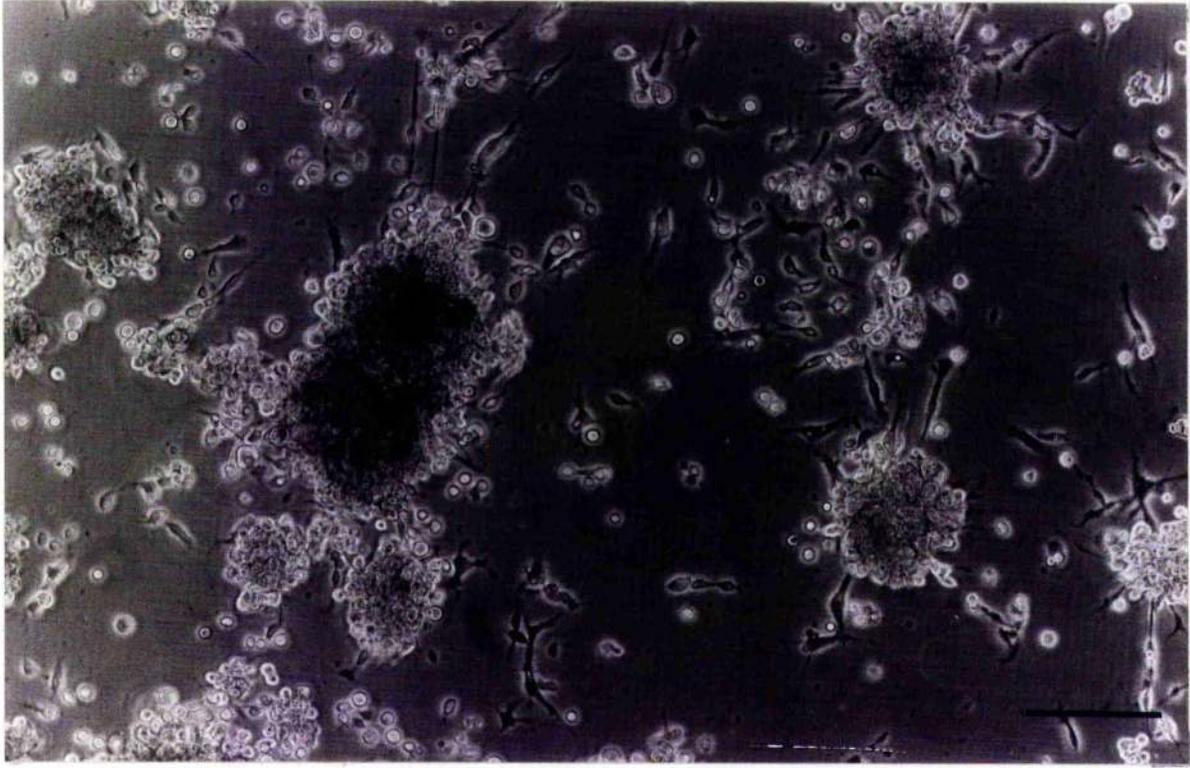
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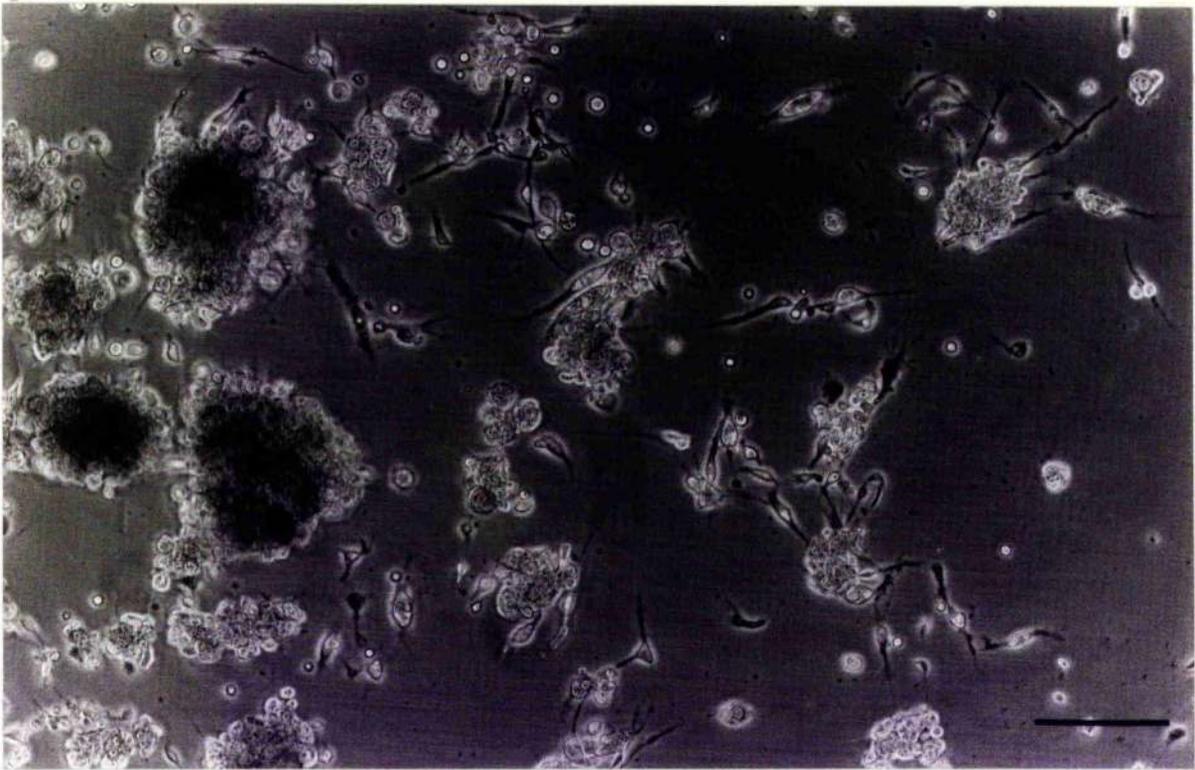


Figure 4.8

Figure 4.8 : Cloned wing cell line Cl. 8+ exposed to titres of 20-HE ranging from 2 ng/ml to 10 ng/ml at 2 ng intervals and left for 7 days. All scale bars = 100 μm .

Fig. 4.8.1 : Control culture, note the degree of aggregation (A) that has occurred.

Fig. 4.8.2 : 2 ng/ml (4×10^{-9} M) of 20-HE, note the reduced degree of aggregation (A) as compared to the control.

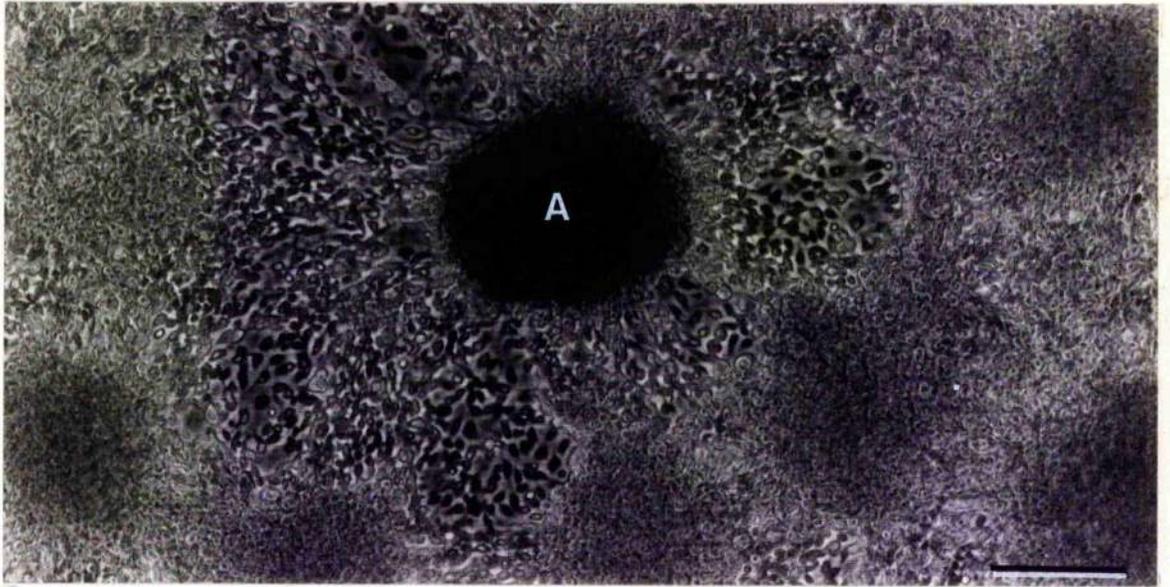
Fig. 4.8.3 : 4 ng/ml (8×10^{-9} M) of 20-HE, note the small foci of aggregating cells (A) and the apparent reduction in cell numbers in the surrounding areas.

Fig. 4.8.4 : 6 ng/ml (1.2×10^{-8} M) of 20-HE, the aggregates (A) are larger and cell processes can be seen extending from them.

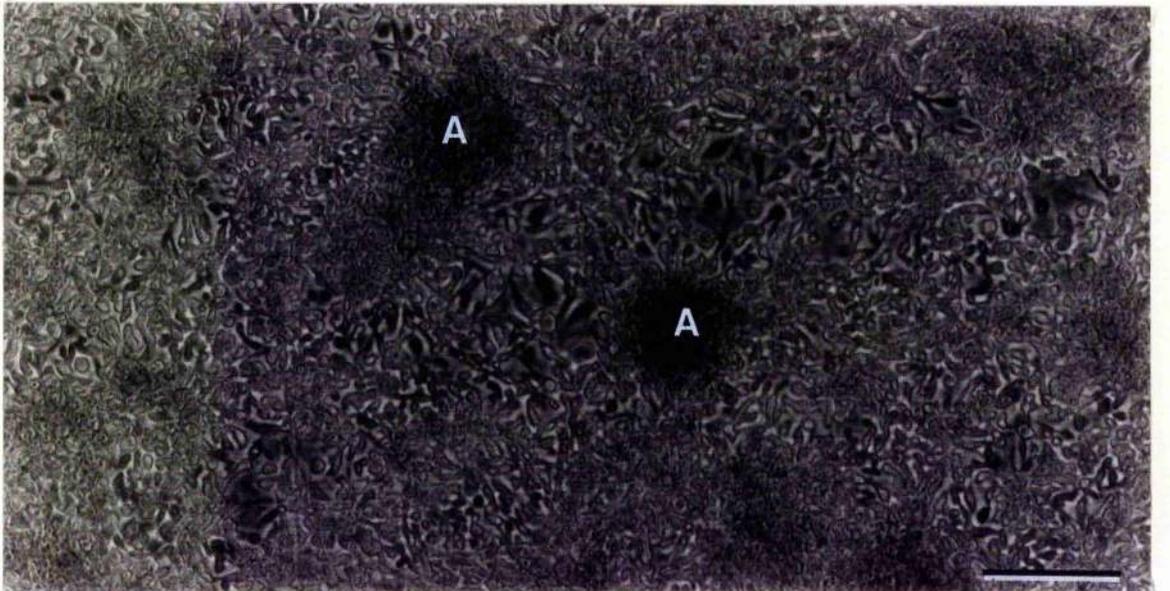
Fig. 4.8.5 : 8 ng/ml (1.6×10^{-8} M) of 20-HE, the areas surrounding the aggregates are now relatively bare of cells and the aggregates (A) now possess long cell extensions (arrowed) radiating outwards.

Fig. 4.8.6 : 10 ng/ml (2×10^{-8} M) of 20-HE, control with similar features as described previously. Aggregates are very distinct with long cell processes (arrowed) extending outwards and the surrounding areas are relatively bare of cells.

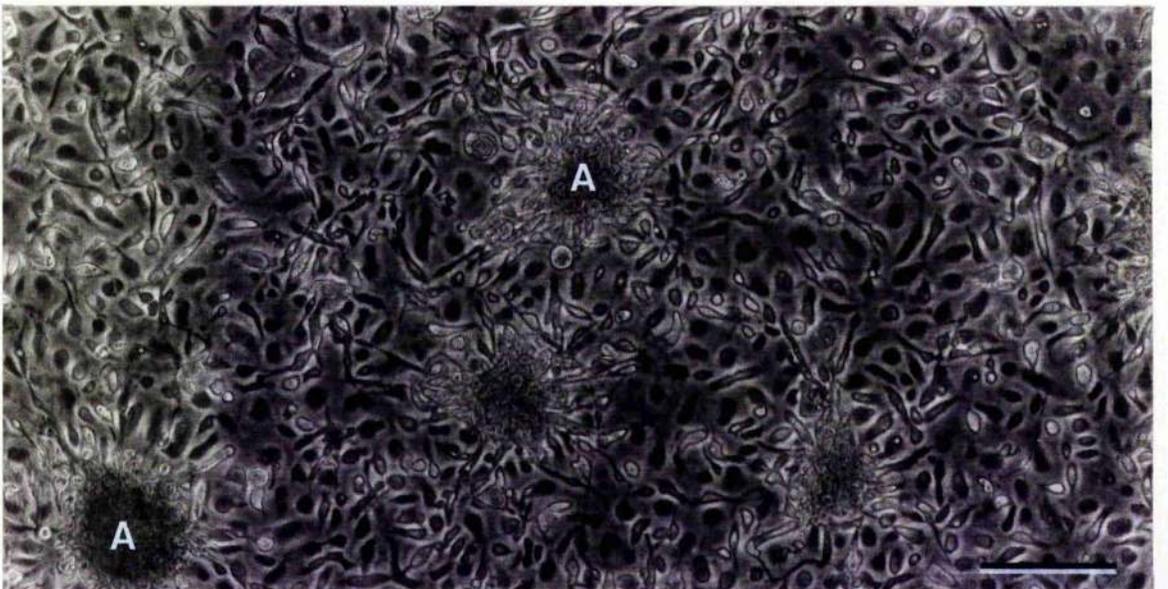
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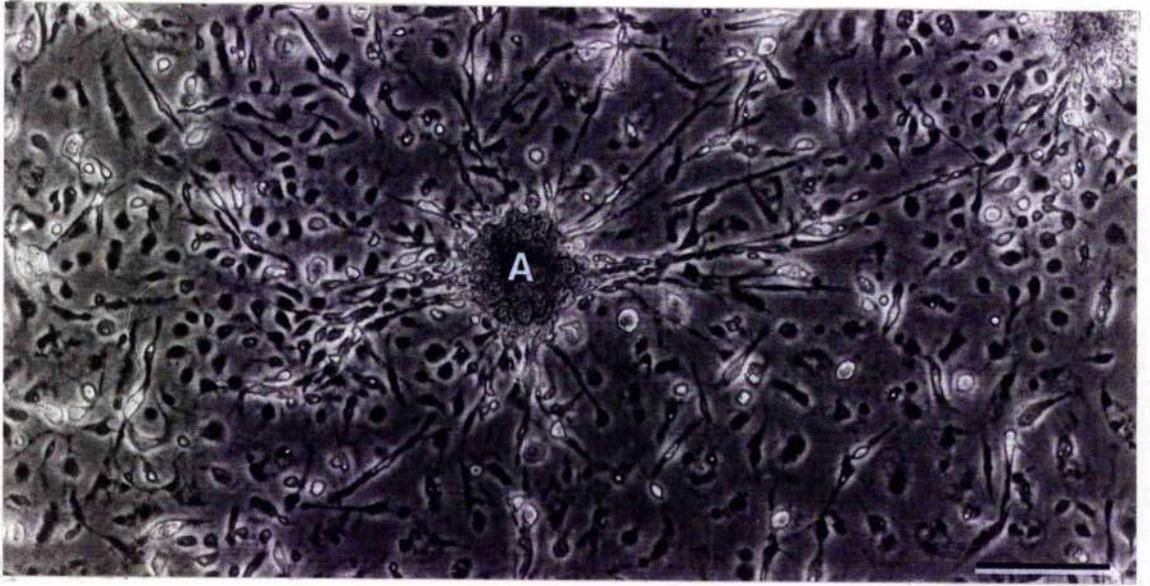
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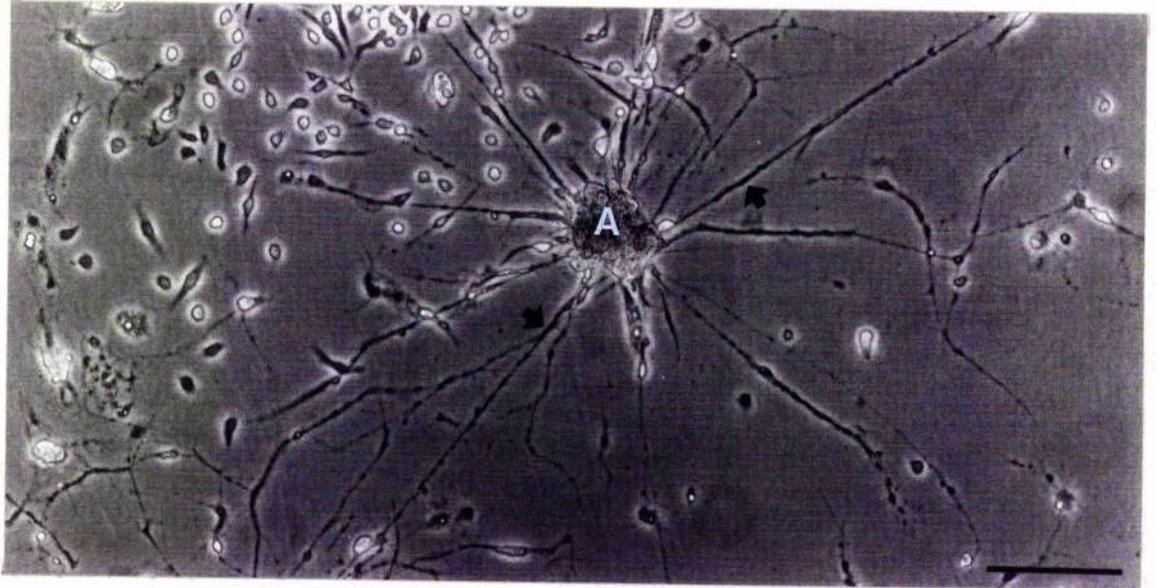
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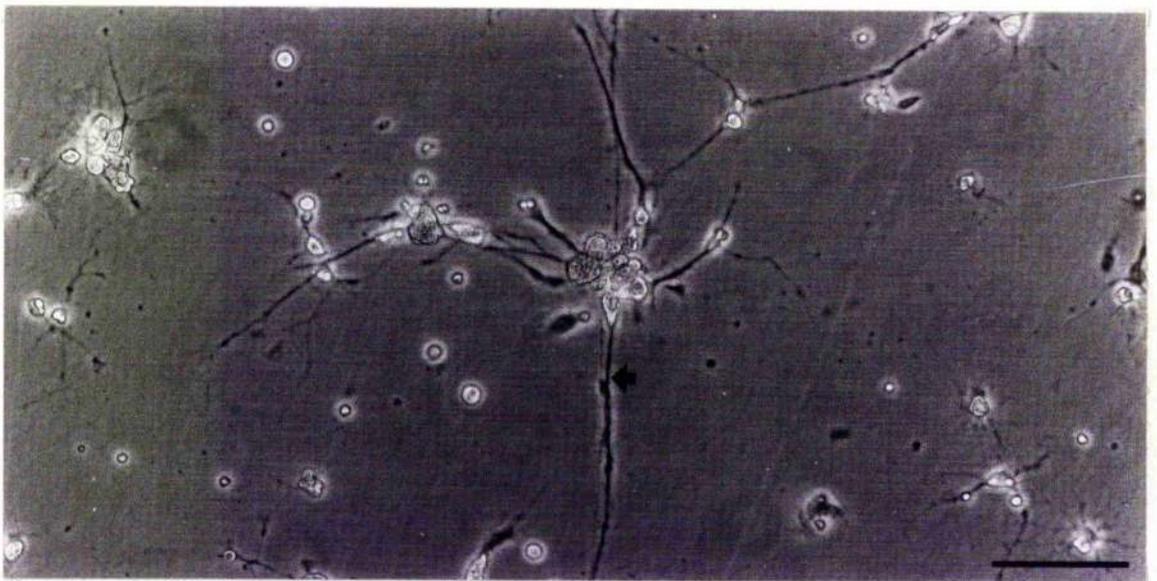


Figure 4.9

Figure 4.9 : Differences in titres of 20-HE that produce a morphological response in wing and leg lines grown for 7 days. All scale bars = 100 μm .

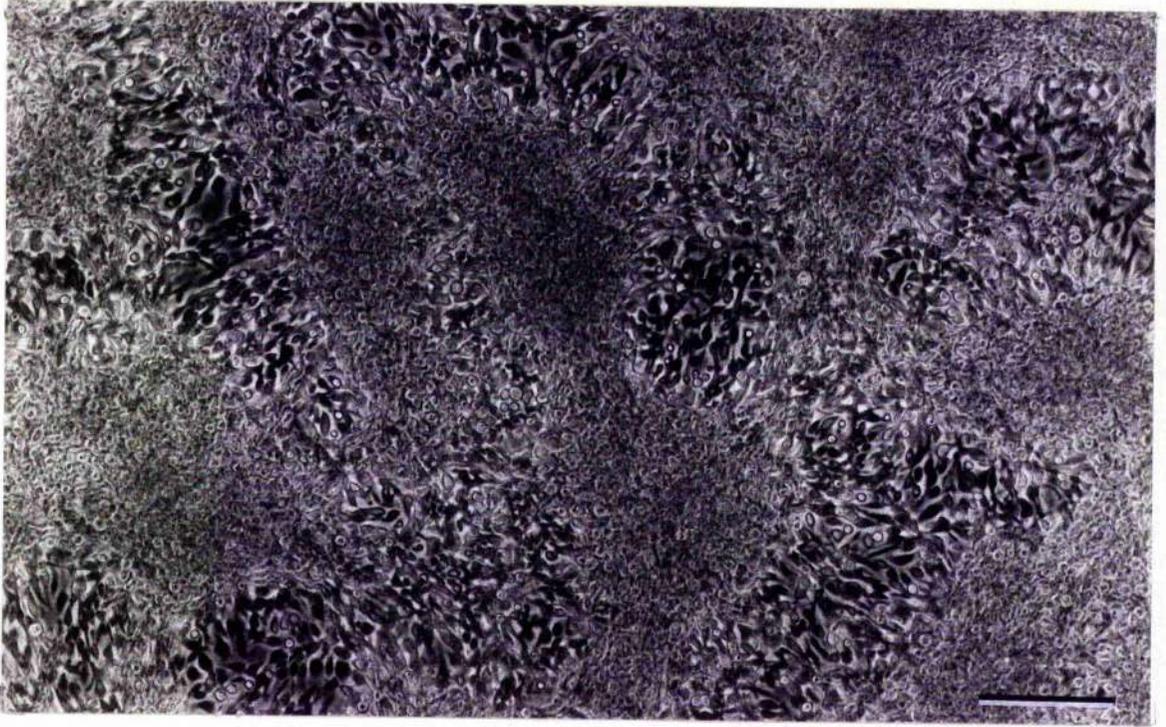
Fig. 4.9.1 : The cloned wing line Cl.8+ without 20-HE.

Fig. 4.9.2 : Cl.8+ treated with 5 ng/ml (1×10^{-8} M) of 20-HE. The cells have aggregated (A) in response to 20-HE.

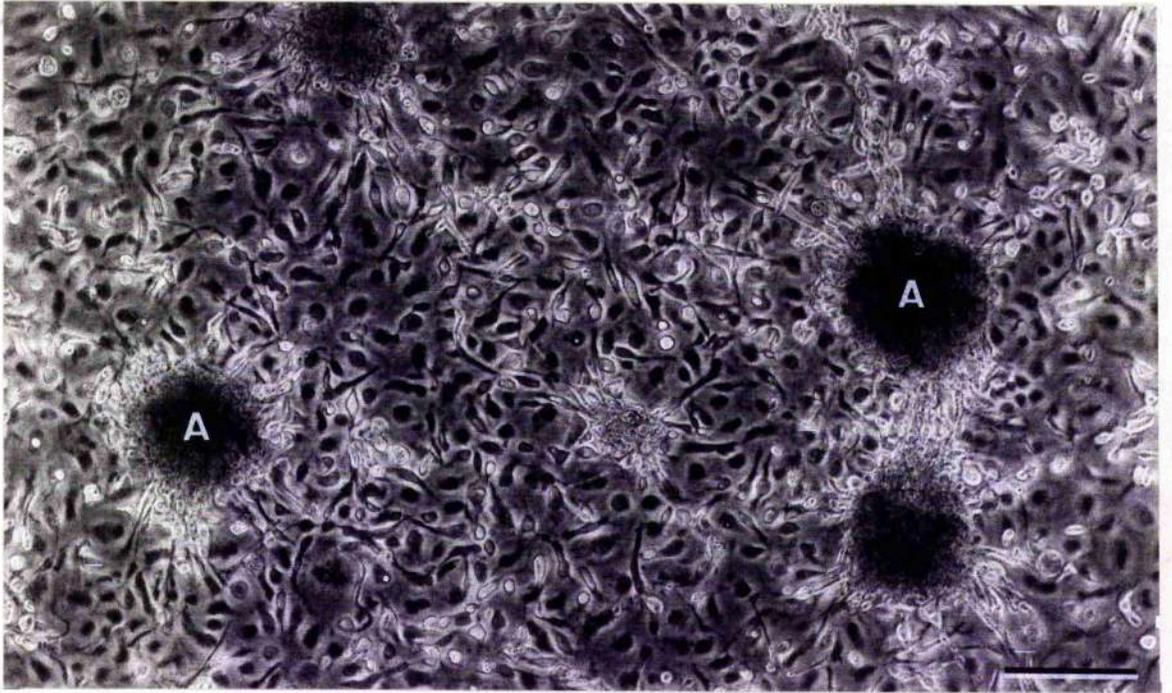
Fig. 4.9.3 : The cloned leg line LIA grown without 20-HE. Note the piece of tanned cuticle (T) in the middle of the centre aggregate of cells.

Fig. 4.9.4 : LIA treated with 5 ng/ml (1×10^{-8} M) of 20-HE. Note the flattened cells (arrowed).

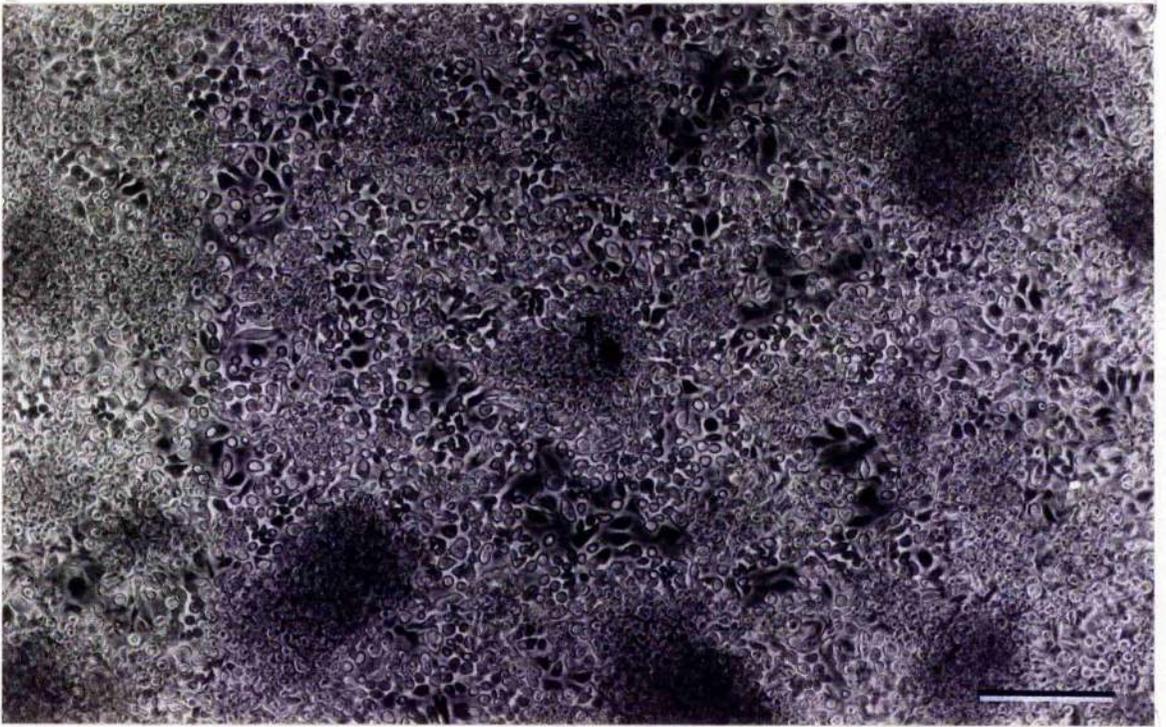
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4

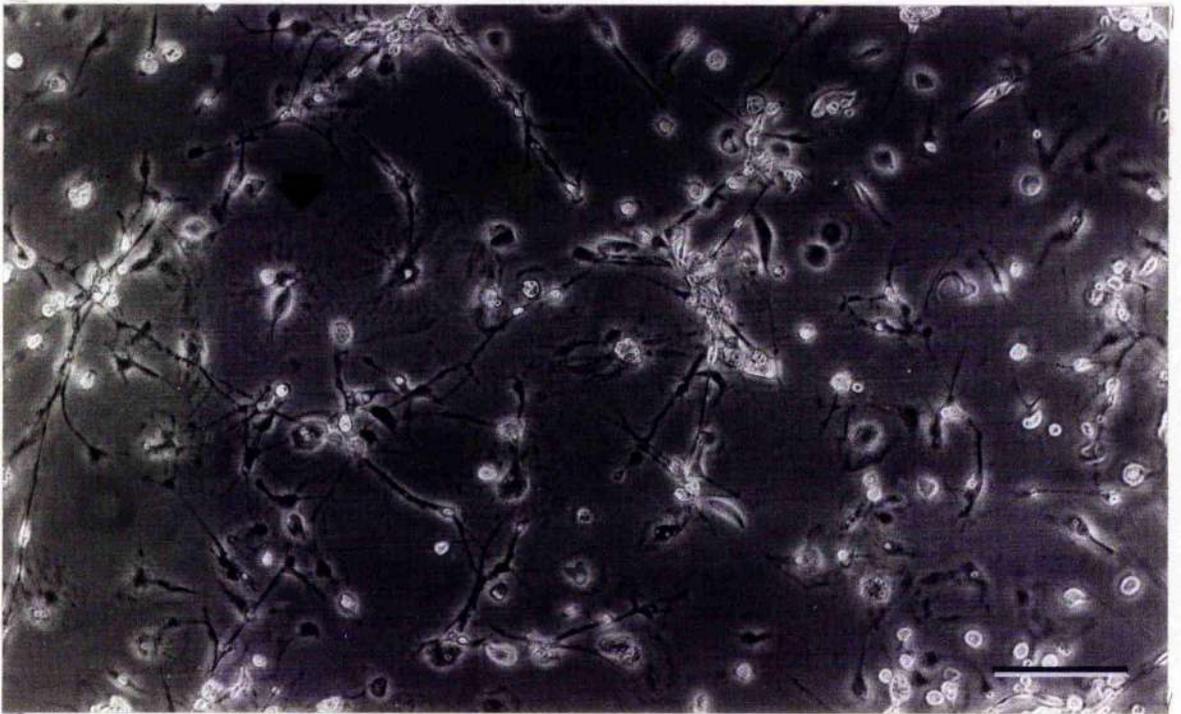


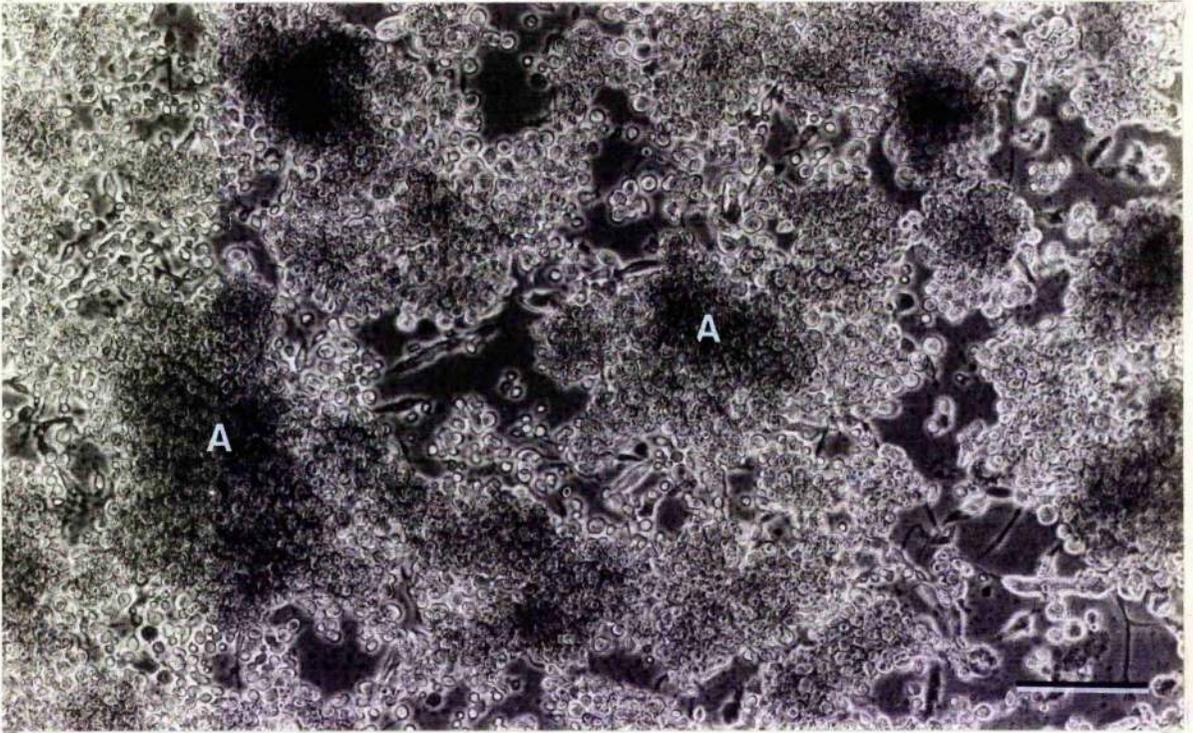
Figure 4.10

Figure 4.10 : Test for 20-HE sensitivity with the cloned wing cell line C9. Cells were incubated for 7 days with or without 20-HE at a dose of 100 ng/ml (2×10^{-7} M). Scale bars = 100 μ m.

Fig 4.10.1 : Control. Note the type of aggregation that is seen with this particular line, the cells have not formed themselves into definite aggregates as in other cloned cell lines but rather form rather diffuse aggregates (A).

Fig 4.10.2 : Experimental with 100 ng/ml of 20-HE. Note the increased cell aggregation (A) and the apparent reduction in cell density outside the aggregates.

1



2

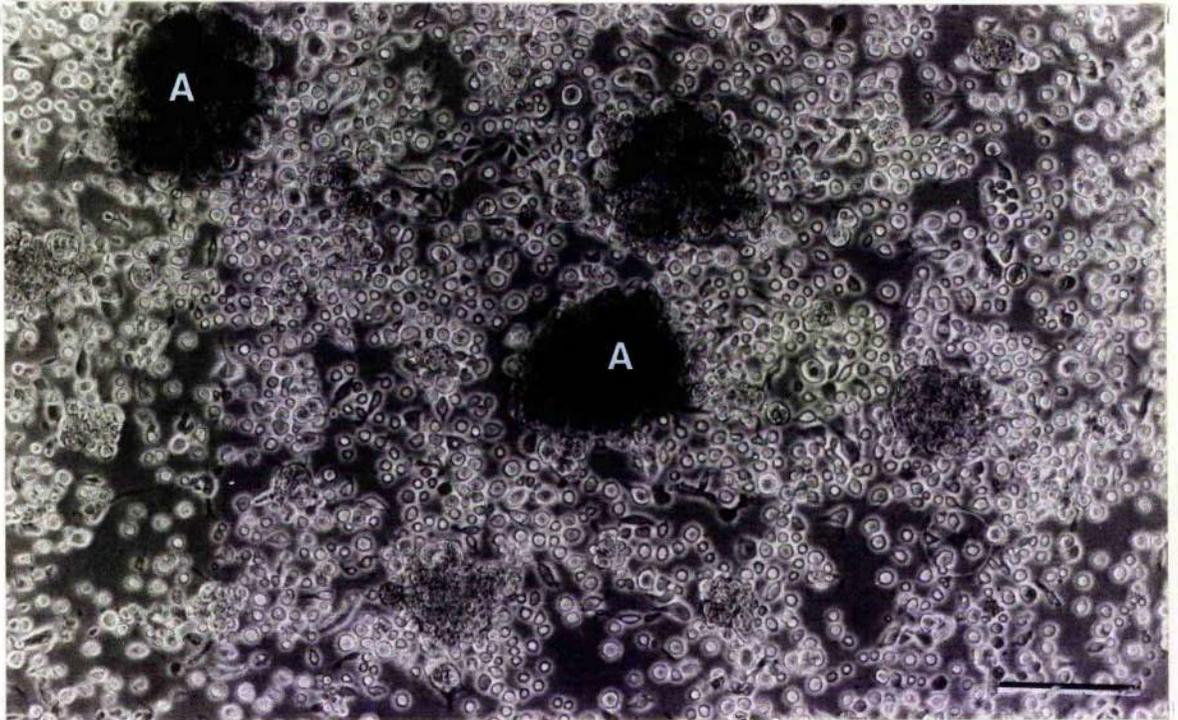


Figure 4.11

and

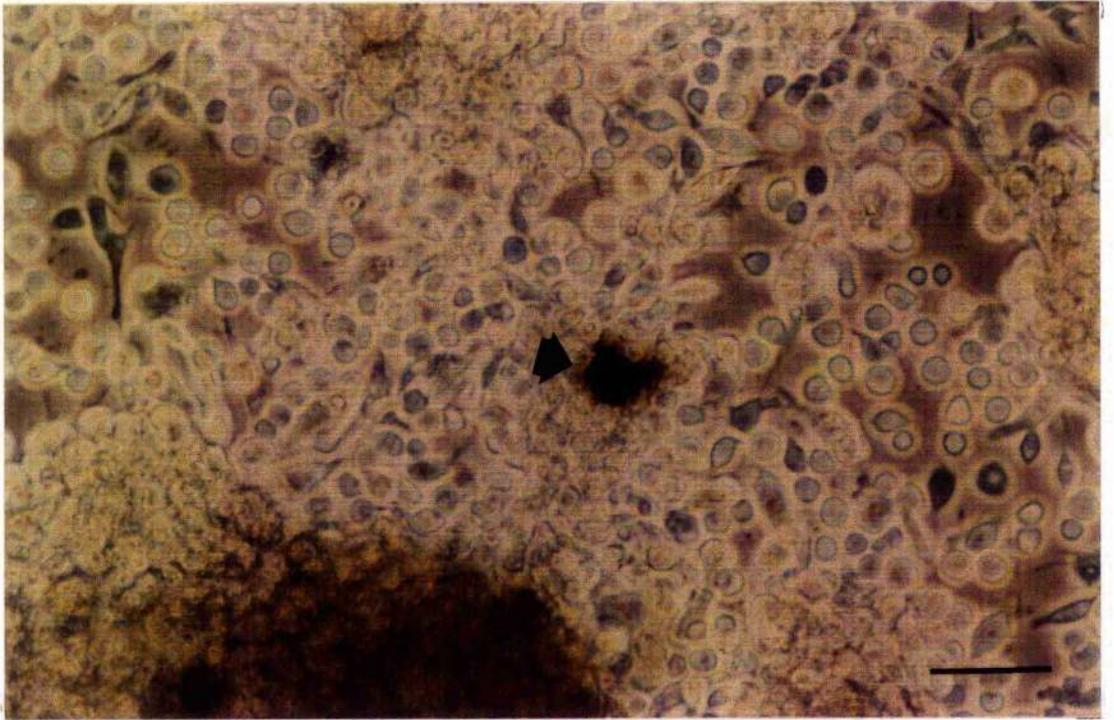
Figure 4.12

Figure 4.11 : Colour print of tanned cuticle in culture.

This picture shows the appearance of occasional patches of tanned cuticle-like material (arrowed) in the cell lines. These patches often appear in small aggregates of cells and most notably in the cloned leg cell line L1A. This picture is from the cloned wing cell line C9 which has been treated with 10 ng/ml 20-HE.

Figure 4.12 : Group of 20-HE resistant cells that have grown up in a culture of wing cells (W2) which had been treated with 200 ng/ml (4×10^{-7} M) of 20-HE.
Scale bar = 50 μ m.

11



12

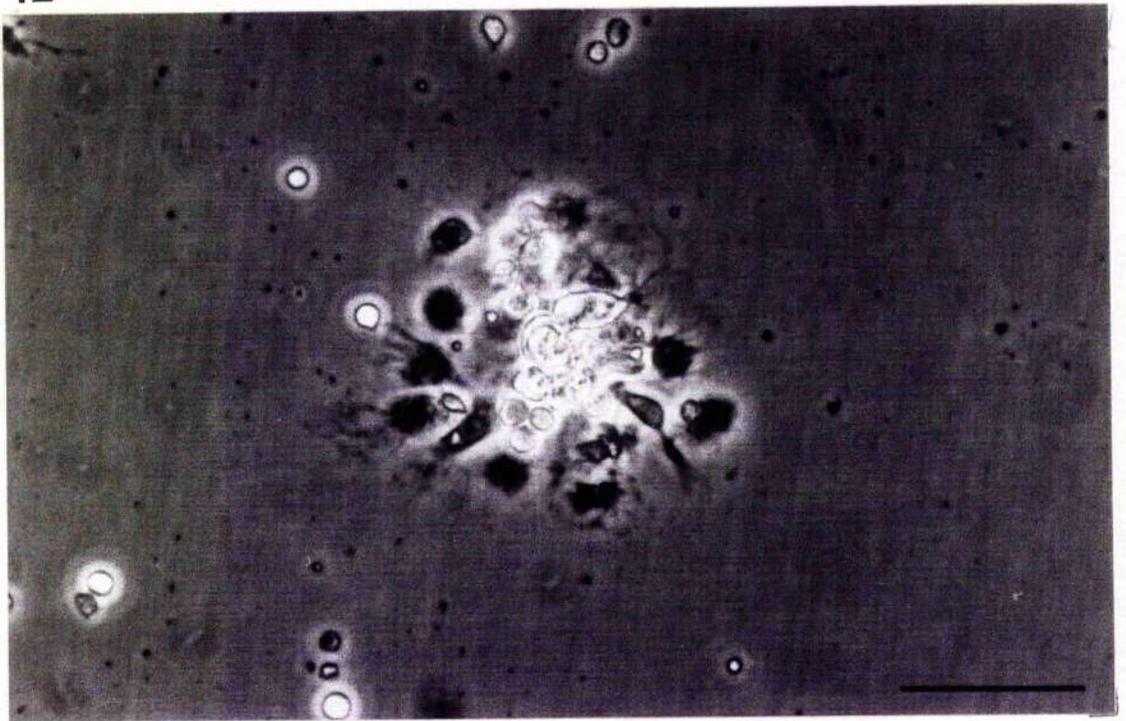


Figure 4.13

Figure 4.13 : Test for 20-HE resistance up to 150 ng/ml (3×10^{-7} M) in the cloned line Cl.8. A subline had been taken from this parent cloned wing cell line which had been selected for resistance to 20-HE, this line was now designated Cl.8 R and the parent cloned line Cl.8+ to distinguish them. The cells were subjected to three treatments of 10 ng/ml (2×10^{-8} M), 50 ng/ml (1×10^{-7} M) and 150 ng/ml (3×10^{-7} M) of 20-HE over a 7 day period. All scale bars = 100 μ m.

Fig. 4.13.1 : Control Cl.8+

Fig. 4.13.2 : 10 ng/ml Cl.8+

Fig. 4.13.3 : 50 ng/ml Cl.8+

Fig. 4.13.4 : 150 ng/ml Cl.8+

Fig. 4.13.5 : Control Cl.8 R

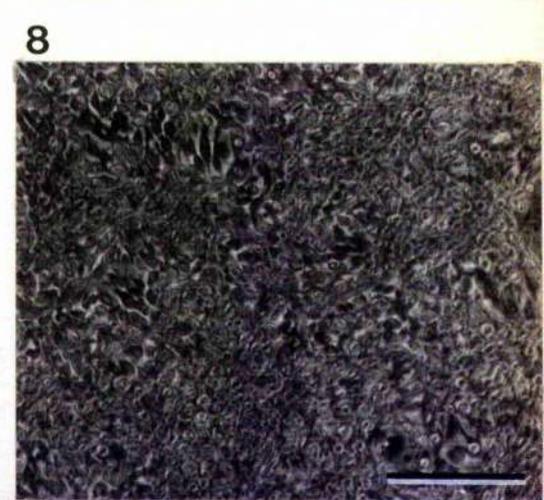
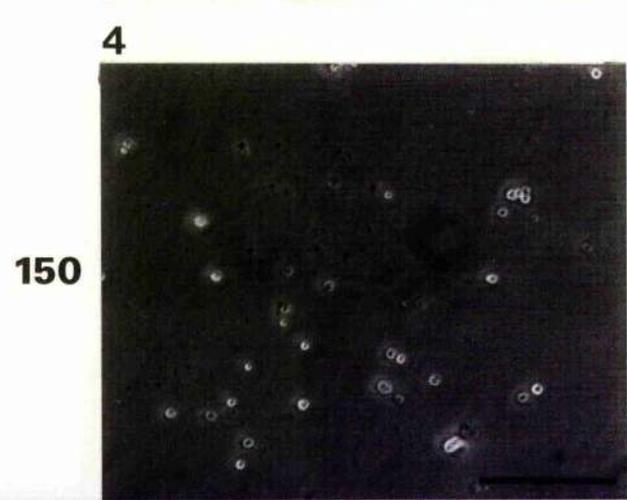
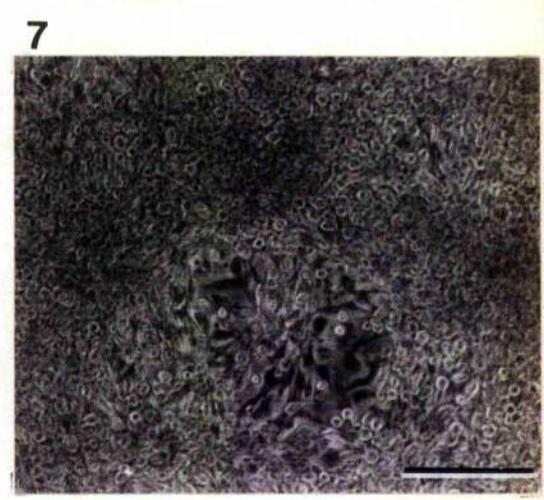
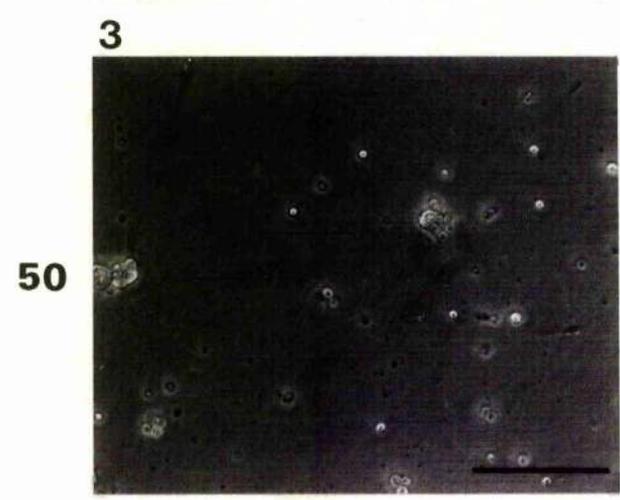
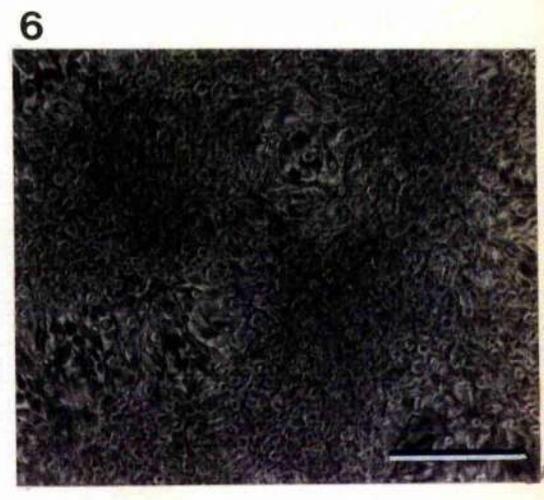
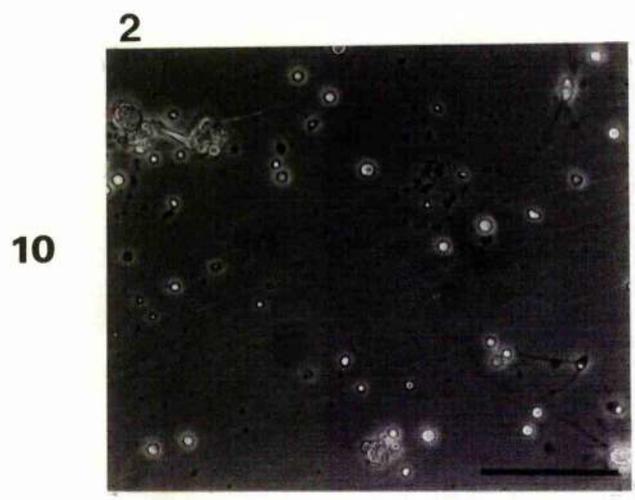
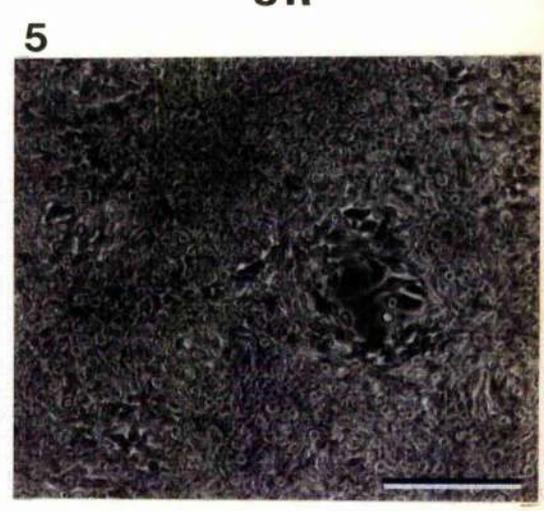
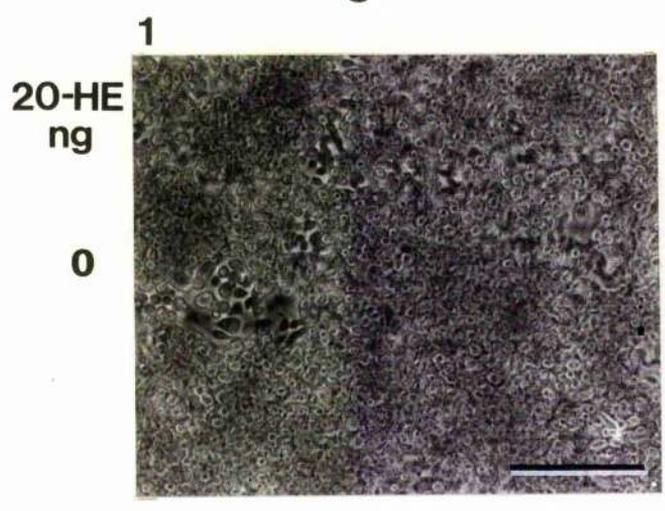
Fig. 4.13.6 : 10 ng/ml Cl.8 R

Fig. 4.13.7 : 50 ng/ml Cl.8 R

Fig. 4.13.8 : 150 ng/ml Cl.8 R

8+

8R



Chapter 5

Biochemical aspects of disc cell lines responses to 20-HE.

Biochemical aspects of disc cell lines responses to 20-HE.

Introduction

Having established the range of morphological responses of disc cell lines to 20-HE, I decided to investigate any concomitant biochemical changes in the cells. This was not really practical in St. Andrews so I spent some time in a laboratory where the biochemistry of ecdysteroid action in insect cell lines was being extensively studied. This enabled me to set up my own assays back in St. Andrews having had some experience in performing them elsewhere and also to perform assays which would have been totally impractical in St. Andrews. To this end I spent 6 weeks in Professor K-D. Spindler's lab in Düsseldorf, Germany. Many of the results presented here were carried out whilst in Germany, especially the assays for ecdysteroid receptor titre in the cell lines and the radio-immunoassay (RIA) of fly extracts. Meanwhile others, notably the chitin synthesis assays were mostly carried out in St. Andrews.

My idea was to look at the biochemical responses of the cells to ecdysteroids in the hope of further characterising the cell lines and also of quantifying more fully the action of ecdysteroids in disc cell lines. The assays carried out included:- ecdysteroid receptor levels in different cell lines, RIA of components of the culture medium, chitin synthesis, acetylcholinesterase (AChE) and dopa-decarboxylase (DDC) determinations.

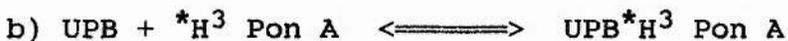
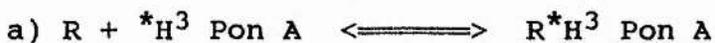
Methods

Ecdysteroid receptor analysis

The assay for ecdysteroid receptor titre in the cell lines was based on a competitive binding assay using tritiated Ponasterone A (Pon A), a phytoecdysone with a thousandfold higher biological activity than 20-HE (Maroy et al 1978).

The cells are processed to produce a crude cytosol preparation. In this preparation there will be some unspecific binding of proteins to labelled hormone (tritiated Pon A). In order to distinguish between this type of binding and specific ecdysteroid receptor binding to labelled hormone a competitive binding assay is performed. This assay is split into two parts, one determining total binding and the other the background unspecific binding. This gives a figure for specific ecdysteroid receptor titre.

In the first part of the assay only tritiated Pon A (H^3 Pon A) is present in addition to the cytosol preparation. In this preparation two different reactions are going on simultaneously. Tritiated Pon A is present and is binding to both unspecific proteins (UPB) and specific ecdysteroid receptor. These two reactions give a figure for total binding.



In part two of the assay Pon A is present in the same concentration as in part one but in addition a 7500x excess of unlabelled 20-HE is also present. Taking into account that Pon A is a thousandfold more active than 20-HE, there is still an

effective 7.5x excess of unlabelled 20-HE. In this assay a more complex set of reactions are occurring

- a) $UPB + 20-HE \rightleftharpoons UPB20-HE$
- b) $R + 20-HE \rightleftharpoons R20-HE$
- c) $UPB + {}^3H^3 \text{ Pon A} \rightleftharpoons UPB{}^3H^3 \text{ Pon A}$
- d) $R + {}^3H^3 \text{ Pon A} \rightleftharpoons R{}^3H^3 \text{ Pon A}$

All these reactions are in equilibrium, the position of the equilibrium depending on the concentrations of their various components, their affinities for each other and the stability of the bound protein-hormone complexes. Ecdysteroid receptor will bind first as it has a high affinity for ecdysteroids, since unlabelled 20-HE is in a 7.5 fold excess receptor will bind to 20-HE first. Specific ecdysteroid receptor binding is more stable and the equilibrium is less dynamic than unspecific protein binding to hormone. The labelled Pon A which can be measured will tend to bind to the unspecific protein binding in the cytosol preparation, as the specific receptor will have formed stable protein-hormone complexes with the excess of 20-HE present. Thus the labelled Pon A will give a measure of the unspecific protein binding that can be found in the cytosol preparation. Subtracting this figure from the figure for total binding found in the first part of the assay will give a value for specific ecdysteroid receptor protein binding.

Cells were harvested by pipetting after being grown up for a week. The cells were then centrifuged down at 1500 rpm for 10 minutes and the total volume of their culture medium

noted. The pellet of cells was resuspended in 1 ml of culture medium and put into 2 ml Eppendorf centrifuge tubes and centrifuged down at 10,000 rpm for 4-5 seconds. The pellet was then resuspended in 3-4 mls of SIII buffer (A.Turberg personal communication). At this stage cell counts were also taken.

SIII buffer

20 mM	HEPES	
25 mM	NaCl	
20%	Glycerol	
0.1 mM	EDTA	
0.5 mM	DTT	
0.5 mM	PMSF	
160 µg/ml	Aprotinin	pH 7.9

PMSF - Phenylmethylsulphonylfluoride

DTT - Dithiothreitol

Phenylmethylsulphonylfluoride (PMSF) and Aprotinin are both protease inhibitors with different specificities, Ethylene-diaminetetra-acetic acid (EDTA) chelates Calcium (Ca^{2+}) ions out of solution which are important in some proteolytic activities. These prevent the cytosol preparation and any ecdysteroid binding proteins in it from being degraded by cellular proteases released when the cells are being homogenised. Glycerol helps preserve the preparation if it needs to be frozen.

The cell suspension, kept on ice, was now homogenised in an all glass homogeniser with a tight fitting plunger approximately 10 times. This disrupted the cell membranes but left the nuclei intact. The suspension was then sonified five times for 3-5 seconds each with the microtip of a 90 W sonifier, this was done on ice in polycarbonate tubes. This treatment ruptures the nuclei so releasing any hormone receptors within the nuclei into solution and making them available for binding.

The suspension was then ultracentrifuged at 40,000 rpm (105,000 g) for 1 hour and 10 minutes. The supernatant was collected, trying to exclude the lipid layer at the top as this could interfere with ecdysteroid binding. Samples of this cytosol supernatant were taken for protein determination using the Bradford method and Bovine Serum Albumin (BSA) as standard. The cytosol preparation was then adjusted (using SIII) so that the protein concentration was in the range of 3-5 mg/ml.

Ponasterone A (Pon A)

The specific activity of the stock tritiated Pon A was 170 Ci/mmol, the concentration of the stock solution Pon A was 3.98×10^{-7} M. By experimentation a concentration of 3.1×10^{-9} M of Pon A was found to be optimal. There are two parts to the assay, and it is important that the same concentration of Pon A is present in both parts of the assay given that a comparison is going to be made between the two. To ensure this the correct concentration of Pon A is prepared and later aliquots taken from this stock. Pon A is dissolved in methanol

and this needs to be removed before incubation can begin. The correct concentration of Pon A is put into a rotary evaporator to evaporate off the methanol. Then the desired quantity of SIII buffer is added to the tube and dissolved by vortexing and sonification in an ice bath for 2-3 minutes. The solution, after centrifuging is ready for use.

Incubation mixture

The incubation mixture consisted of H^3 Pon A and cytosol for part one of the assay and H^3 Pon A, cytosol and 20-HE for part two of the assay. The amount of cytosol and SIII buffer were adjusted so that there were comparable protein levels in each part of the assay of between 3-5 mg/ml. The final volume of each incubation mixture was 650 μ l. This solution was incubated for 3-4 hours at 4°C to prevent any protease activity.

After 3-4 hours the reactions will have reached equilibrium. In order to measure the bound Pon A, the unreacted Pon A has to be removed from the reaction mixture. This has to be done quite quickly, as a gradual reduction in any one of the components of the reaction equations would cause the equilibrium to be upset and the position of the equilibrium would change. In order to achieve this a charcoal solution is used. Charcoal has the ability due to its very porous structure to absorb molecules out of solution. However charcoal can absorb a wide range of molecules, in order to limit the size of molecules it can absorb, it is coated with a Dextran sugar of known molecular weight (Dextran 60 MW=60,000). These sugar molecules will block all the larger

holes and pits in the particles of charcoal so that only the smaller holes are left. This has the effect of restricting the range of molecules that the charcoal particles can absorb to smaller molecules. This shift in the size range of molecules that the charcoal particles can absorb means that all the unbound labelled Pon A can still be absorbed out of solution by the charcoal whereas the Pon A that has bound to protein is now too large a molecule to be absorbed by the charcoal. This results in the selective removal from solution of the unbound Pon A, leaving only the bound Pon A to be measured.

The charcoal solution is made up by adding
20 g of Norit A
2 g of Dextran 60

to 160 mls of SIII buffer without glycerol. After being left overnight, to enable the Dextran 60 to coat the charcoal particles, the solution is centrifuged down a couple of times and new buffer added. The solution is stirred continuously overnight before use in order to keep the charcoal particles evenly distributed in solution.

The reactions in the incubation mixture are stopped by taking 190 μ l aliquots from the incubation mixture and adding them to 45 μ l of charcoal solution. This removes the free hormone in the solution so upsetting the equilibrium, which will try to re-establish itself by dissociating hormone-protein complexes. In *Chironomus* cells at room temperature after 20 minutes the binding will be reduced by 50%, so all these procedures are carried out quickly and at 4°C. The charcoal and cytosol are mixed on a vortex and allowed to

react for 2 minutes and then centrifuged in an Eppendorf microcentrifuge for 4 minutes at 14,000 rpm. After centrifugation 170 μ l of the supernatant is taken and put in a borosilicate scintillation vial and 3.5 mls of scintillant added and counted in a scintillation counter.

The scintillation counter gives a series of readings giving figures in mean counts per minute (cpm) of the sample. These figures are arranged in 2 groups, the first giving total binding values and the second unspecific binding values. Subtracting one from the other gives a value for specific binding. By a series of calculations total receptor content can be found and from that the number of binding sites per cell determined.

CALCULATIONS

Example from assay one from the experiment on the 2nd Dec.

Wing cell line (Clone 7)

Mean cpm with s.d.

1.) H 462 \pm 18 Total binding

K 333 \pm 32 Unspecific

Specific binding = 129

Other data

Culture volume = 73 ml

Cell nos. = 1.39×10^9

Protein content of cytosol mg/ml = 3.12

Total cytosol volume/ml = 2.96

The value of 129 gives the no of cpms in 170 μ l of the sample. However in the assay (DDC) there was 190 μ l of cytosol +50 μ l DDC therefore the need to convert to cpm/ml

$$\frac{129 \times 240}{170} = \text{cpm in 190 } \mu\text{l of cytosol} = 182.1$$

$$\frac{182.1 \times 240}{190} = 958.5 \text{ cpm/ml}$$

Molarity of Pon A present = 5.3×10^{-18} mol/cpm
(from molarity and specific activity figs)

Assuming 1 mol Pon A binds to 1 mol Receptor, you can obtain a figure for the molarity of Pon A present from the cpm/ml value ---> to give a direct value of how much receptor there is present/ml of cytosol preparation.

$$\begin{aligned} \text{Molarity of Pon A present} &= 958.5 \times 5.3 \times 10^{-18} \\ &= 5.08 \times 10^{-15} \text{ mol/ml} \end{aligned}$$

$$\text{therefore mol. of receptor} = 5.08 \times 10^{-15} \text{ mol/ml}$$

Total cytosol volume = 2.96 ml

Total protein content of cytosol = 9.235 mg

Protein content of cytosol = 3.12 mg/ml

Protein content in incubation medium = 3 mg/ml

Receptor content in the incubation medium mol/mg protein

$$= \frac{5.08 \times 10^{-15}}{3} \text{ mol/ml} = 1.69 \text{ mol/mg protein}$$

This figure of 1.69×10^{-15} mol gives a value for the amount of receptor present in 1 mg of protein taken from the cytosol preparation.

$$\begin{aligned}\text{Total Receptor content} &= 1.69 \times 10^{-15} \times 9.235 \\ &= 15.6 \text{ femtomoles of receptor}\end{aligned}$$

$$\text{Cell numbers} = 1.39 \times 10^9$$

$$\text{Total Receptor} = 15.6 \times 10^{-15}$$

To find out the no. of binding sites/cell

$$\text{Total Receptor} = 15.6 \times 10^{-15} = 11.22 \times 10^{-24} \text{ mol}$$

$$\text{Total Cell no.} = 1.39 \times 10^9$$

Receptor/cell

Using Avogadro's number to convert the molarity to the no. of binding sites present you get a figure for the no. of binding sites/cell

$$11.22 \times 10^{-24} \times 6.022 \times 10^{23} = 6.75 \text{ sites/cell}$$

Chitin Synthesis

Chitin is a biopolymer of N-acetyl-D-glucosamine, and is a major structural component of the exoskeleton of invertebrates and also of the cell wall of fungi. Chitin is a very abundant molecule, being almost as common as cellulose, it has been estimated that approximately a billion tonnes are synthesised every year (Nicol 1991). The assay for chitin

synthesis used is based upon the incorporation of radioactively labelled glucosamine (GlcN) into chitin. Glucosamine can also be incorporated into other molecules within the cell such as glycoproteins. In order to distinguish between this incorporation and that of chitin, after the cells incubation with glucosamine they are subjected to extensive alkali hydrolysis. This solubilises the glycoproteins in the cell sample, leaving only the insoluble residue of chitin which is very resistant to degradation. The insoluble fraction is then filtered out and the radioactivity present counted. In order to further characterise the incorporated product as chitin, the filtered product was subjected to further treatments before being counted in the scintillation counter. The filters were incubated with or without chitinase enzyme (from *Serratia marcescens*, Sigma) at 8 mg/ml in 0.05 M acetate buffer pH 5.2 for 48 hours at 37°C (Oberlander et al 1978). After this period the filters were again washed as before (10 x 1 ml absolute alcohol and 6 x 1 ml 1.5 M KOH), dried, scintillant added and placed in the scintillation counter.

The cells were passaged and cultures set up at 2×10^6 cells in a 3cm Petri dish in 1.5 mls of medium, to which the various ecdysteroid treatments had been added. Four replicates were set up for each treatment. To each Petri dish was added 20 μ l of cold (non-radioactive) glucosamine and 45 μ l of radioactively labelled H^3 glucosamine (H^3 GlcN). The cells were then grown for a week and harvested. The cells were pipetted off the culture dish and centrifuged in a microcentrifuge at 10,000 rpm for 5 minutes, washed in PBS and centrifuged down again. The supernatant was taken off and 100

μ l of 0.1 M NaOH was added to the pellet to solubilise the proteins. When the pellet had been thoroughly dissolved, a 10 μ l sample was taken for protein determination. Then 410 μ l of 1.5 M KOH was added to the original sample and allowed to cook for 2 hours at 100° C in a water bath.

After 2 hours the samples are taken out and after allowing to cool are filtered onto wet Whatman GF/F filters (glass microfibre filters with a very fine mesh). These are then washed successively 6 times each with 1 ml of 1.5 M KOH and 10 times with 1 ml of absolute alcohol. The filters are then dried for 2 hours at 50° C, put into a scintillation vial together with 3.5 mls of Optiphase scintillant and the samples counted.

The scintillation counter gives results as cpms and these are combined with the protein level values for each sample to give a figure for cpm/ μ g protein.

Dopa decarboxylase (DDC)

DDC is an enzyme which converts Dopa into Dopamine and is involved in sclerotisation and tanning of the cuticle. The assay for DDC is based on an assay developed by McCaman *et al* (1972). A cell homogenate is prepared and incubated with a mixture of cold and radioactively labelled Dopa. DDC activity will convert this into Dopamine. At the end of the incubation period an ion exchanger called di-iso-octylphosphate (DEHP) is used. DEHP selectively absorbs any amine products from the incubation mixture, including any recently produced Dopamine. DEHP is only soluble in non-polar solvents such as chloroform,

when a solution of DEHP in chloroform is added to the aqueous incubation mixture two non-miscible layers are formed, one of which is selectively absorbing amine products from the mixture. When the organic chloroform phase is measured for radioactivity only the recently converted Dopamine is measured giving a value to the activity of DDC.

The cells were harvested and centrifuged down into a pellet, washed with PBS and centrifuged down again. Depending on the amount of cell material present between 200 and 500 μl of homogenising buffer was added, this consisted of 100 mM phosphate buffer pH 7 with 0.3 M sucrose. The suspension was then homogenised in an all glass homogeniser and centrifuged at 5,000 rpm for 15 minutes at 4°C. The supernatant was taken and used for enzyme measurement.

The reaction mixture consisted of 25 μl of Dopa (0.2 mg/ml in 0.1 phosphate buffer pH 7), 10 μl of pyridoxalphosphate (0.1 M in 0.1 M phosphate buffer pH 7) and 10 μl of C^{14} Dopa (L-3,4-Dihydroxyphenyl (3- C^{14}) alanine, 1.8×10^{-3} μM in phosphate buffer pH 7). To start the test 60 μl of cytosol preparation was added and incubated for 45 mins in a 25°C water bath. To stop the reaction 100 μl of distilled water and 200 μl of 0.1 M DEHP in chloroform was added. The tubes were then mixed on a vibrator shaker for 5 mins and centrifuged for 2 mins at 5,000 rpm. The upper aqueous phase was removed. This was repeated twice more each time adding 200 μl of 50 mM phosphate buffer pH 7 and separating off the aqueous phase. Then the tubes were placed in an oven at 60°C for 2 hours to dry. After this 1 ml of scintillant was added and the sample counted.

Acetylcholinesterase (AChE)

AChE is one of a number of esterase enzymes and this assay is based on a microfluorometric test for cholinesterases (Parvari *et al* 1983). Esterase activity in the sample releases thiocholine from the substrate which reacts with a chemical to produce a highly fluorescent blue product which can be measured in a fluorimeter. There are two parts to the test, in one part total esterase activity is measured and in the other a specific inhibitor of AChE is included in the test. Specific AChE activity is measured by taking the value for activity with inhibitor away from the total esterase activity.

The cells were grown for a week in various ecdysteroid treatments, pipetted off the culture surface, centrifuged down, washed in PBS and centrifuged again. The pellet of cells was then resuspended in 100 μ l of homogenising buffer, this consisted of 50 mM Tris HCl, 2 M NaCl and 2% Triton X 100 adjusted to pH 7.5. AChE is membrane bound and the triton detergent releases it enabling it to be measured. Samples (20 μ l) are taken from this and added to two series of reactions labelled A and B, these contained 470 μ l of substrate buffer (50 mM Tris HCl, 0.12 M NaCl and 1 mM EDTA), the difference being that B contained 10 μ l of Eserine hemisulphate (1×10^{-2} M in substrate buffer). This was left for 20 minutes at 25 °C. Samples were taken for protein determination by the Lowry method, which was used rather than the Bradford method due to the presence of Triton X 100 which interferes with protein dye binding in the Bradford method. The reaction was started by adding 10 μ l of Acetylthiocholine iodide (AThCh) at a

concentration of 0.1 M in substrate buffer to both A and B. This was then incubated for 60 minutes at 25° C. The reaction was stopped by taking 100 µl samples from the reaction mixture and adding them to tubes containing 20 µl of coumarinylphenylmaleimide (CPM) at a concentration of 0.16 mg/ml in isopropanol. Then 2 mls of 1% Triton X 100 in buffer was added. The mixture can now be measured in a fluorescence photometer at an emission of 473 nm and an excitation of 389 nm, the machine is set up at an accelerating voltage of 240 Hz.

Radioimmune assay (RIA)

The radioimmune assay for ecdysteroids depends on the competitive binding of a known amount of radioactive ecdysteroid and an unknown amount of ecdysteroids in the sample with an anti ecdysone antibody which also shows cross reactivity with other ecdysteroids. This antibody-antigen complex can then be precipitated out of solution and its radioactivity counted. When compared with the radioactivity levels found for several standard concentrations of ecdysone the concentration of ecdysteroids can be read off from a standard curve.

Before samples can be used in this sort of method, they have to be extensively purified so that nothing interferes with the essential antibody-antigen reaction. Firstly 1 ml of sample is mixed with 2 mls of distilled methanol, which precipitates out all the proteins, This was left overnight at -20° C to allow a better precipitation to occur. After leaving

overnight it was then centrifuged at 10,000 rpm for 20 minutes at 4 °C. The supernatant was removed and put into another tube, 1 ml of methanol was added to resuspend the pellet and again centrifuged down as above. This supernatant is added to the above supernatant. These are then dried in an oven at 40 °C overnight, this being hot enough to dry off the methanol but not hot enough to cause any oxidation. After drying, 2 mls of distilled water was added bringing the dried residue back into solution. Then 2 mls of chloroform was added from the lower phase of a water/chloroform bottle. The tubes were mixed again on a vortex mixer and then centrifuged for 5 minutes at 5,000 rpm. The lower chloroform phase was thrown away. This was repeated twice. After drying 2 mls of water was added to the dry matter to solubilise it and then 2 mls of hexane added from the upper hexane layer of a hexane/water bottle. This was mixed and centrifuged, the upper hexane layer then being discarded. This was repeated twice. The aqueous phase was dried overnight and then dissolved in 1 ml of methanol, from this aliquots were taken for RIA.

In order to measure the levels of ecdysteroids in these aliquots a standard curve using known concentrations of ecdysone is needed. The same procedure is carried out both for the stock concentrations of ecdysone and for aliquots of unknown ecdysteroid samples. For the unknown samples, aliquots (10,50,100 μ ls) were pipetted into different Eppendorf tubes and dried in an oven at 40 °C. For the standard samples, 0,20,40,80,150,300 and 600 picograms of ecdysone were added to different tubes and dried in an oven. From now on the tubes are treated in the same way. Radioactive tritiated (H^3)

ecdysone (50 μ l) was added to the tubes at a concentration of 3000 cpm in 50 μ l dissolved in borate buffer made up of 0.1 M Boric acid, 0.075 M NaCl and 0.05 M of anhydrous disodium tetraborate and adjusted to pH 8.4. Next 50 μ l of a mixture of rabbit anti ecdysone antibody (0.015%) with 5% normal rabbit serum in borate buffer is added to the tubes and they are incubated overnight at 4°C to allow the antibody-antigen reaction to occur. Next morning 100 μ l of saturated ammonium sulphate solution is added to each sample, this will precipitate out all the antibody-antigen complexes. This is then left for 30 minutes at 4°C. Then the samples are centrifuged at 5,000 rpm for 5 minutes and the supernatant removed. Next 40 μ l of distilled water is added to the pellet, mixed and then 600 μ l of scintillant added to each sample and the radioactivity counted.

This produces cpm values for the different samples from which a standard curve of cpm values against ecdysone concentration can be made. From this curve the ecdysteroid concentration in the unknown samples can be determined.

Results

Ecdysteroid receptor analysis

Ecdysteroid receptor analysis was carried out on a wide range of disc cell lines in order to define any differences between them. I was especially interested to see whether or not the lines that had been selected for ecdysteroid resistance showed this resistance due to a lack of receptor in

the line or whether there was some other mechanism leading to resistance. The protocol used was based largely on the method used to determine levels of ecdysteroid binding protein (receptor) in a *Chironomus tentans* cell line (Turberg et al 1988), the only difference being that the salt concentration in the S III buffer used is higher (25 mM) than that used for *Chironomus* cells (10 mM).

Firstly in order to define better the conditions for optimal binding, an experiment was carried out using 3 different concentrations of radioactively labelled H³ Pon A (see Table 1). This was conducted with the cloned leg cell line LIA. From this experiment it can be seen that the concentration of Pon A that appears to give the best binding conditions is 3.1×10^{-9} M, this concentration was used in all further experiments.

Table 1

Cell line name	Pon A ($\times 10^{-9}$ M)	Receptor content f.mol/mg protein	Receptor binding sites/cell
LIA	1.4	6.8	98
leg	3.1	8.2	119
cell	9.5	6.7	97
clone			

It appeared that freezing the cytosol preparation had quite a detrimental effect on the binding capacity of that cytosol. There appeared to be on average about a 40% reduction in binding on freezing. This figure was taken from two cell

lines LIA and Cl.8+ whose values were averaged from experiments involving the same cytosol used fresh and then frozen, from different cytosol preparations and also from cytosol preparations frozen from either 1 or 15 days, length of time frozen did not make any difference. So in order to make all my results comparable as they involved using both fresh and frozen cytosol preparations from previous determinations, all my results using frozen samples were raised by 40%. This enabled me to make direct comparisons between fresh and frozen preparations.

Table 2

Cell line name	Days frozen	Receptor content f.mol/mg protein	Receptor binding sites/cell
C1.8 +	-	3.8	67
C1.8 R	-	0.3	4
L1A	1	3.3	47
C1.7	1	1.69	7
W2	1	5.71	50
20C6	1	8.82	65
C9+	1	8.90	123
C9R	1	8.60	100
C1.8+	1	5.11	42
C1.8R	1	0	0
C1.9	1	3.40	21
C1.8+	10	2.67	47
C1.8R	10	0	0
L1A	15	4.47	64

Table 2 shows the results of several determinations from a number of different cell lines. There was quite a range of values for binding sites per cell between the different cell lines. Some of these results are also represented in Figure 5.1. The lowest value achieved was 10 per cell for C1.7 to 172 for the line C9+. Two sets of cell lines had been selected for 20-HE resistance, C1.8 and C9. These were designated C1.8+ and C9+ for the parent lines and C1.8R and C9R for the resistant lines selected from these parent lines. When the ecdysteroid

titres of these different cell lines were measured there was a substantial difference between them. Both the parent lines (Cl.8+, C9+) had significant levels of ecdysteroid receptor. However of the resistant lines Cl.8R showed no significant specific binding above unspecific binding indicating that there was no specific ecdysteroid receptor present. The other resistant line C9R still had a significant level of specific binding and therefore of ecdysteroid receptor, even though the level of receptor in this line was slightly lower than in the parent line (140 as compared to 170). For the other cell line Cl.8, in order to further investigate whether or not there was any receptor in the subline Cl.8R the conditions in the binding assay were changed. This was carried out to see if there had been a change in affinity for the receptor. In order to see whether this had happened the protein concentration of the cytosol was increased and also the concentration of H³ Pon A was changed. Table 3 shows the results of these changes in the binding conditions.

Table 4 To further investigate binding of the cells especially the selected lines by changing the conditions in the incubation medium namely increasing the protein concentration and hormone concentration.

a.) Increasing protein concentrations to 5 mg/ml in the incubation medium

Cell line name	Days frozen	Receptor content f.mol/mg protein	Receptor binding sites/cell
C9R	1	8.76	102
Cl.8+	1	4.54	80
Cl.8R	1	-	-
LIA	1	7.0	100

B.) Increasing the concentration of H³ Pon A to a level of 5 x 10⁻⁹ M in the incubation medium

Cell line name	Days frozen	Receptor content f.mol/mg protein	Receptor binding sites/cell
C9R	1	9.4	110
Cl.8R	1	-	-

The results from these analyses show that increasing the protein concentration in the cytosol preparation does increase the figure for specific binding sites/cell in the normal lines (Cl.8+, LIA, C9R), however there still is not any significant binding for Cl.8R line. Again in the experiment which increases the H³ Pon A concentration in the incubation mixture

the figure for specific binding with the C9R line is increased as compared with results from Table 2. However there is still a lack of any specific binding in the Cl.8R cell line. Even changing the conditions of binding to increase specific binding as in Table 3 did not produce any specific binding in the Cl.8R line indicating that there is a true lack of receptor rather than a change in affinity of that receptor. The C9R line even though it has been selected for resistance to 20-HE still shows an appreciable titre of specific ecdysteroid binding not unduly different from its parent line.

The results from ecdysteroid receptor analysis reveal the presence of ecdysteroid binding (receptor) activity in all the cell lines tested with the exception of one. In this line which had been selected for resistance to the effects of 20-HE there is a total lack of any specific binding. In another line which was also resistant to the effects of 20-HE, ecdysteroid receptors are still present. This shows that in one line (8R) resistance appears to be due to a lack of receptor whereas in another line (C9R) resistance to 20-HE seems to be due to another mechanism.

In all these experiments what is being measured is an ecdysteroid binding protein in a crude cytosol preparation. In order for this to be characterised as a receptor, a number of criteria have to be satisfied. In the first experiment (Table 1) using different concentrations of Pon A it can be seen that the highest degree of binding was achieved at a Pon A concentration of 3.1×10^{-9} M. When the Pon A concentration was trebled to 9.5×10^{-9} M the measurable binding did not increase at all and indeed fell off slightly. This shows two

things, firstly that the binding has a very high affinity and secondly that it is very specific. If binding was due to some unspecific cytosolic proteins then it would be much more difficult to saturate it out and certainly not at such a low concentration. These features point to there being a specific ecdysteroid receptor in the cell lines.

Chitin synthesis

Chitin synthesis as measured by the incorporation of radioactive glucosamine into a cell fraction resistant to extensive alkali hydrolysis appeared to be hormone dependent. Treatment of the cells with 20-HE dramatically increased the incorporation of GlcN. The product that was left after alkali hydrolysis was further characterised by incubating the product with chitinase enzyme which produced a 50% reduction in labelled product as compared to control (Figure 5.2). This showed that the labelled product was indeed largely composed of chitin.

Figure 5.3 shows the results from an experiment looking at the dose dependency of the chitin response for 2 cell lines. The greatest stimulation of chitin synthesis seems to occur at 10 ng/ml (2×10^{-8} M) of 20-HE in the Cl.8+ cell line. This line possesses ecdysteroid receptors (see previous section) whereas the Cl.8R line is devoid of any measurable receptor activity. This is reflected in the lack of stimulation of chitin synthesis in this cell line by 20-HE. This indicates that the stimulation of chitin synthesis is

directly receptor mediated, the first time that this has been demonstrated in a receptor positive and negative cell line.

In the above dose response test 10 ng/ml (2×10^{-8} M) 20-HE was found to be optimal for stimulating chitin synthesis. Levels of incorporation were also tested using lower concentrations of 20-HE than 10 ng/ml (2×10^{-8} M) and it was found (Figure 5.4) that concentrations even down to 4 ng/ml (8×10^{-9} M) gave quite considerable stimulation of incorporation and in these experiments the optima for ecdysteroid stimulation appeared to be around 8 ng/ml (1.6×10^{-8} M). In all my experiments 10 ng/ml (2×10^{-8} M) was used as a standard ecdysteroid treatment.

Figure 5.5 shows the time course of induction of chitin synthesis by 10 ng/ml (2×10^{-8} M) 20-HE. This shows that stimulation of chitin synthesis is fairly linear in its time course accelerating at 3 days but not reaching a plateau even after 7 days, which for other experiments was the usual incubation period before harvesting. Chitin synthesis even after 7 days had not reached a plateau, but this was taken as a reasonable incubation period to measure synthesis. The deposition of chitin, an insoluble product is not under the same constraints as other reactions, no equilibrium is reached and the deposition will continue as long as the cells are viable. Indeed in primary cultures often several layers of chitin-like material is seen (Cullen and Milner 1991).

When a whole range of cell lines were tested for stimulation of chitin synthesis (Figure 5.6) it can be seen that there was quite a difference between individual cell

lines. All lines tested except one (20C6) showed a 20-HE induced stimulation of chitin synthesis.

Acetylcholinesterase (AChE)

These results were partly obtained from samples which were grown and processed in St. Andrews and then shipped to Germany for fluorimeter and protein determinations. Figure 5.7 (A) shows a clear dose response curve for AChE activity with increasing concentrations of 20-HE for the cell line Cl.8+. It can be seen that there is very little endogeneous AChE activity present in the cell line (control value) and that stimulation with 20-HE causes an induction of activity, the optimal level of 20-HE for induction being 50 ng/ml (1×10^{-7} M). Comparing these values with a similar experiment using the receptor negative Cl.8R cell line (see Fig. 5.7.B) it can be seen that the response is not so clear cut. Interestingly in this cell line there is quite a high level of endogeneous AChE activity (control value). Incubating the cells with 20-HE does not show a well defined dose response as in Fig. 5.7 (A). Treatment with 50 ng/ml (1×10^{-7} M) 20-HE causes approximately a two-fold stimulation above the control indicating that this level of 20-HE is having some effect. Thus it is clear from comparing the two cell lines that induction of AChE activity is at least partly receptor mediated even though Cl.8R cells without any appreciable receptor still show some response. Apart from receptor mediated steroid hormone action, ecdysteroids have also been shown to have diverse effects on membranes (Koolman and Spindler 1983), and as AChE is membrane bound this may explain

some of the activity that is induced in the receptor negative cell line.

AchE activity first shows an appreciable induction after 3 days of hormone application (Fig. 5.8). With 50 ng/ml (1×10^{-7} M) of 20-HE the level of AchE induction appears to increase in a linear manner never reaching a plateau, at least in the 7 days of this experiment. However at 10 ng/ml (2×10^{-8} M) of 20-HE, a suboptimal concentration for AchE induction, a plateau in AchE activity is reached at 4 days. Figure 5.9 shows the degree of stimulation of AchE activity in a number of different cell lines all subjected to 10 ng/ml (2×10^{-8} M) of 20-HE for 7 days. It can be seen that there is quite a wide variation between different cell lines in the degree of stimulation by 20-HE though unlike the chitin synthesis experiments on different cell lines all the cell lines respond positively to 20-HE.

Dopa-decarboxylase (DDC)

Despite repeating the method several times and using a positive control in the shape of *Chironomus tentans* cells no DDC activity was found in the cell lines.

Discussion

Ecdysteroid receptor analysis

Ecdysteroid receptor analysis revealed the presence of a high affinity, saturable ecdysteroid binding protein

(receptor) in the disc cell lines. Analysis showed that the concentration of binding sites per cell, assuming that one receptor has one binding site, was approximately 100 sites per cell, ranging from a low of 10 to a high of 172. Studies of ecdysteroid receptors from intact imaginal discs give a figure for binding sites per cell of approximately 100 (Siegel and Fristrom 1978; Yund et al 1978). Indeed analysis of ecdysteroid receptors in a number of different tissues and species yields a figure for binding sites per cell of approximately 1000-2000 (Spindler-Barth and Spindler 1987). Clearly the concentration of ecdysteroid receptors measured in the disc cell lines is far below this figure. However the figures obtained in these assays may well be an underestimate of the actual ecdysteroid receptor content for a number of reasons. Firstly these assays were performed using a method developed for the measurement of ecdysteroid receptors in *Chironomus* cells (Turberg et al 1988). Differences between the receptors of the two species might be reflected in a decreased binding of *Drosophila* receptors in this assay. This reflected itself in the fact that since this assay was performed exclusively in Düsseldorf, due to the scarcity of labelled Pon A, attempts to optimise binding for *Drosophila* imaginal disc cell lines were limited. An indication of this can be seen in Table 4 where changes in the binding conditions in the incubation mixture (higher protein concentration, slightly higher Pon A concentration) did result in a slight increase in binding. Optimising all these variables might well have yielded a higher figure.

Published figures for binding sites per cell generally rely on a Scatchard analysis, this is produced from a number of different binding results using different concentrations of labelled hormone. Generally this type of analysis produces a higher figure for binding sites per cell than just taking a figure from a single saturation binding result. So Scatchard analysis of disc cell binding to labelled Pon A may well have produced a higher figure for binding sites per cell.

Nevertheless despite the reduced binding seen in this study of disc cells, comparative results between cell lines show some interesting differences. In the cloned wing cell line Cl.8 a subline from this which was selected for resistance to 20-HE shows a lack of receptor. Despite changing the conditions of binding (see Table 4) to see if there had been a change in affinity of the receptor rather than a complete loss, no detectable receptor was found. This indicates that there was a complete loss of receptor in this cell line which was probably the basis of the resistance to ecdysteroids.

The cloned wing cell line C9 shows a limited response to 20-HE characterised by an increase in aggregation of the cells. When cells from this line were subjected to increased concentrations of 20-HE in parallel to the Cl.8 line a C9R subline was selected which appeared to show no response to 20-HE, not even the weak response shown by the parent C9 line. Yet when both the C9R line and the now designated C9+ parent line were subjected to ecdysteroid receptor analysis both lines which showed very little if any response to 20-HE showed a very high titre of ecdysteroid receptors. This was in

contrast to the other cloned and uncloned cell lines in which 20-HE showed a dramatic effect and yet had generally a lower ecdysteroid receptor titre than the C9+, C9R line.

Two cell lines, both of which are resistant to the action of 20-HE appear to show different mechanisms for their resistance. One shows a lack of receptor possibly indicating that a mutation occurred in this cell line enabling it to overcome the growth inhibiting properties of 20-HE. The other cell line showed resistance to 20-HE both within the selected line and the parent line (to a lesser degree) and yet had an adequate level of receptor. Obviously these cells had a different mechanism for resistance, the ecdysteroid receptor was present and was able to bind to hormone as measured in the assay. Perhaps the hormone-receptor complex was unable to bind to DNA and affect transcription.

Ecdysteroid receptor analysis of embryonic cell lines also reveals that changes in receptor levels in cell lines are associated with insensitivity to 20-HE. K_C cells in response to 20-HE undergo a cessation of division and a morphological transformation (Cherbas et al 1980). K_C cells continuously exposed to hormone after 5 days escape the mitotic arrest and re-enter the cell cycle (Stevens et al 1980) and are refractory to any subsequent 20-HE stimulation for periods of up to 5 months. The inability of these cells to respond to 20-HE is correlated with a 50-70% decrease in the numbers of intracellular ecdysteroid receptors. In other words exposure to 20-HE results in a down regulation of ecdysteroid receptor titre. Hormone resistant cells that have undergone down regulation of their ecdysteroid receptors do not show a

complete loss of receptor titre and those that are left are indistinguishable in their binding characteristics from those of hormone sensitive cells (Stevens and O'Connor 1982). Some sublines of K_C (Br^-) are permanently ecdysteroid resistant and have lost any detectable ecdysteroid receptors (Stevens and O'Connor 1982). Clearly this is similar to the situation in the Cl.8R disc cell line where there is no detectable ecdysteroid receptor. These cells could not have merely down regulated their ecdysteroid receptor content. The cells had been selected for resistance to hormone approximately 4-5 months before ecdysteroid receptor analysis was carried out and since that period had been grown without hormone. Such a length of time in culture without 20-HE should have brought about some recovery from the down regulation of ecdysteroid receptors. In K_C cells 5 months is necessary for a full recovery to titre levels similar to control cells (Stevens and O'Connor 1982). The cloned cell line Cl.8R was still insensitive to 20-HE at least 18 months after selection (p. 20) for resistance ruling out down regulation as a mechanism for the ecdysteroid resistance seen in this cell line.

Chitin synthesis

The results from analyses of chitin synthesis in disc cell lines show that chitin synthesis is regulated by the hormone 20-HE. Cells exposed to 20-HE responded by a greatly increased incorporation of GlcN into chitin.

Intact imaginal discs cultured *in vitro* also respond to 20-HE by a stimulation of chitin synthesis (Oberlander et al

1978). Some insect cell lines also synthesise chitin, but generally the response of different cell lines to 20-HE can vary greatly from stimulation to inhibition and those variations do not always correspond with the supposed origin of the cells in culture. Marks and Ward (1987) surveyed 14 *Dictyopteran* and *Lepidopteran* cell lines, of which 4 showed evidence of chitin production, one of which was set up from imaginal discs and the rest were embryonic. Only one of these, an embryonic cell line showed a quantifiable stimulation of chitin synthesis by 20-HE (Ward et al 1988). The cell line set up from imaginal discs produced chitin but was found to be difficult to quantify any effects of 20-HE on synthesis (Marks and Ward 1987).

An epithelial cell line from *Chironomus tentans* (Wyss 1982) and the K_C cell line from *Drosophila* both show evidence of chitin synthesis (Londerhausen et al 1988) and show opposite responses to 20-HE. In the *Chironomus tentans* cell line 20-HE inhibits chitin synthesis and in the K_C cell line 20-HE stimulates synthesis. Clearly there is quite a difference as regards chitin synthesis between different cell lines and their response to 20-HE. In all the imaginal disc cell lines tested in this study both from leg and wing all showed evidence of chitin synthesis and 4 out of 5 showed a 20-HE induced stimulation of synthesis. Thus these imaginal disc cell lines show much greater conformity in their response to 20-HE as regards chitin synthesis than other established insect cell lines where as mentioned above only a few respond positively to 20-HE. This represents a much clearer response to 20-HE in these cell lines than in derived from embryonic

material and is similar to the response of intact imaginal discs to 20-HE. These results indicate that chitin synthesis is under direct receptor mediated hormonal regulation. The type of response that is seen in these disc cell lines show that the cells are of an epithelial origin.

Acetylcholinesterase (AChE)

AChE activity in disc cell lines is induced by 20-HE in a dose dependent manner. AChE is thought of as an enzyme of the nervous system acting on the neurotransmitter acetylcholine at synaptic junctions and indeed its inhibition is the basis of the action of a number of nerve gases and insecticides. Clearly it is considered to be a marker of neural tissue. Therefore it is perhaps quite surprising that AChE activity induced by 20-HE is present in disc cell lines.

AChE activity has also been found in *Drosophila* embryonic cell lines where it is also induced by 20-HE (Cherbas et al 1977; Berger et al 1980). This activity was detected in a search for something to measure in order to quantify the cells biochemical responses to 20-HE and also to try and elucidate the origin of the embryonic cells seen in culture. Indeed the presence of AChE in embryonic cell lines such as K_C and others whose cellular origin is unknown led some authors to suggest that these cells were neural in origin (Cherbas et al 1977; Best-Belpomme et al 1978). However a number of findings seem to suggest that the induction of AChE activity in *Drosophila* cell lines is not an indication of their neural origin. The level of AChE activity seen is much

lower than that seen in the *Drosophila* nervous system, Cherbas et al (1977) finding only 2% of the activity seen in the nervous system. Berger and Wyss (1980) showed by electrophoresis that the AchE found in Schneider 3 cells differs from the enzyme of the *Drosophila* central nervous system. Also in these cell lines there was no induction by 20-HE of a second important neural enzyme choline acetylcholinesterase (Berger et al 1978). Along with the enzyme AchE, the enzyme β -galactosidase was also found to be induced by 20-HE in embryonic cell lines (Best-Belpomme et al 1978). β -galactosidase is an enzyme which is not thought of as a marker for neural tissues, it breaks glycosidic bonds between galactose, an important component of glycolipids, glycoproteins and other molecules.

Hormonal induction of AchE activity occurs in both embryonic and disc cell lines. In disc cell lines 5 out of the 5 cell lines tested proved positive for 20-HE induction of AchE activity. In embryonic cell lines the response is perhaps not so clear cut, not all the cell lines tested prove positive for 20-HE induction of AchE (Berger et al 1980). An epithelial cell line from *Chironomus tentans* also shows an induction of AchE by 20-HE. Clearly AchE is not acting in this system as would be expected from a neural specific enzyme.

Localisation of AchE activity in vertebrates has shown that AchE is present in many non-neuronal and embryonic tissues and that it is expressed in neuroblasts long before synapses are formed in early neurogenesis (Layer 1990). Drews (1975) in a survey of AchE expression in several different systems found AchE activity to be widespread in such different

situations as gastrulating sea urchin embryos and chick embryos, and postulated a role for AchE during morphogenetic movements. AchE may well have other functions besides breaking up the neurotransmitter acetylcholine. AchE has been shown to hydrolyse substance P, a vasoactive intestinal peptide (Chubb *et al* 1980). It has also been shown that AchE acts on substrates that are normally associated with aryl acylamidase enzymes and that AchE may exhibit weak proteolytic activity (Balasubramanian 1984; Greenfield 1984). All these studies indicate that AchE has a more widespread substrate specificity than is commonly thought and this may be related to the wide polymorphisms seen of the AchE molecule (Massoulie and Bon 1982). Therefore AchE is a membrane-bound enzyme that may well be exhibiting other enzymic activity besides its role in breaking up acetylcholine, including proteolytic activities and that it is seen in a number of sites where cells are actively rearranging.

In intact imaginal discs proteases are produced during 20-HE induced evagination, a process that involves cell shape changes and cell rearrangement (Pino-Heiss and Schubiger 1990). In imaginal disc cell lines 20-HE causes aggregation and cell shape changes producing the characteristic morphological transformation seen in the previous chapter. Along with these morphological changes, AchE is induced by 20-HE and this together with the fact that AchE may have some proteolytic activity suggests that membrane bound AchE may play a role in the cell shape changes and rearrangements that are seen *in vitro*. Indeed as was mentioned in the previous chapter, membrane bound proteases localised to pseudopodia

have been implicated in cell movement, degrading extracellular matrix (ECM) material (Chen and Chen 1987). A cell adhesion molecule called neurotactin has been shown to have an extracellular domain similar to cholinesterases (Barthalay et al 1990; De la Escalera et al 1990). This molecule is found mainly in neural tissue, the mesoderm and imaginal discs and is only detected during cell proliferation and differentiation (De la Escalera et al 1990). So perhaps what at first appears to be a deranged response, that is the 20-HE induction of AchE, an enzyme that is associated with the nervous system may have some role to play in the dramatic changes that are seen on 20-HE application to disc cell lines.

Dopa-decarboxylase (DDC)

DDC is an enzyme involved in the sclerotisation and tanning of the cuticle, converting Dopa into Dopamine which in turn is converted into sclerotin and many other molecules associated with the cuticle. DDC has been considered a marker for epidermal cells as it is involved in hardening and tanning the cuticle. Disc cell lines failed to show any DDC activity whereas an epithelial cell line from *Chironomus tentans* did prove positive for DDC. A leg cell clone LIA was primarily used for these studies due to the prevalence in this particular line of occasional patches of what appeared to be tanned cuticle (see Chapter 4). However even with this line no DDC activity was observed. The problem with the assay to detect DDC was that it required a large protein concentration in the sample of about 2 mg/ml. This proved difficult to

obtain when trying to measure activity in cells that had been treated with 20-HE due to its effects on cell proliferation and cell death. If the cells had been cultured with a continuous level of 20-HE then the protein level of the sample that would be harvested at the end would be inadequate for DDC determination. To overcome this, cells were grown without 20-HE allowing an adequate cell density and hence protein level to build up and then a brief pulse (1 day) of 20-HE added. Even so no DDC activity was measured either in 20-HE treated cultures or controls whereas the *Chironomus* cells a basal level of DDC activity could be measured without 20-HE stimulation, which was increased on 20-HE addition.

It is quite interesting that an obviously epithelial cell line from *Chironomus* that grows as single-layered multicellular vesicles displays DDC activity whereas imaginal disc cell lines do not show any activity. However studies on the *in vivo* expression and spatial distribution of DDC activity using antibodies raised against the DDC fusion peptide show an interesting pattern. DDC is found in the epidermis and central nervous system of the larva and adult (Konrad and Marsh 1987) but is absent from mature 3rd instar imaginal discs. Another study shows an interesting regulation of DDC activity that differs between the mature larval epidermis and imaginal discs that go to form the adult structures. On exposure to 20-HE, a rapid increase in levels of DDC was found in larval epidermis. DDC activity was not detected in imaginal discs cultured in the continuous presence of hormone but a late induction was observed when 20-HE was added and then taken away (Clark et al 1986). This makes sense

when one considers that at the onset of pupariation the larval epidermis hardens to form the case of the puparium whereas the underlying imaginal discs need to undergo complex morphogenetic movements to take up the form of the adult. These would be hampered if the response of the disc cells to the first pulse of hormone was produce a hardened cuticle hindering cell shape changes and rearrangement, whereas the larval epidermal cells need to undergo rapid hardening to protect the underlying tissues during pupation.

Hence it is perhaps not surprising that DDC activity was not detected in cell lines isolated from late 3rd instar imaginal discs but was observed in an epithelial cell line derived from embryonic cells. The later response of the imaginal discs *in vivo*, that is the formation of the hardened adult cuticle and a consequent increase in DDC levels would be difficult to mimic *in vitro* due to the more primary effects of 20-HE on cell proliferation. These would limit cell growth in culture and produce a harvested preparation that was of too low a protein concentration to enable a determination of DDC using this assay system.

Figure 5.1

Figure 5.1 : Ecdysteroid receptor analysis of several different imaginal disc cell lines. The graph gives figures for the number of binding sites per cell.

Cell lines

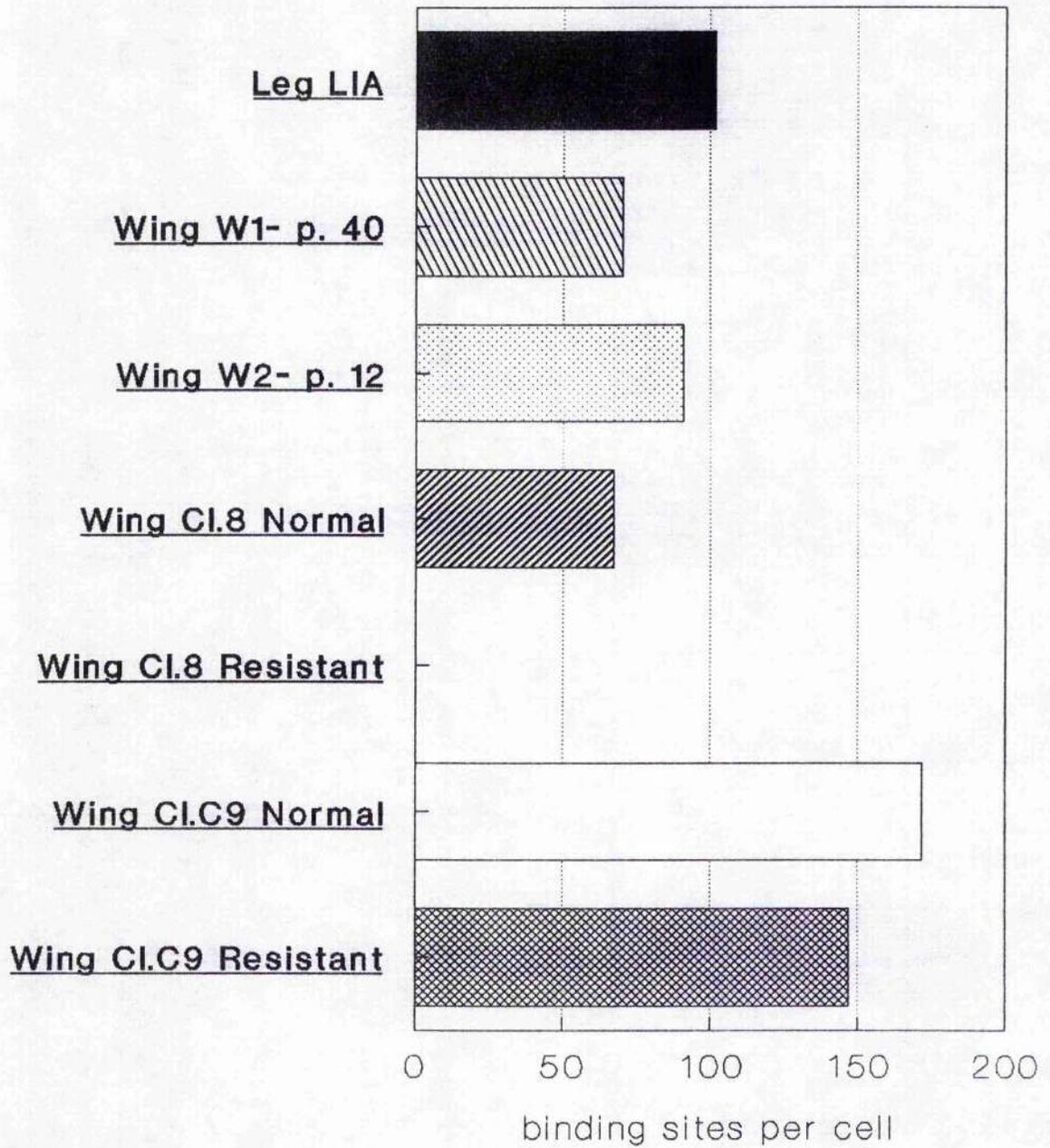


Figure 5.2

Figure 5.2 : Chitin characterisation. Radiolabelled product collected on a glass fibre filter was digested with or without chitinase enzyme, washed again and counted for radioactivity. In all chitin determinations four replicates of each treatment were taken. Error bars = Standard error of the mean (S.E.).

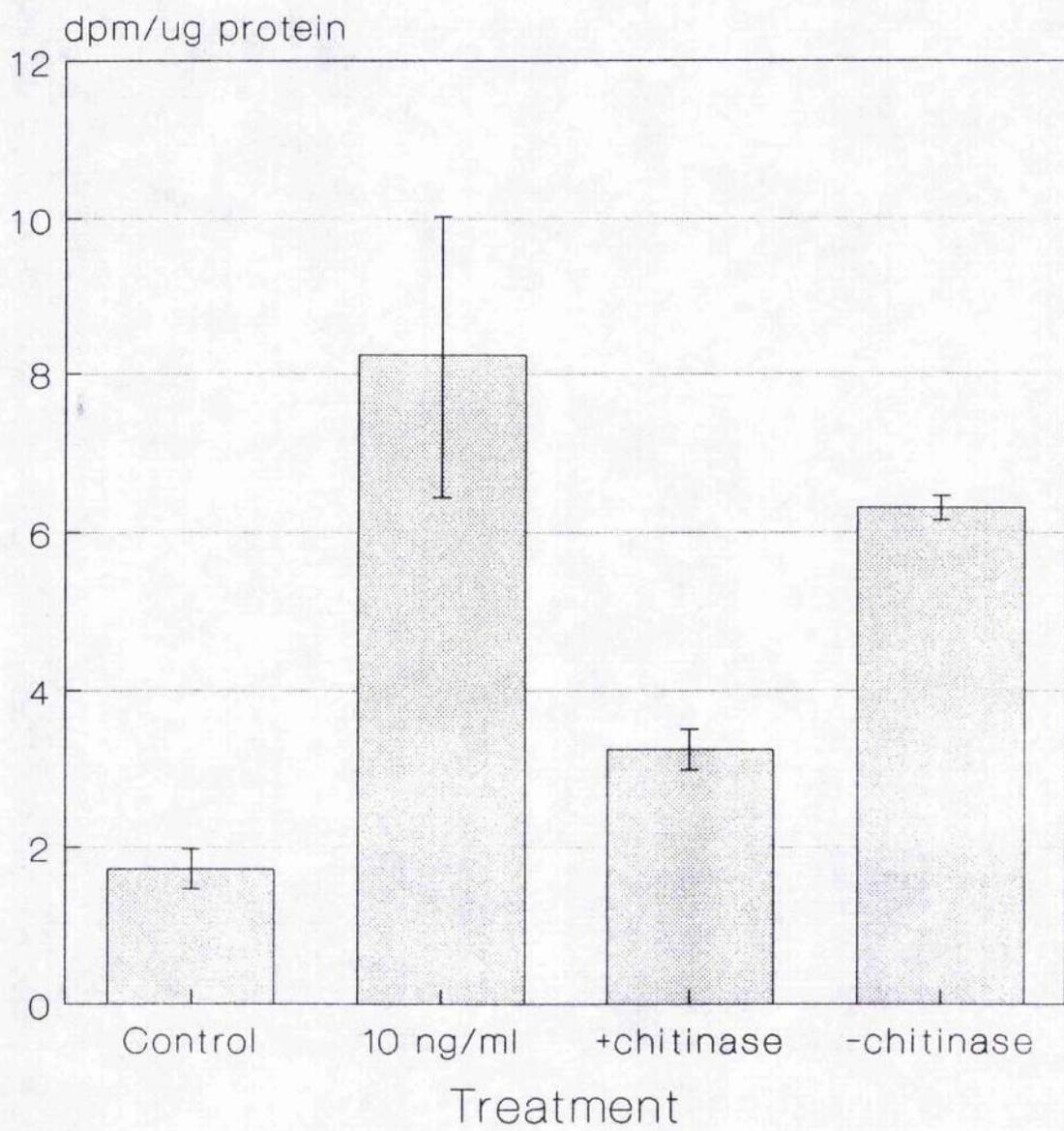


Figure 5.3

Figure 5.3 : Dose response experiment for two cloned wing lines Cl.8+ and Cl.8R, which have been demonstrated to be receptor positive and negative respectively. Error bars = \pm S.E.

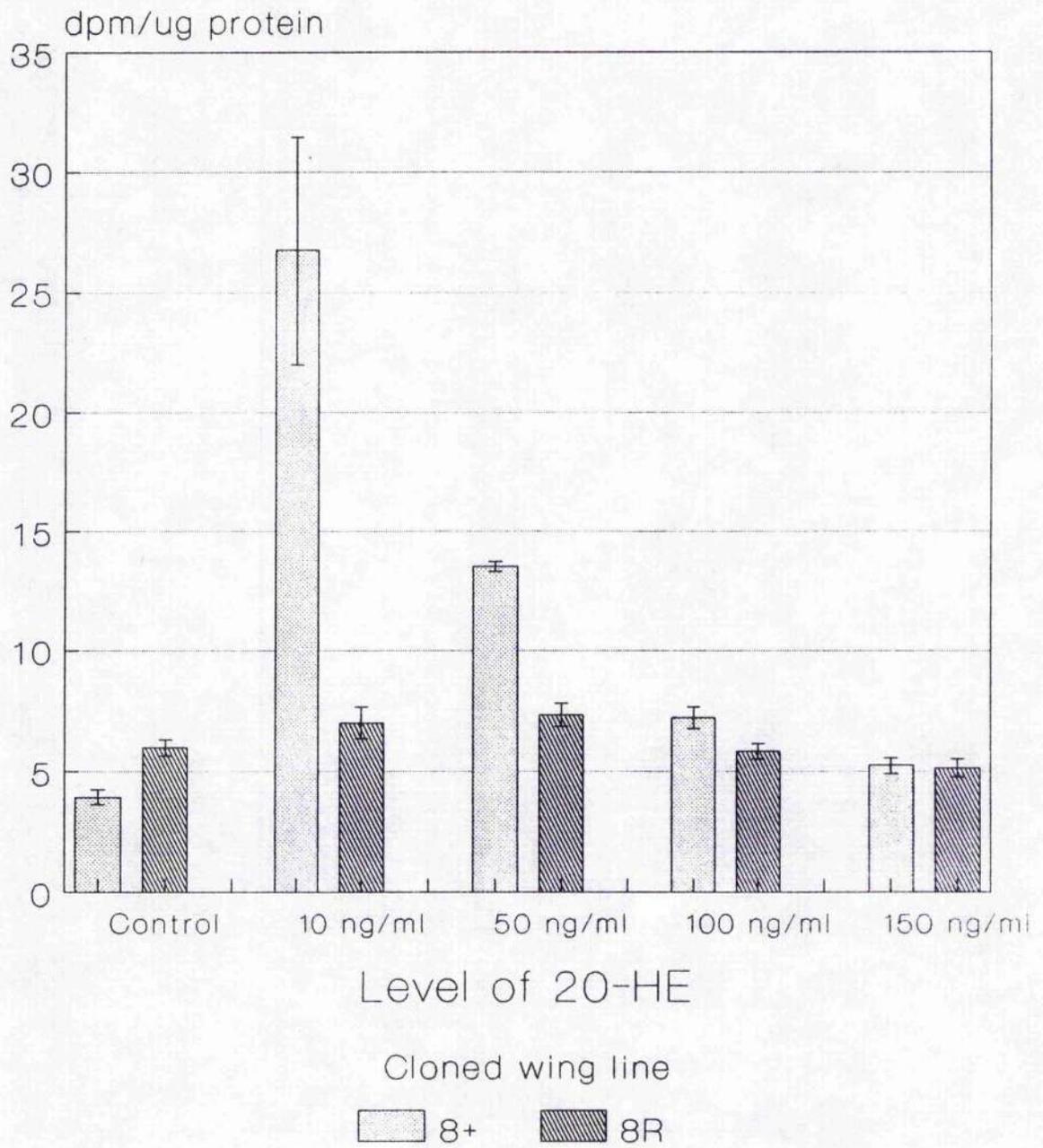
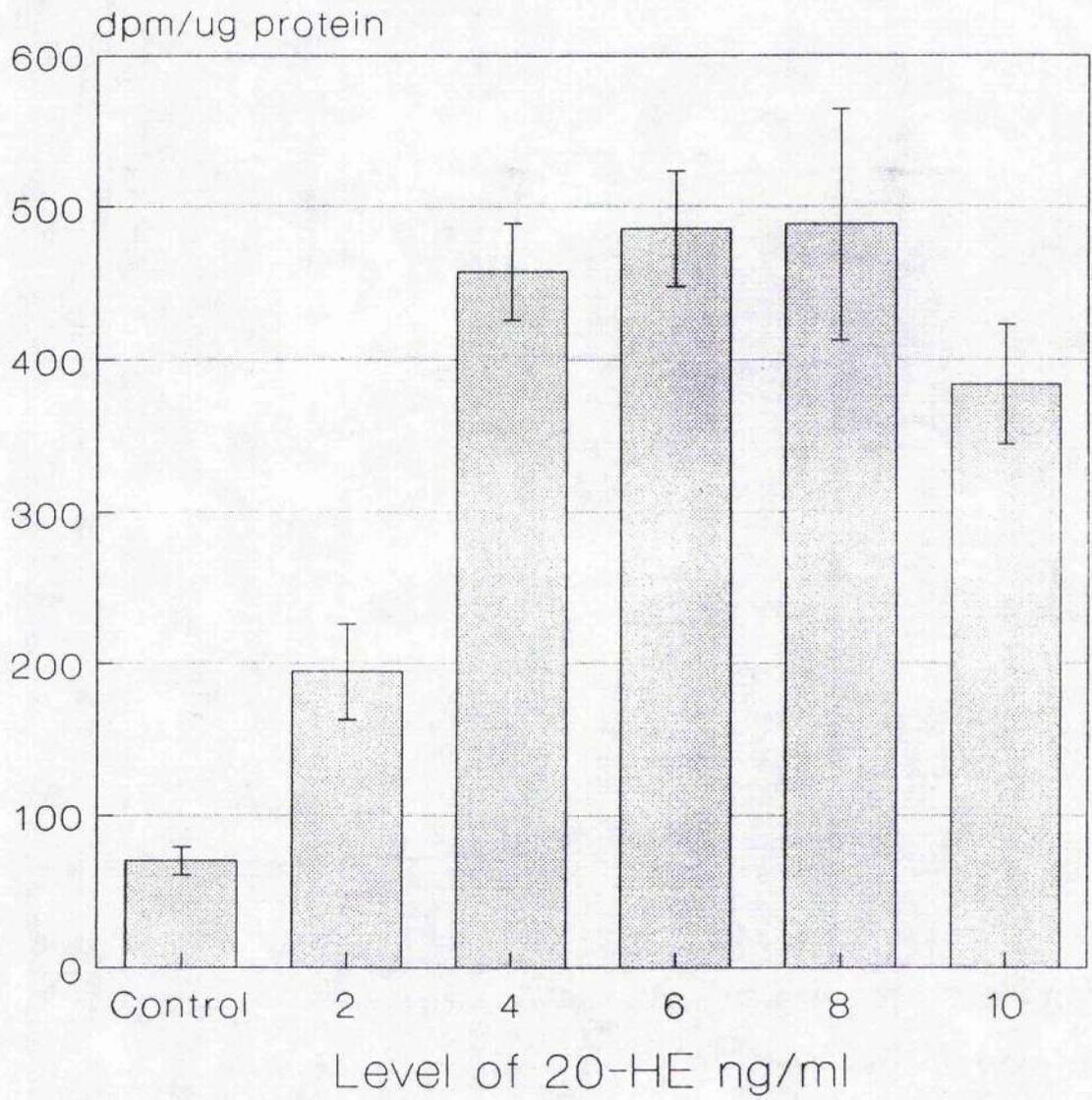


Figure 5.4

Figure 5.4 : Dose response curve for lower levels of 20-HE.

The cloned wing cell line Cl.8+ was grown for 7 days in culture medium containing titres of 20-HE ranging from 2-10 ng/ml. Error bars = \pm S.E.



Cloned wing line

8+

Figure 5.5

Figure 5.5 : Time course of induction of chitin synthesis over a 7 day period. The cloned wing cell line Cl.8+ was exposed to 10 ng/ml of 20-HE and harvested at daily intervals. Error bars = \pm S.E.

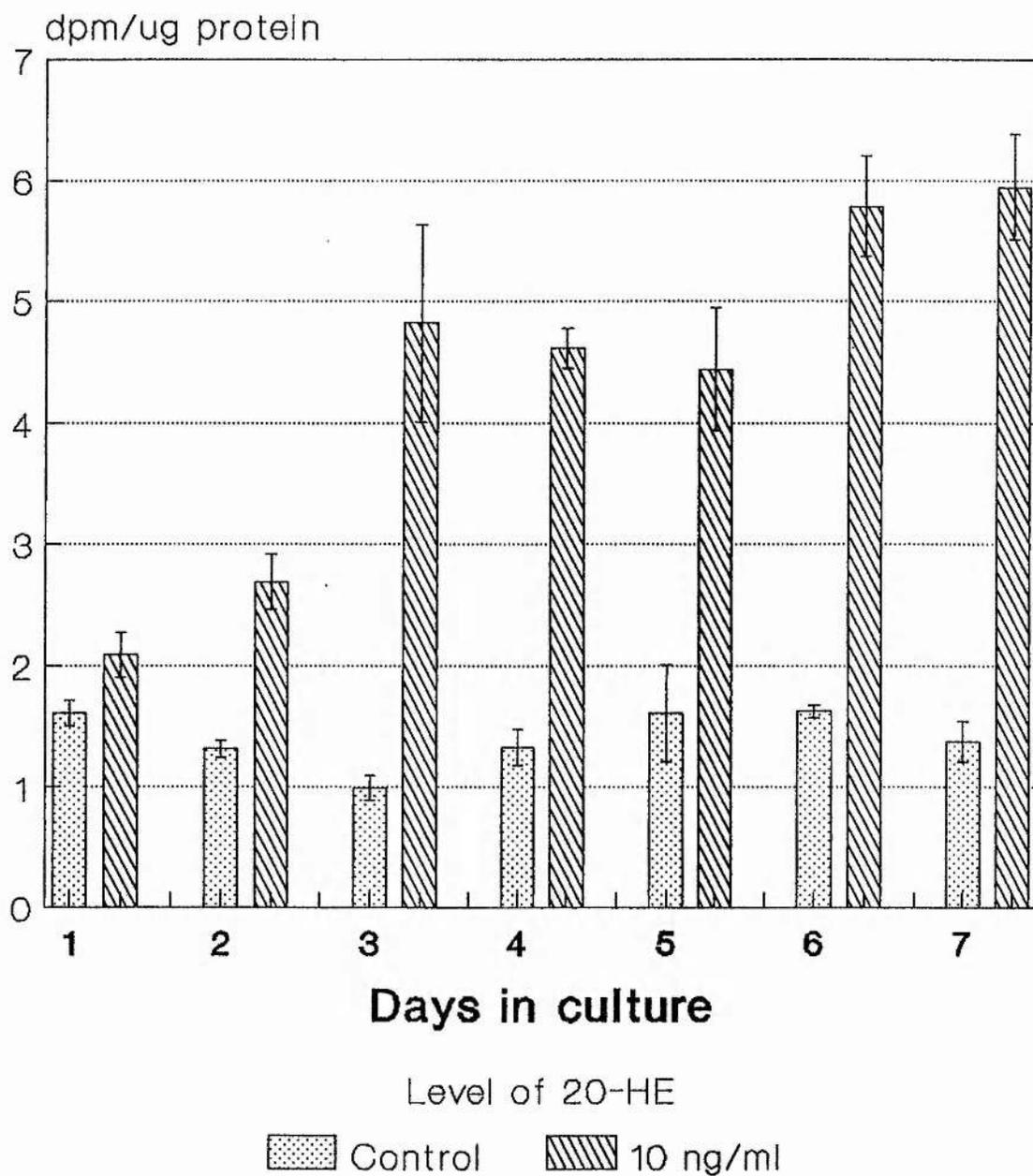
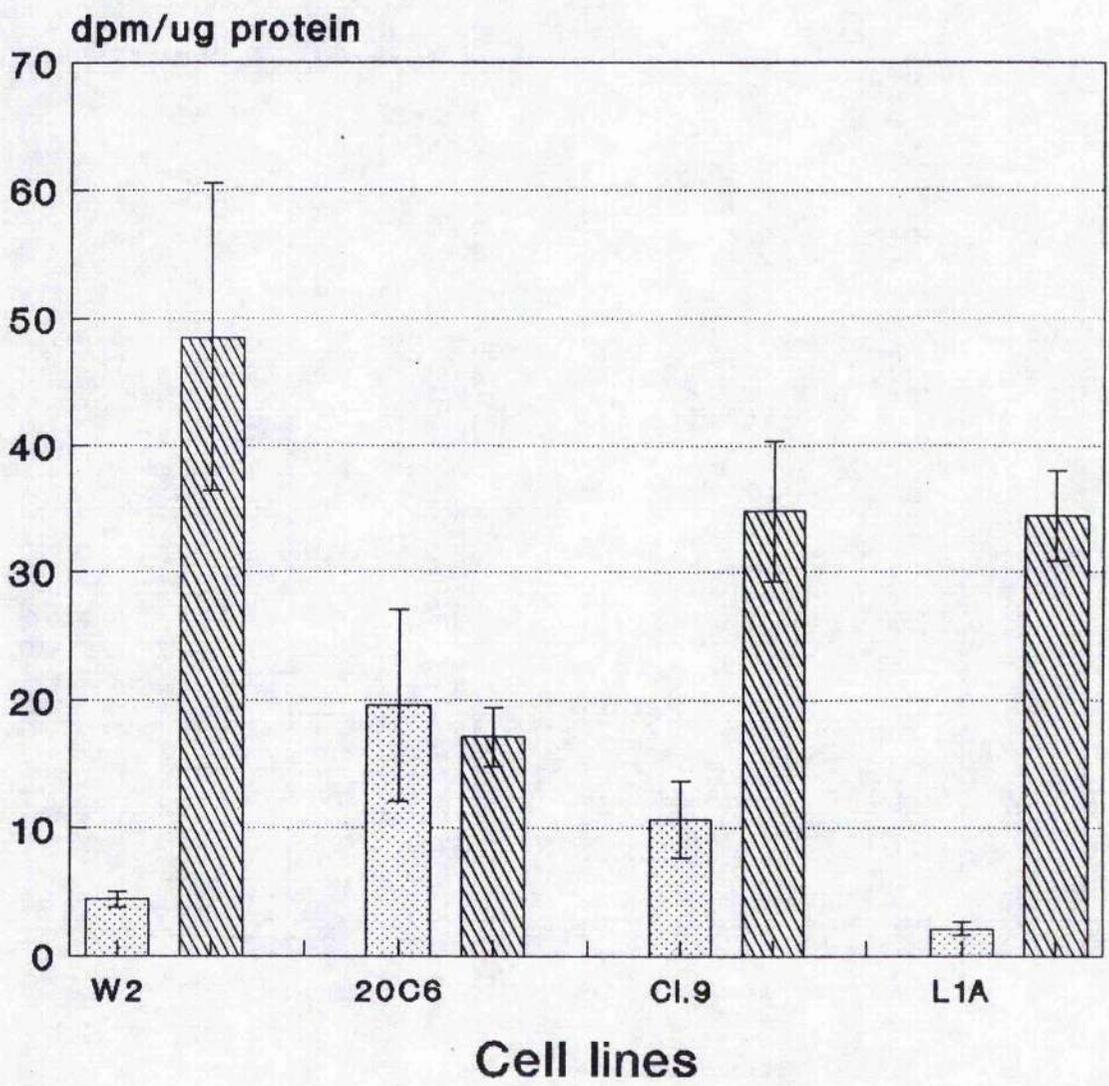


Figure 5.6

Figure 5.6 : Analysis of chitin synthesis in several different disc cell lines. Error bars = \pm S.E.



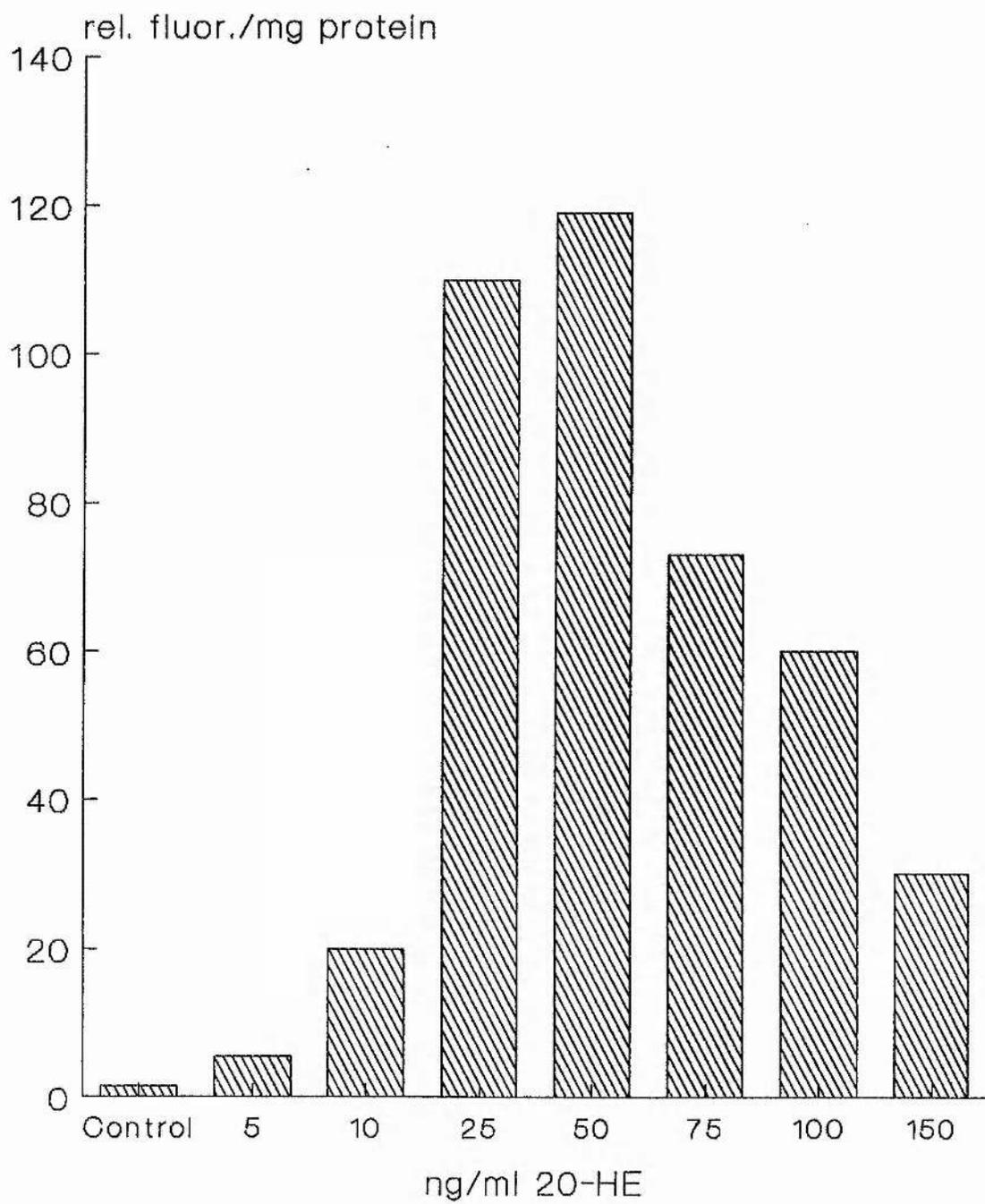
Level of 20-HE

Control 10 ng/ml

Figure 5.7

Figure 5.7 : Acetylcholinesterase (AChE) assay. AChE activity of the cloned wing cell lines Cl.8+ (A) and Cl.8R (B) exposed to a range of levels of 20-HE.

A



B

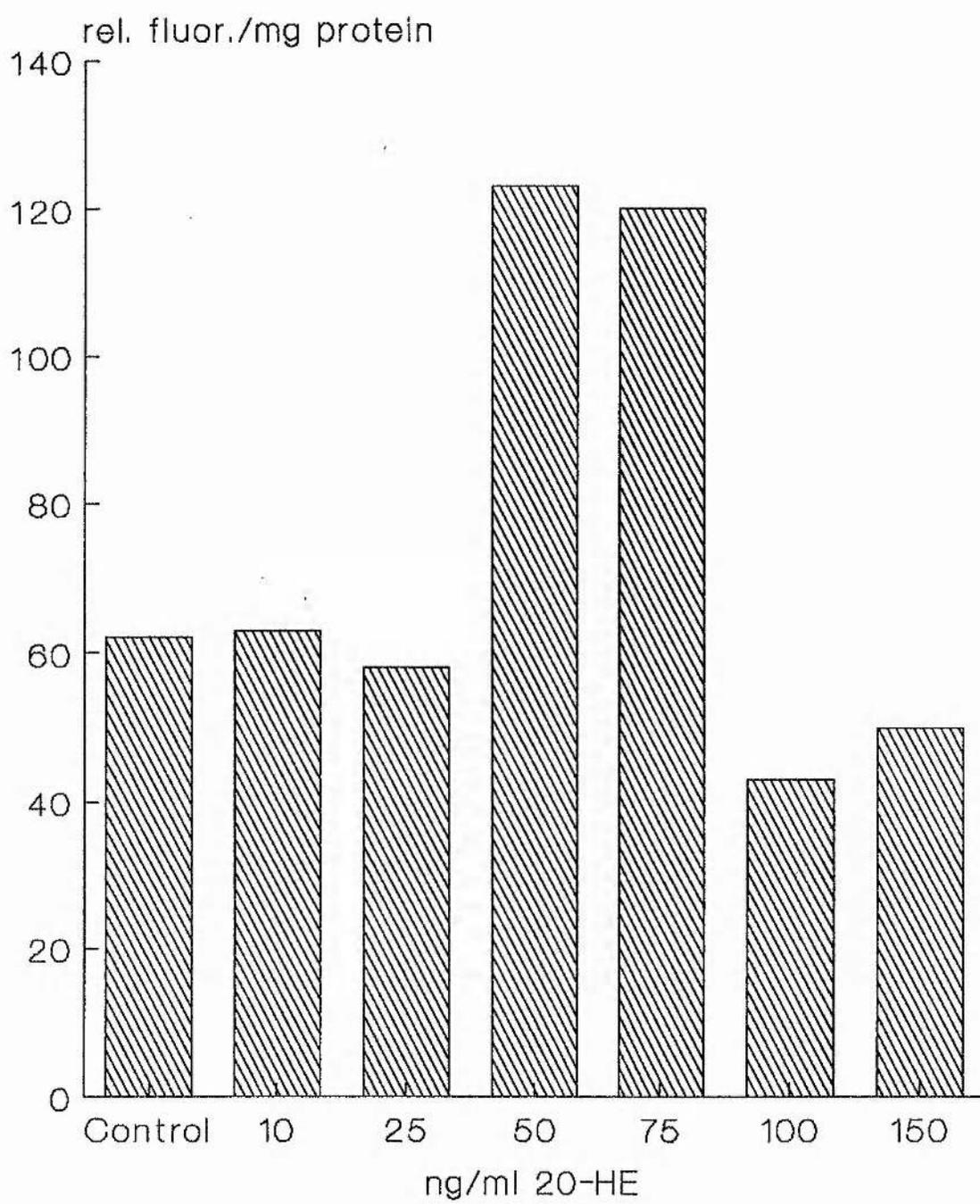
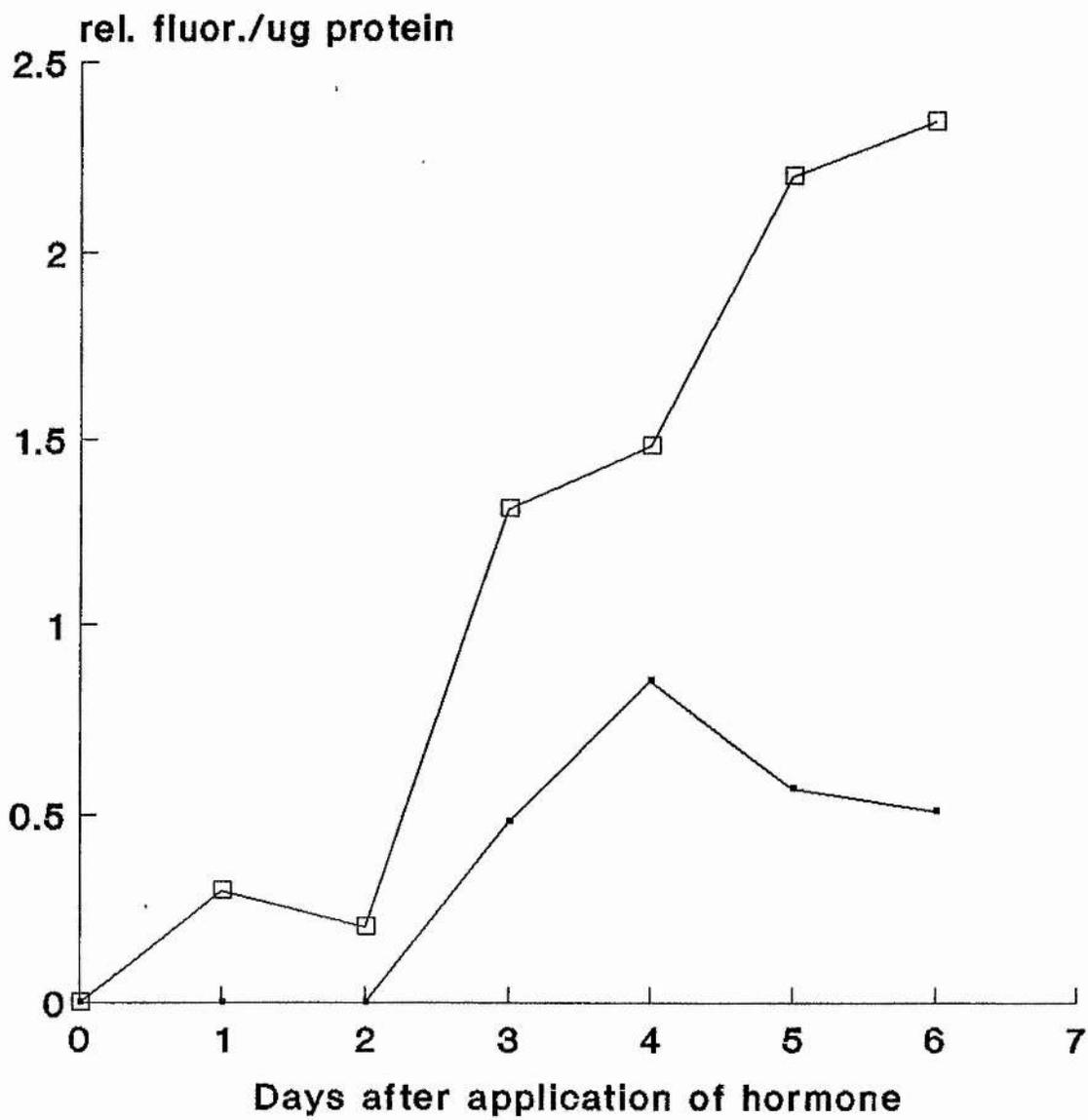


Figure 5.8

Figure 5.8 : AchE assay. Time course for induction of AchE activity in the cloned wing cell line Cl.8+ exposed to 10 ng/ml (2×10^{-8} M) and 50 ng/ml (1×10^{-7} M) of 20-HE over a 7 day period.

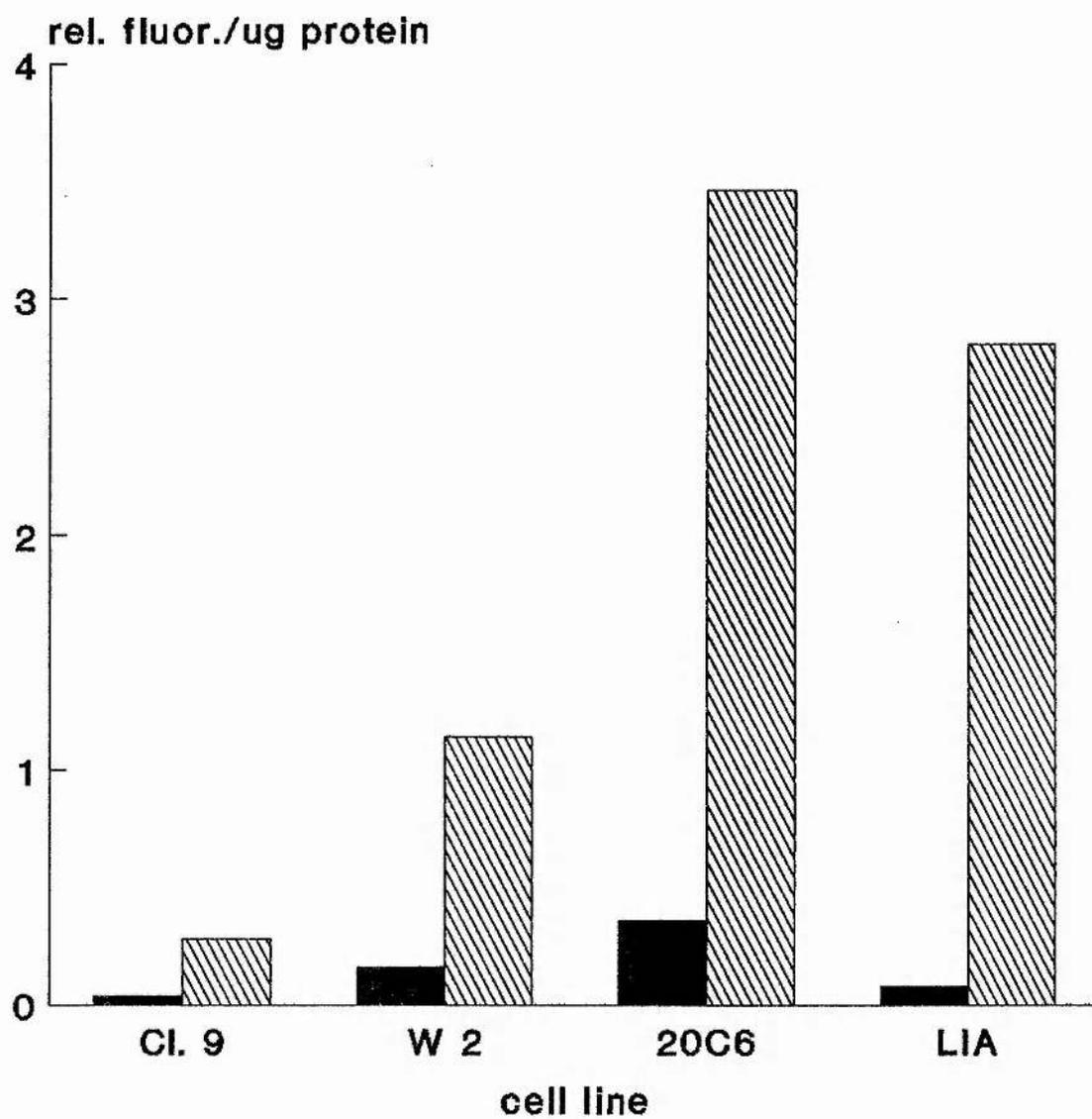


Level of 20-HE

—●— 10 ng/ml —□— 50 ng/ml

Figure 5.9

Figure 5.9 : AchE activity in several different disc cell lines exposed to 10 ng/ml (2×10^{-8} M) of 20-HE.



Level of 20-HE

■ Control ▨ 10 ng/ml

Chapter 6

Aggregation in imaginal disc cell lines

Aggregation in imaginal disc cell lines

Introduction

Imaginal disc cells growing as continuous cell lines show a very characteristic pattern of aggregation between passages (Peel et al 1990). This results in the formation of large multicellular aggregates. In order to try and understand the process of aggregation in culture several different aspects were studied. At the ultrastructural level the overall pattern of aggregation was examined with the SEM and the internal structure of the aggregates with the TEM. Along with this ultrastructural analysis the role of cell processes was studied by looking at patterns of microtubule deployment in aggregating cells with anti-tubulin immunofluorescence.

As well as looking at the role of cell processes and changes in the structure of the cells a more molecular approach was taken by looking at the expression of cell surface molecules known to be important in adhesive mechanisms. The Position Specific (PS) integrins were studied using monoclonal antibodies generously supplied by Dr. Michael Wilcox of the Laboratory for Molecular Biology at the MRC, Cambridge. These molecules were found to be invertebrate equivalents to vertebrate integrins (Bogaert et al 1987; Leptin et al 1987,1989), cell surface molecules which bind to components of the extracellular matrix and interact with the cortical cytoskeleton. The PS2 integrin has been found to be analogous to the vertebrate fibronectin receptor (Bogaert et al 1987) and this molecule was chosen for most of the studies. The changes in expression of PS integrins were studied at

various stages of culture using immunocytochemistry to see if there was any correlation between its expression or localisation and aggregation in the cell lines.

Methods

For both immunofluorescent and immunocytochemical studies on the cell lines Labtek culture chamber slides were used. These allowed cells to be grown on a glass bottomed microscope slide which had been tissue culture treated. The cells could be grown in these chamber slides and all the antibody and staining procedures carried out on the cells *in situ*. After staining, the chambers of the slide could easily be snapped off and a microscope coverslip directly placed over the preparation. Using these culture chamber slides enabled all the complicated incubations, washings and staining procedures to be carried out on the cells with the minimum of disturbance. This proved valuable as imaginal disc cells growing as continuous cell lines do not adhere strongly to tissue culture plastic or glass. Indeed as aggregation occurs in culture cells become more adherent to each other and less so to the culture dish surface. At later stages of culture when the cells have formed large multicellular aggregates, they can very easily detach themselves from the tissue culture surface under the force of surface tension.

Immunofluorescence with anti-tubulin antibody

Cells were plated out at 7.5×10^5 cells/ml in 0.4 mls of medium per chamber, grown for 7 days and processed as follows. The cells were permeabilised for 10 minutes using a cytoskeletal buffer consisting of 100 mM NaCl, 300 mM Sucrose, 10 mM PIPES, 3 mM $MgCl_2$, 0.5% TRITON X 100 and 1.2 mM Phenylmethylsulfonylfluoride (PMSF) pH 6.8. The solution was then diluted 1:1 with 4% paraformaldehyde in PBS and fixed for 20 minutes in this mixture. After two five minute washes in PBS 1% BSA, the cells were incubated overnight at 4°C on a shaker in primary antibody (monoclonal anti- α tubulin (Sigma T-9026)), which was diluted in PBS 1% BSA at 1:500. After washing as before the cells were incubated for 1 hour at room temperature in biotinylated anti-mouse IgG (Vector) diluted 1:200 in PBS 1% BSA. The cells were again washed as before and incubated for 1 hour at room temperature in Avidin FITC (Vector) diluted 1:250 in PBS 1% BSA.

Alternatively, cells were washed and fixed in 4% paraformaldehyde in PBS for 10 minutes and then permeabilised in PBT buffer (PBS with 0.01% BSA, and 0.01% Triton X-100) to which was added 0.02% horse serum for 3 hours. The cells were then processed as above for antibody incubations except that PBT buffer was used instead of PBS 1% BSA. This method gave better visualisation of more isolated cells whereas the permeabilisation before fixation method was superior for the examination of processes within and between aggregates.

After washing in PBS the cells were mounted in 70% glycerol, 30% 0.1M TRIS pH 9 with 2% n-propyl gallate to reduce bleaching. The samples were viewed using a Leitz

Diavert inverted fluorescent microscope and photographed with 1600 ASA film using a Wild photoautomat.

Immunocytochemistry

Cells were plated out at differing densities based around the figure of 7.5×10^5 cells/ml which was found to be the optimal cell concentration for the Labtek culture chamber slides. The cells were washed with PBS and then fixed for 10-15 minutes with 4% paraformaldehyde in PBS. After two five minute washes in PBS 1% BSA the cells were incubated for 2 hours at room temperature in primary antibody (anti-PS2 mouse IgG, CF2C7 and HC2) which was diluted in PBS 1% BSA at 1:250. The cells were washed extensively in PBS 1% BSA as previously and then incubated for 1 hour at room temperature in diluted biotinylated anti-mouse IgG (Vectastain ABC kit). After 1 hour the cells were washed twice for 10 minutes each time in PBS 1% BSA. Then the cells were incubated for 1 hour at room temperature in ABC reagent (Vectastain kit). The cells were then washed as before in PBS 1% BSA and then in PBS alone.

The cells were stained for horse radish peroxidase (HRP) as follows. The cells were incubated for 2-5 minutes in a substrate solution consisting of 3.8 mls 0.05 M acetate buffer pH 5.0, 0.2 ml stock 3-amino-9-ethylcarbazole (AEC) made up as 20 mg AEC in 2.5 mls dimethylformamide and finally 20 μ l of 3% H_2O_2 (freshly diluted from a stock solution of 30% H_2O_2 with double distilled water) was added to the substrate solution. The reaction with HRP produces a red insoluble reaction product, the reaction was terminated by washing with double

distilled water. The cells were then counterstained for 5 minutes in a 1:1 dilution of stock Mayers' haematoxylin (BDH) in PBS and then washed in running tap water for five minutes to blue the haematoxylin staining. After staining, the slides were partially wiped dry and mounted in an aqueous mounting media (Aquamount) which was sealed with nail varnish to prolong the life of the preparation. An aqueous mounting media had to be used when AEC was used as substrate as the red reaction product is soluble in non aqueous mounting media.

Alternatively the preparations could be stained with diaminobenzidine (DAB) as a substrate. The cells were incubated for 10-15 minutes in a substrate solution consisting of 25 μ l of a DAB solution (20mg DAB/ml double distilled water) and 2 μ l of 30% H_2O_2 per ml in PBS with the addition of 30 μ l of a solution of 1% $NiSO_4$ and 1% $CoCl_2$ to enhance staining. After staining the cells were treated as before except that the preparations could be mounted in a non-aqueous mounting media such as Histomount.

Western blotting

Cells grown at different densities were harvested and cell protein samples run on an Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to nitrocellulose, probed with antibody and levels of antigen present determined by scanning densitometry.

Cells were grown in 5cm Petri dishes at different densities ranging from 1×10^5 cells to 9×10^6 cells per 5cm

dish. Cell densities were measured with a haemocytometer and protein levels determined using the Bradford method (see general methods). The cell samples were then adjusted so that each sample from the different cell densities had the same protein concentration (50 μ g). The samples were mixed 1:1 with double strength sample buffer consisting of 30 mls stacking gel buffer (0.5 M TRIS, 0.4% SDS), 10 mls glycerol with 10% mercaptoethanol and adjusted to pH 6.8. The samples were boiled for 3 minutes and were then ready for use.

The protein samples were then separated on a 7.5 % SDS-PAGE gel. After electrophoresis the gel was carefully removed, sandwiched together with a sheet of nitrocellulose (Gelman) and several sheets of 3M chromatography paper and then placed between two metal plates, one of which was the anode and the other the cathode. The gel was placed nearest the cathode plate and overlain by the nitrocellulose which was closer to the anode and would bind proteins electrophoretically transferred from the gel which would run in the direction of the anode. This sandwich of electrode plates was then placed in a blotting tank full of circulating buffer at pH 8.3 consisting of 25 mM TRIS, 192 mM glycine and 20% methanol. The gel was then blotted for 1 hour at room temperature.

After blotting, the nitrocellulose membrane was carefully removed from the sandwich and washed in a solution containing 0.1% Tween 20 in Tris buffered saline (100 mM TRIS, 0.9% NaCl pH 7.5) called TTBS. After washing the sheet was incubated in TTBS for 2-3 hours sometimes with the addition of 1% non fat dried milk (Marvel). The sheet was then transferred

to a solution of the primary antibody (anti-PS2 mouse IgG, clone Hc2) at a dilution of 1:1000 in TTBS and incubated overnight on a shaker at 4°C. After washing with 3-4 changes of TTBS over 30 minutes the sheet was transferred to a solution of biotinylated anti-mouse IgG antibody (Vectastain kit) in TTBS for 2-3 hours at room temperature on a shaker. The nitrocellulose sheet was then washed as previously and transferred to the Vectastain ABC reagent made up in TTBS and left for 1 hour on the shaker at room temperature. After incubation the membrane was washed as before and placed in a substrate solution for HRP. This consisted of 200 µl of DAB stock (40 mg/ml DAB tetrahydrochloride), 50 µl of NiCl₂ (80 mg NiCl₂/ml double distilled water) and 30 µl of 3% H₂O₂ (freshly prepared from 30% stock) in 10 mls of 100 mM TRIS pH 7.5. The nitrocellulose membrane was incubated in this solution for 15 minutes, washed with 2 changes of double distilled water over 10 minutes and allowed to air dry in the dark. After drying the membrane was ready for scanning densitometry analysis. Molecular weight markers (97 kD, 58 kD) that were biotinylated (Sigma) were used to determine the molecular weight of the stained PS2 band which was found to be around 115 kD. This is near the figure quoted for PS2 integrins of approximately 110 kD (Brower et al 1984)

Scanning densitometry

Scanning densitometry analysis on the stained nitrocellulose was carried out on a Shimadzu scanning densitometer on reflective mode. The optimal wavelength for

scanning was found to be around 490 nm. The bands of stained PS2 antigen were scanned by setting the densitometer to scan a set distance spanning the bands and to calculate the area of any specific stained band found. Each band was scanned across the middle and the figure multiplied by the length of band, this was done to try and standardise the results as often the bands were not uniform all the way across. This figure was taken as the total amount of antigen and expressed as area of antigen peak per μg of protein against the seeding density of the different samples.

Enzyme immunoassay (ELISA)

Cells were grown at differing densities in 3 cm Petri dishes. Cells were harvested by pipetting approximately 4-5 days after seeding when the cultures were in active growth. The cell samples were then counted in a haemocytometer. The cells were washed by centrifuging the cells at 1,000 r.p.m. for 5 minutes, resuspending them in PBS and pelleting the cells again. Then the cells were fixed by resuspending the pellet in 4% paraformaldehyde in PBS for 5-10 minutes and then washed twice with PBS 1% BSA, centrifuging them down in between. The samples were then split up into controls and experimentals. The experimental aliquot was incubated in the primary anti-PS2 mouse IgG antibody (CF2C7) used at a concentration of 1:250 in PBS 1% BSA. The cells were incubated in Eppendorf tubes for 1 hour on the shaker at room temperature. Control aliquots omitted the antibody but were

incubated with PBS 1% BSA alone. After incubation the cells were centrifuged and washed as before.

The secondary antibody incubation consisted of a 1:1000 dilution of anti-mouse IgG HRP conjugated antibody (Sigma) in PBS 1% BSA. Both experimentals and controls were prepared and the tubes were again incubated for 1 hour on the shaker at room temperature. The cells were then washed as before in PBS 1% BSA and resuspended in the substrate solution. The substrate solution consisted of a 50 mM citrate/phosphate buffer pH 5.3 with 0.015% H_2O_2 to which was added 0.1 mg/ml of 2,2 azino di (3 ethylbenzthiozoline 6 sulfonic acid) commonly called ABTS. The ABTS solution reacts with HRP to produce a green soluble reaction product which can be read in a spectrophotometer. The cells were incubated in this substrate mixture for 15 minutes at room temperature after which the cells were centrifuged down. The incubation period depended on observations as to when the control samples began to change colour and so could sometimes vary. The clear supernatant was taken and its absorbance measured in a spectrophotometer at 405 nm. Results were taken as the difference in absorbance between the experimental and control per cell or per μg protein.

Results

Figure 6.1. (a-e) shows the process of aggregation that occurs in an imaginal disc cell line after passaging. As can be seen in Fig. 6.1. b the cells quickly achieve confluency 2-3 days after plating out, and they then continue to divide piling up into aggregates. These separate from each other and

finally form large multicellular aggregates separated by areas that are left fairly bare of cells (Fig. 6.1. c). These large aggregates often have strands (Fig. 6.2. d,e) consisting of aligned cells streaming from one aggregate to another.

These large multicellular aggregates were sectioned and examined under the TEM. These sections revealed that the cells are closely packed and interspersed with numerous cell processes (Fig. 6.2). On closer examination these processes can be seen to be full of microtubules (Fig. 6.2. c,d).

Cell processes can best be visualised by immunofluorescence with anti-tubulin antibodies. Figure 6.3 shows cells in various stages of aggregation stained with anti-tubulin antibodies. Figure 6.3. a shows cells in a culture which has yet to reach confluency. A cell process which can be seen to extend over several cell diameters is arrowed. In cultures where cells have started to aggregate (Fig. 6.3. b,c) long cell processes can be seen extending towards aggregates from small groups of cells (Fig. 6.3. b) and also in large strands (Fig. 6.3. c).

Therefore from ultrastructural and immunofluorescent studies on aggregating cells two things become apparent. Firstly as cell density increases and cells grow beyond confluency they become more adhesive to each other than to the substrate. This results in the formation of large multicellular aggregates on the culture surface often separated by areas relatively bare of cells. Secondly that cell processes and the microtubules present therein are ubiquitous in aggregating cells.

During the aggregation that is seen in culture, disc cells appear to become less adherent to the tissue culture plastic surface and more adherent to each other. This change in the growth pattern of the cells could either be a cell-cell or a cell-substrate phenomenon. Deposition of extra-cellular matrix (ECM) by the cells during early stages of culture could result in the changes that are seen during aggregation in culture. Alternatively these changes may be the result of a purely cell-cell process. In order to test the hypothesis that changes in the substrate brought about the pattern of aggregation *in vitro* cells were plated out onto culture dishes that were subjected to different treatments. Cells were plated out onto fresh culture dishes and also onto dishes that had previously grown cultures for one week and hence had acquired any ECM material that might have been secreted during that period. If substrate influences were important in aggregation then cells plated onto a used culture surface would be expected to aggregate more rapidly than cells plated onto a surface lacking any ECM material. Used culture dishes were prepared by growing cells for one week then vigorously pipetting the cells off the plastic and removing all the medium whilst leaving the surface damp. Then fresh cells were plated out onto these used culture dishes.

The cells that were plated out onto used culture dishes showed some initial differences in binding to the tissue culture surface. The cells initially did not bind as evenly as control cells plated onto fresh tissue culture plastic. This may well have been something to do with the electrostatic charge that is part of the tissue culture treatment that

plastic dishes are given to promote cell growth and attachment. After being used once, this charge may be somewhat different to that of a fresh dish resulting in an initial difference. However as the cultures progressed no differences were seen in the way that cells grew and started to aggregate. Therefore secretion of ECM material does not seem to affect aggregation, indicating that it is more likely to be dependent on a cell-cell process than a cell-substrate mechanism.

In a further attempt to characterise the aggregation response of the disc cells in culture the expression of the PS2 integrin was analysed during aggregation. Figure 6.4 shows the staining pattern seen in aggregating cells. Aggregates of cells show heavy staining with PS antibodies. Indeed as can be seen in Figure 6.4 staining appears to correlate with aggregation. Single cells appear to show little or no staining whereas heavy staining occurs where cells are aggregating. Figures 6.4.a and b clearly show heavy staining of aggregates whilst nearby single cells show little or no staining. Figure 6.4.b shows a close up of the edge of an aggregate where the cells in that aggregate show diffuse staining all over their surface whereas a cell nearby which has not any firm connections with the aggregate does not show any staining.

In more isolated groups of cells staining with PS2 antibodies can clearly be seen in areas of cell-cell contact. Figures 6.4.c,d and e clearly show staining in areas of cell-cell contact. Figure 6.4.c shows a sheet of cells all showing staining where they contact their neighbouring cells. Figure 6.4.e shows very clearly this type of staining in areas of

cell contact where a chain of cells all show staining at the points where they meet.

These immunocytochemical studies gave the impression that PS2 integrin expression was very dependent on the degree of aggregation seen in culture and hence on cell density. This hypothesis was further tested by using more quantitative methods of antigen detection. Cell samples from cultures at different densities were fractionated by a SDS-PAGE gel and then the proteins blotted onto a nitrocellulose sheet. This allowed detection of fractionated PS2 integrins with antibodies and subsequent staining (see Fig. 6.5). These bands of PS2 integrin gave an indication of the quantity of antigen present in each cell sample and this was quantified by using a scanning densitometer. The quantity of each stained band was taken as the area underneath the antigen peak (see Fig. 6.6). Figure 6.7 shows the results from one such analysis. The graph gives the area of these antigen peaks expressed per μg of protein against the different densities that the cells had been seeded at. The graph shows that levels of antigen increase rapidly with increasing cell density but it appears not to be an exponential rise. At the two lowest cell concentrations levels of antigen are very low and then there is a dramatic rise, this may well correlate with the increasing levels of aggregation that is seen as seeding density increases.

Figure 6.8 show the results from ELISA tests on cells grown at different densities. Figure 6.8.1 gives the results as the difference between the absorbance of the experimental minus the control against the density at which the cells were

seeded. Figure 6.8.2 shows the results of another similar experiment expressed against the density of the cells when they were harvested. Both of these graphs show that there is no increase in levels of antigen as cell density increases and indeed there appears to be a slight decrease. These results from ELISA experiments contradict the results achieved using Western blot analysis where levels of antigen dramatically increase with increasing cell density.

Discussion

Aggregation

The aggregation that is seen in culture is very characteristic of the growth of imaginal disc cells *in vitro*. This aggregation process ends up in the production of large multicellular aggregates which are separated by areas of plastic which have only a few cells. Examination of these aggregates shows the presence of numerous cell extensions both within and between aggregates which is confirmed by immunofluorescence with anti-tubulin antibodies. This also reveals the presence of long cell extensions in more isolated cells that have not yet aggregated, perhaps suggesting a role for these cell extensions in initiating aggregation.

Aggregation in culture could occur due to differential expression and laying down of ECM material, this would result in areas of the tissue culture plastic becoming more adherent than others, causing cells to form large multicellular aggregates above such accumulations. In disc cell aggregation

this seems unlikely for a number of reasons. As aggregation proceeds, cells appear to become more adherent to each other and less so to the tissue culture plastic. In later stages of aggregation when cell density is high, large areas of substrate become relatively devoid of cells. Aggregates of cells are often very large and pile up on one another such that only a few cells maintain contact with the substrate. Experiments comparing aggregation of cells on used and new culture dishes show no final difference in aggregating ability. Hence previously laid down ECM material does not affect aggregation.

Some other mechanism must be initiating aggregation in these cell lines. Aggregation has been shown to occur *in vitro* in fibroblasts cultured on a collagen gel (Harris et al 1984). Aggregates are formed, connected by bridges of stretched and aligned cells very reminiscent of aggregation in imaginal disc cell culture. These aggregates are formed by a process of traction which brings neighbouring cells in contact, which then adhere to form an aggregate. This process is dependent on cells being able to exert a force on their substrate and pulling themselves together. This aggregation therefore only occurs when the cells are distributed on collagen gels and not when they are plated on plastic tissue culture surfaces alone (Harris et al 1984). These conditions did not exist in aggregating disc cells. In our system, cells were plated onto bare tissue culture surfaces with no added substrate, and also the results from one above mentioned experiment suggested that any previously laid down ECM did not affect aggregation. Therefore it seems unlikely that aggregation was achieved by a

substrate dependent traction based mechanism as described by Bard (1990). This suggests that substrate influences are minimal and that cell-cell phenomena are more important.

The presence of large numbers of cell processes full of microtubules in aggregating cells begs the question as to what their role is, if any. The presence of these processes may be analogous to the development of epidermal feet in the epithelial cells of *Calpodes*, a lepidopteran (Locke and Huie 1981; Delhanty and Locke 1989). In this system epidermal basal cell processes or feet elongate and then shorten, producing a contraction in the epidermis as the cells become columnar. This process occurs in preparation for moulting and metamorphosis (Delhanty and Locke 1989). Epithelial cell extensions have also been implicated in the rearrangement of scale cells in the developing moth wing (Nardi and Magee-Adams 1986). Indeed basal epithelial cell extensions have been observed in many actively rearranging epithelia (Fristrom 1988).

Cell extensions could represent one mechanism for achieving the aggregation that is seen in imaginal disc cell lines. Cell processes extending across the culture surface and contacting other cell processes could result in a coming together, providing a focus for further aggregation. Other cells with processes contacting such an aggregate could end up pulling themselves or being pulled inwards and hence produce a growing aggregate. This would result in the formation of large multicellular aggregates appearing to be dragging other cells inwards. Aligned bridges of cells would result from cells pulling or being pulled in two directions. This method of

aggregation however relies on one basic assumption. That is that cells prefer to adhere to other cells rather than plastic. When cells are first plated out and up until 3 or 4 days thereafter they do not form large regularly spaced multicellular aggregates. Instead cells are fairly evenly spread out on the surface. At later stages cells are gathered into large multicellular aggregates and intervening patches of plastic become relatively devoid of cells. Therefore there seems to be an increase in cell-cell adhesiveness in culture that stabilises the formation of these large multicellular aggregates. Epithelial cell extensions may well have a role in initiating aggregation and bringing about cell contact in disc cell lines but other adhesive mechanisms appear to be operative in stabilising these aggregates. To test this hypothesis of increased adhesion in aggregates the role of PS integrins in aggregation was studied.

Position Specific (PS) integrins

Position specific (PS) antigens were originally identified in a monoclonal antibody screen as cell surface glycoproteins which showed spatially restricted distributions in the mature wing imaginal disc (Wilcox et al 1981; Brower et al 1984). Three classes of monoclonal antibodies were isolated, these being identified as the PS1, PS2 and PS3 antigens. PS1 and PS2 antigens were defined by their preferential binding to cells of the dorsal and ventral compartments respectively. PS3 antigens appeared to show a common determinant with both PS1 and PS2. Further analysis of these antigens revealed that they are homologues of the vertebrate integrins (Bogaert et al 1987; Leptin et al 1987).

Integrins are a family of integral membrane glycoproteins that are thought to mediate cell-ECM and cell-cell interactions by acting as receptors for ECM ligands (Hynes 1987). Figure 6.9 shows an idealised model of an integrin molecule. Integrins consist of two subunits, α and β which on their extracellular side have a binding site for ECM ligands often involving the tripeptide Arg-Gly-Asp referred to as RGD (Ruoslahti and Pierschbacher 1987). On their cytoplasmic side integrins have been found to co-localise with various components of the cytoskeleton notably talin (Horwitz 1986), actin and α actinin (Burrige 1988).

The PS1 and PS2 antigens share a common β subunit which is recognised by PS3 antibodies and each have unique α subunits. The common β subunit is encoded by the gene *myspheroid* (Mackrell et al 1988; Leptin et al 1989) and the PS2 α subunit by the *inflated* locus (Wilcox et al 1989). This

genetic knowledge of the PS integrins has enabled a functional analysis to be undertaken using viable mutant alleles and mosaic analysis of lethal alleles. PS integrins appear to have widespread roles in embryonic tissues, as might be expected from molecules involved in cell adhesion (Zusman et al 1990; Leptin et al 1989). PS integrins seem to be required for proper wing development, lack of these molecules leads to blistering of the apposing dorsal and ventral wing epithelia (Wilcox et al 1989; Brower and Jaffe 1989).

Imaginal disc cells in culture show an interesting pattern of staining with PS antibodies. Immunocytochemistry reveals that staining with PS2 antibody appears to correlate with the aggregation that is seen in culture. From the results it appeared that staining corresponded with cell-cell contact, cells in aggregates showed heavy staining whereas single cells often nearby these aggregates failed to stain. Small groups of cells often showed patterns of staining restricted to areas of cell-cell contact.

As already mentioned PS antigens were originally identified as antigens that showed spatially restricted expression patterns on mature wing imaginal discs. Thus, not all the cells of a disc were stained by all the different antibodies. In the late third instar disc from which these cell lines were derived the pattern of PS1 and PS2 antigen staining is related to the dorsoventral lineage restriction (Brower et al 1985). PS1 antibodies stain dorsal cells and PS2 antibodies ventral cells. Therefore you might expect that ordinary uncloned lines from these discs would be heterogeneous in their PS antigen staining and that cloned

lines would show a more homogeneous but split phenotype. However all the lines that were tested proved positive for both PS1 and PS2 suggesting that the cells had lost their identity as either dorsal or ventral. The dorsoventral lineage restriction pattern of PS antigen staining is not apparent throughout the whole development of the wing disc (Brower et al 1985). It only appears during the third instar and is approximately coincident with the appearance of a zone of reduced cell division along the wing margin separating the dorsal and ventral areas (O'Brochta and Bryant 1985). Therefore perhaps in culture where no such area separates the dorsal and ventral cells such a distinction between dorsal and ventral becomes less valid for the cells.

The correlation between PS antigen expression and aggregation that was seen with immunocytochemistry suggested that PS integrins might have some role to play in aggregation. From immunocytochemistry it seems clear that aggregated cells express more PS integrin than single cells. In order to further analyse this correlation more quantitative methods were used to look at levels of antigen expressed on cells grown at different densities. It was hoped that this would provide a clearer understanding of the role of cell-cell contact in PS integrin expression. However the results were somewhat contradictory. Western blot assays and densitometry of those blots seemed to show a dramatic increases in integrin expression with increasing cell density. These results appeared to confirm the results achieved with immunocytochemistry.

However ELISA assays contradicted these results. The reasons for these inconsistencies are not clear. One possibility is that it is due to the way the samples were prepared. In the ELISA samples cells were stained directly. Antibodies against PS integrins were incubated and allowed to react directly with PS integrin molecules available for binding on the cell surface only. In the Western blotting samples the cells were lysed and the proteins denatured by SDS, and in these samples all of the antigen will be available for binding including that present on the cell surface and also that present in any intracellular pool. In Western analysis all the cellular PS integrin was being measured and not just cell surface integrin, some studies suggest that as much as 50-90% of integrin molecules may exist in an intracellular pool rather than as cell surface molecules (Akiyama et al 1990). In the ELISA samples it is also possible that some of the antigen present on the cell surface may not be available for binding due to masking of the active site by ECM material or other cell surface molecules. However this problem is the same for the results from immunocytochemistry where interactions could possibly hamper binding but the results suggest that it is not much of a problem. In the Western analysis any such interaction will be broken up by SDS denaturation.

Another possible problem in interpreting this quantitative data is turnover. Integrin molecules present on the surface of cells are in a constant state of turnover as are all other cellular proteins. Turnover means that proteins are continually being manufactured, integrated into the

cellular membrane and then degraded. For transmembrane molecules such as these integrin molecules this natural turnover could be disrupted if the molecules are stabilised by binding to ECM molecules in the immediate extracellular environment and also to molecules immobilised on neighbouring cell membranes. This stabilisation would be more prominent in cells in aggregates where interactions between cells and accumulation of ECM material are more prevalent. This could account for some of the observed increased staining seen in aggregates. Turnover of integrin molecules could better be analysed by pulse chase labelling with radioactive methionine to label newly synthesised proteins. This could more reliably determine if cell-cell contact was inducing expression of PS integrins.

Immunocytochemistry of aggregating disc cells suggests that increased cell-cell contact in aggregates results in a higher expression of PS integrins. Indeed it suggests that single cells do not express integrin but that only cells in aggregates do. This leads to the conclusion that cell-cell contact induces expression of integrin. More quantitative analysis of integrin expression in relation to increased cell density provide more contradictory results. These results could in turn be affected by such mechanisms as turnover. So perhaps it is a bit premature to suggest that increased cell-cell contact induces expression of integrins, but that there does seem to be some correlation of PS integrin staining and aggregation.

As already mentioned, aggregates of cells showed heavy diffuse staining with PS antibodies whereas single cells

lacked any staining. Besides single cells and large multicellular aggregates there were other groups of cells in culture. These consisted of small groups of relatively few cells which had joined up together but had not yet got to the stage of an aggregation. These more isolated groups of cells showed staining at regions of cell-cell contact very reminiscent of the type of staining that is seen with cadherin adhesion molecules.

Cadherins are a family of glycoproteins involved in Ca^{2+} dependent cell-cell adhesion (Takeichi 1988), whereas integrins are more generally thought of as molecules mediating cell-ECM interactions. The results with PS integrins suggest that they are involved in cell-cell adhesion in *Drosophila* development. PS integrins were originally identified as most homologous to the vertebrate fibronectin receptor family (Leptin et al 1987). This suggests that these receptors should be interacting with ECM molecules containing the RGD recognition sequence that is found in fibronectin and is common to many integrin ligands (Ruoslahti and Piersbacher 1987). Studies looking for PS integrin binding to immobilised RGD peptides failed to show any binding activity (Leptin et al 1987) and as yet no ligands have been found for PS integrins (M. Wilcox pers. comm.).

Not all integrins are restricted to cell-ECM interactions. Integrins are also involved in cell-cell interactions especially in the immune system. These integrins, notably the LFA-1 lymphocyte adhesion receptor are involved in cell-cell adhesive mechanisms between lymphocytes (Hynes 1987; Springer 1990). LFA-1 interacts with two distinct cell surface

receptors, termed intercellular adhesion molecules-1 and 2 (ICAM-1 and -2) to mediate cell-cell adhesion (Makgoba 1988; Dustin 1990). The lymphocyte integrin, VLA-4 acts as both an ECM and a cell receptor, it binds to a molecule called VCAM-1, a member of the immunoglobulin superfamily and also to a distinct domain of fibronectin (Springer 1990). Another lymphocyte integrin VLA-3 has been localised to cell-cell contact sites and so seems to be involved in cell-cell adhesion (Kaufman et al 1989). Therefore integrins can participate in cell-cell adhesive interactions.

Apart from evidence of the role of integrins in cell-cell interactions in the immune system, recently other integrin molecules have also been found to be important in mediating cell-cell adhesion in epithelial cells (Carter et al 1990; Larjava et al 1990). These studies revealed integrin staining at cell-cell contact sites very similar to that seen in imaginal disc cell lines. Supposedly either integrin molecules are interacting directly between adjacent cells or there is an as yet unidentified ligand molecules that can bridge cell-cell interactions. Information from lymphocyte integrins suggests that ligand molecules may be involved.

Therefore, integrin molecules can mediate cell-cell interactions not only in lymphoid cells but also in epithelial cells. In order to try and compare the binding seen with PS integrins disc cells were also stained with antibodies against the *lethal 2 giant larvae (l(2)gl)* protein, which is a putative *Drosophila* cadherin molecule (Klamt et al 1989). This was done to try and compare the PS integrin staining with molecules well characterised as cell-cell adhesion molecules

(Takeichi 1990). However no staining was achieved and there does seem to be some doubt as to the role of *l(2)g1* as a cadherin molecule given the lack of a transmembrane domain (Klamt et al 1989; P. Bryant pers. comm.). Integrin molecules in vertebrates have been found to be involved in cell-cell as well as cell-ECM interactions. The pattern of staining achieved with PS integrins on disc cell lines and the apparent correlation with aggregation suggests that PS integrins do have some role to play in cell-cell adhesion.

In conclusion, disc cells show a characteristic pattern of aggregation in culture, the presence of large numbers of cell processes ramifying through aggregates and also in cells about to aggregate suggests that they have some role to play in bringing about that aggregation. However the maintenance and growth of large multicellular aggregates would require some other processes to be acting in order to retain cells within an aggregate and it would seem that increased cell-cell adhesion is part of that. The expression of a well characterised *Drosophila* adhesion molecule was analysed and found to correlate with the aggregation seen although the exact role of cell-cell contact in inducing expression of PS integrins is not certain. This clearly demonstrates that PS integrins have a direct role to play in cell-cell adhesion, something that has only been inferred from previous studies.

Figure 6.1

Figure 6.1 : Aggregation in wing cell lines.

(a) Cells of line W1, one day after plating out. Scale bar = 50 μm .

(b) W1 cells 2 days after the start of a passage, reaching confluency. Some cells are beginning to pile up. Scale bar = 50 μm .

(c) 4 days after plating out the cells have reached and gone beyond confluency and are beginning to separate out. Scale bar = 50 μm .

(d) 6 days after plating the cells have continued to aggregate into large multicellular aggregates (A) which begin to form connecting strands (S) separated by areas of tissue culture plastic which are relatively free of cells. Scale bar = 50 μm .

(e) These aggregates (A) form connecting strands (S) of cells often suspended above the surface of the plastic. Scale bar = 30 μm .

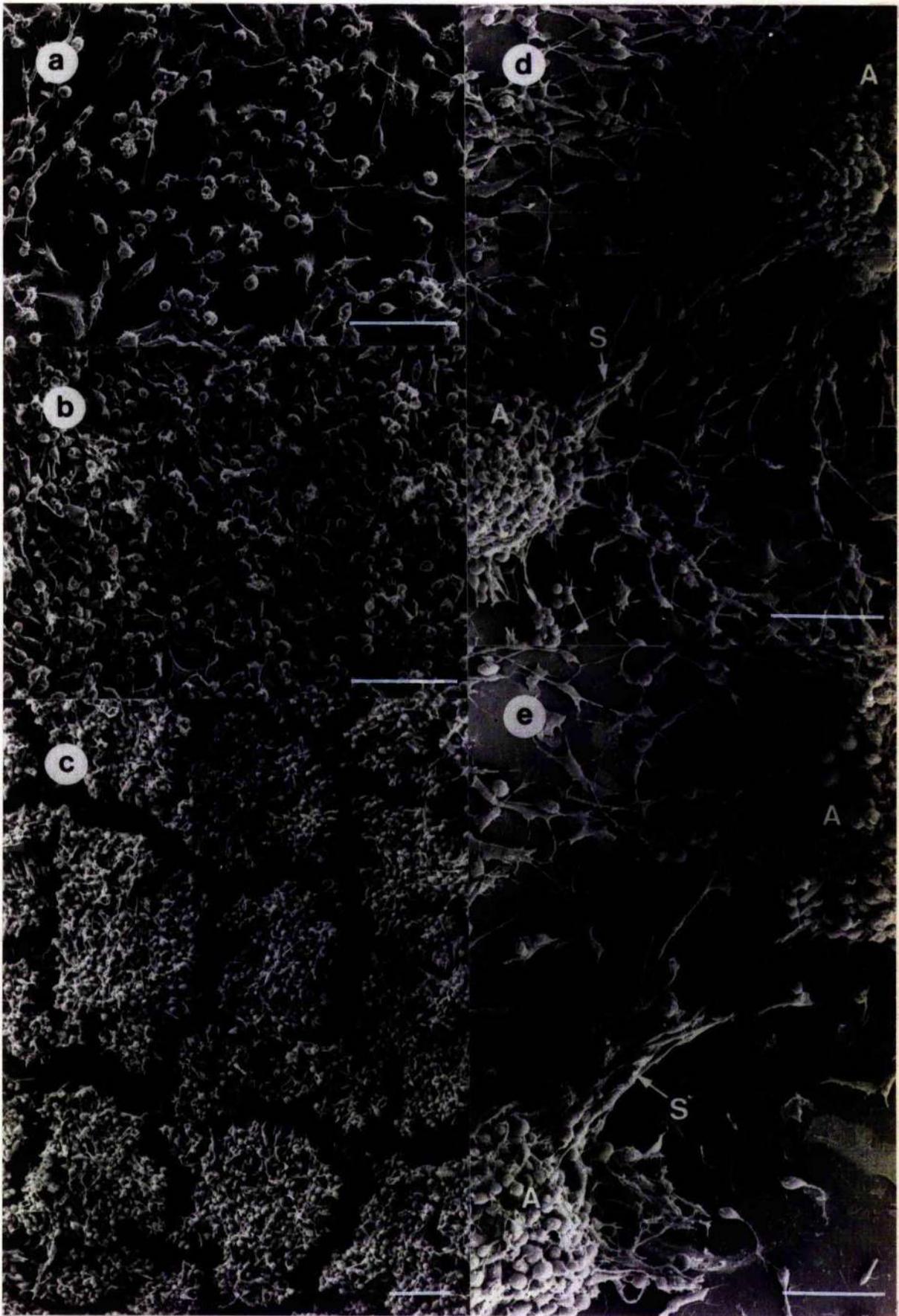


Figure 6.2

Figure 6.2 : The ultrastructure of cells in aggregates.

(a) Viral particles are sometimes found in the cells. In these cells of the wing line W1 viral-like particles (V) are found in the nucleus. Scale bar = 0.5 μm .

(b) Overview of a sectioned aggregate in the leg cell line L1. Numerous cell extensions (CE) can be seen extending through the aggregate. Scale bar = 1 μm .

(c) LS and (d) TS of cell extensions (CE) in aggregates of the wing cell line W1 showing numerous microtubules (MT) within them. In (d) virus-like particles (V) may be present in the cytoplasm of the cell. (c) Scale bar = 0.2 μm ;(d) Scale bar = 0.4 μm .

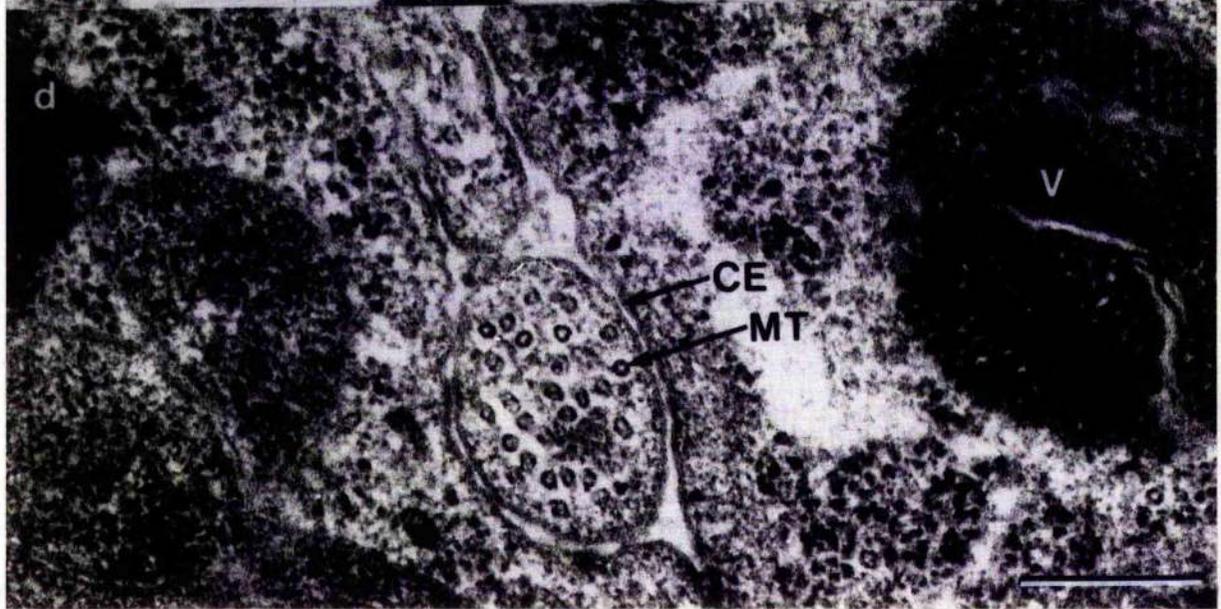
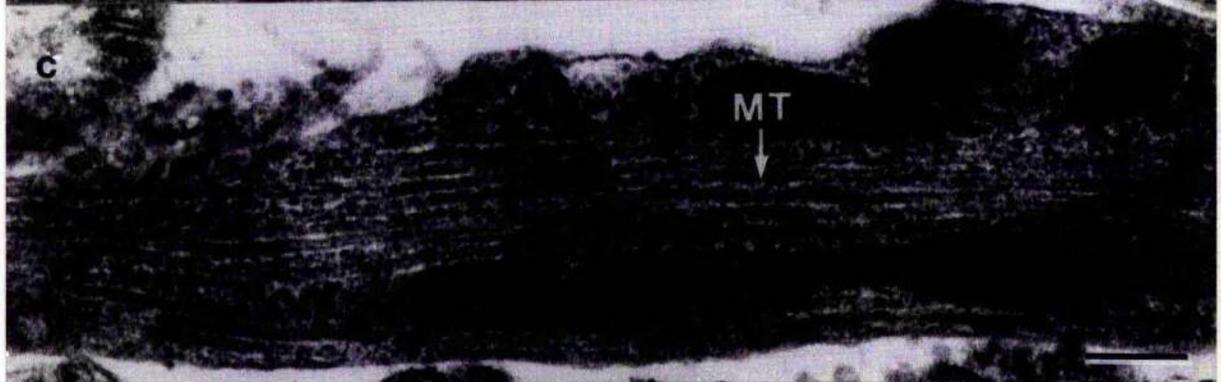
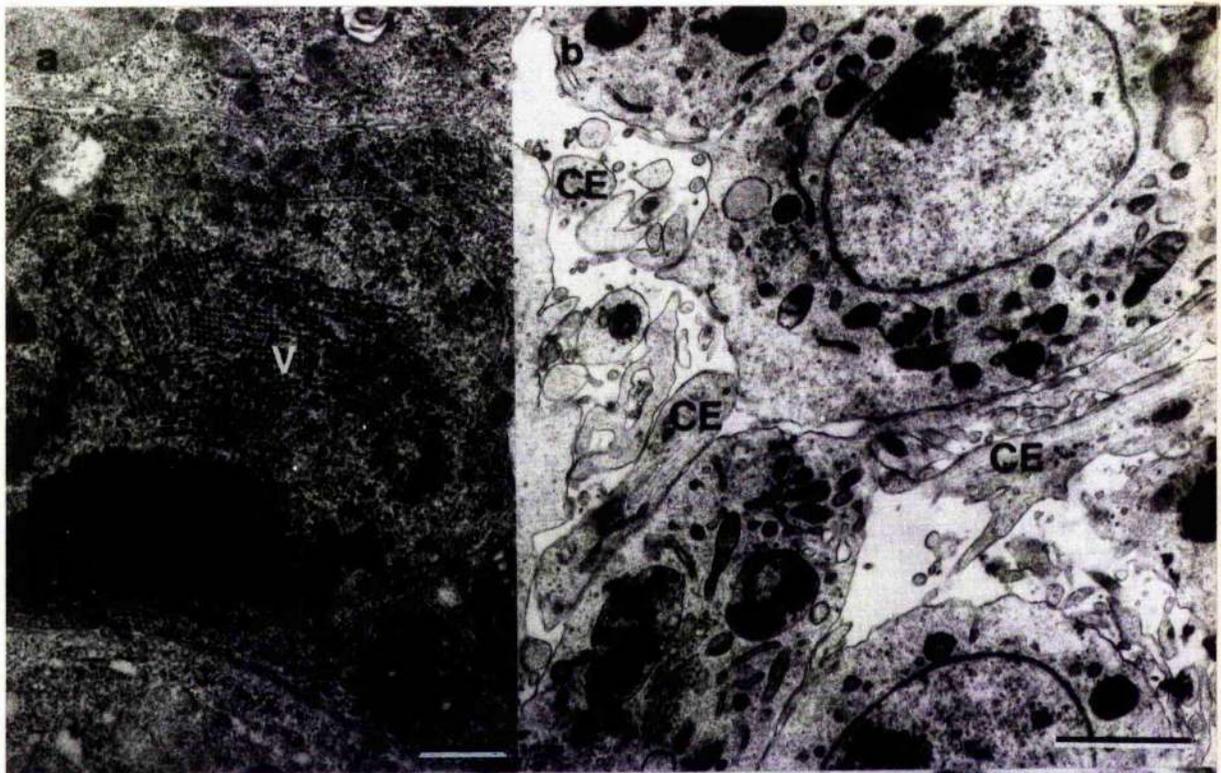


Figure 6.3

Figure 6.3 : Immunofluorescence using anti- α tubulin antibody of cell processes in cloned wing cell lines (a-clone C9; b,c,d-clone 13).

(a) A long cell process (arrowed) 6 days after culture initiation before confluency and aggregation has occurred. Scale bar = 5 μm .

(b) Small group of cells connected by cell processes (arrowed) to a large aggregate (A) 8 days after culture initiation. Scale bar = 20 μm .

(c) Two aggregates joined by a cellular bridge containing a number of cell processes (arrowed) 8 days after culture initiation. Scale bar = 20 μm .

(d) As in (c) two aggregates joined by cellular bridge with other connections radiating outwards. Scale bar = 20 μm .



d



Figure 6.4

Figure 6.4 : The distribution of PS2 antigen (red reaction product) in cells of the cloned wing line Cl.9 (p. 42) at 3 days post passage counterstained with Mayers haematoxylin (blue).

(A) Preferential staining of aggregates (A). Single cells interspersed between the aggregates show little staining.

(B) Two unstained cells (C) in close proximity to an aggregate which expresses PS2 uniformly.

(C) A sheet, (D) a clump and (E) a chain of cells expressing PS2 at points of cell-cell contact only. Bar represents 100 μm in (A), 20 μm in (C) and (D), and 10 μm in (B) and (E).

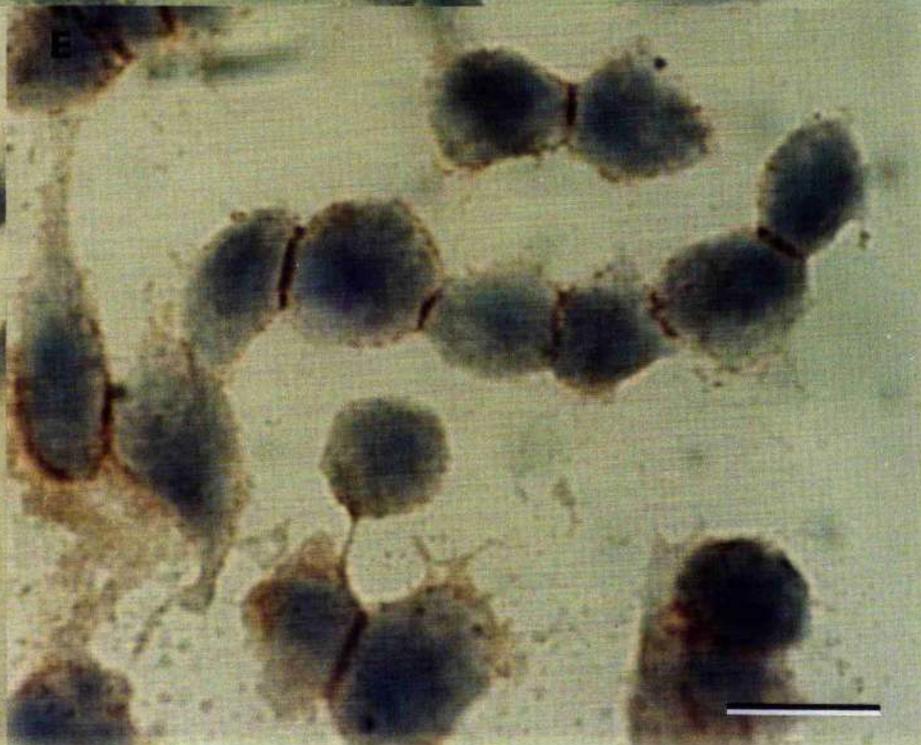
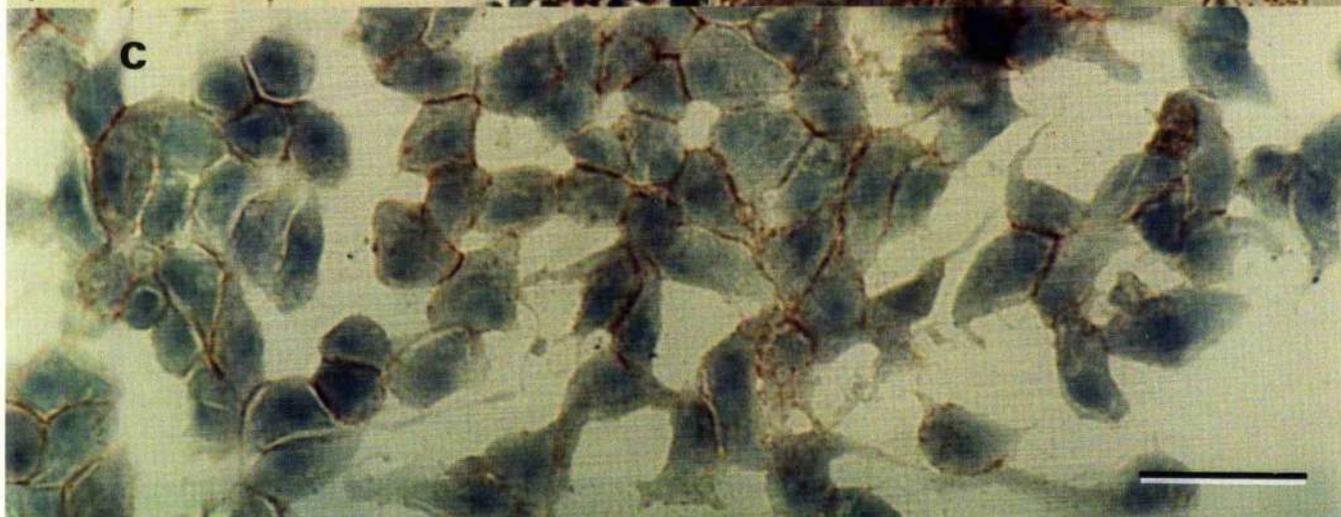
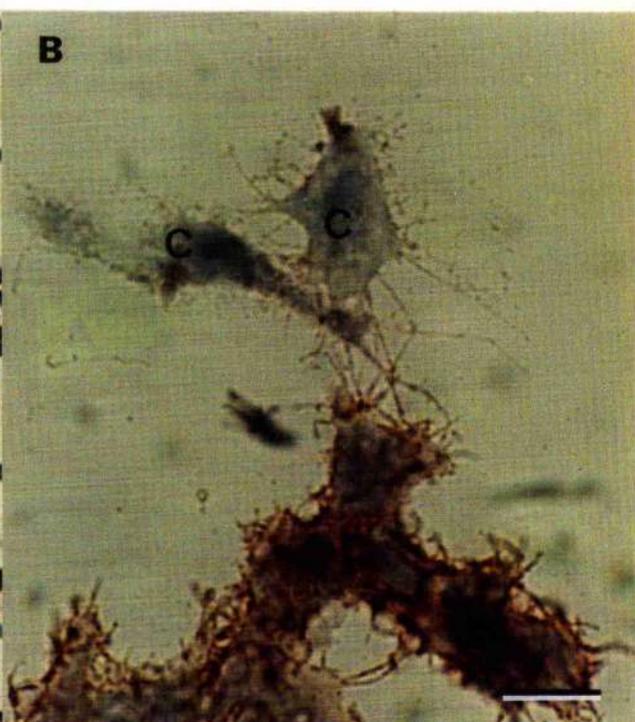
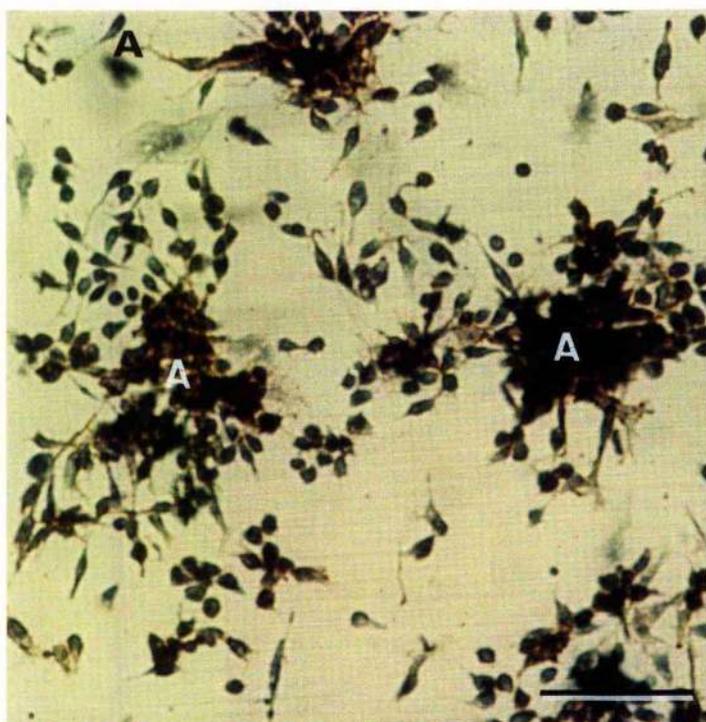


Figure 6.5

Figure 6.5 : Western blot of fractionated cell proteins probed with PS2 antibody. Molecular weight markers (Mr) consist of phosphorylase b at 97.4 kiloDaltons (kD) and catalase at 58.1 kD. Lanes extend from (a)-(f).

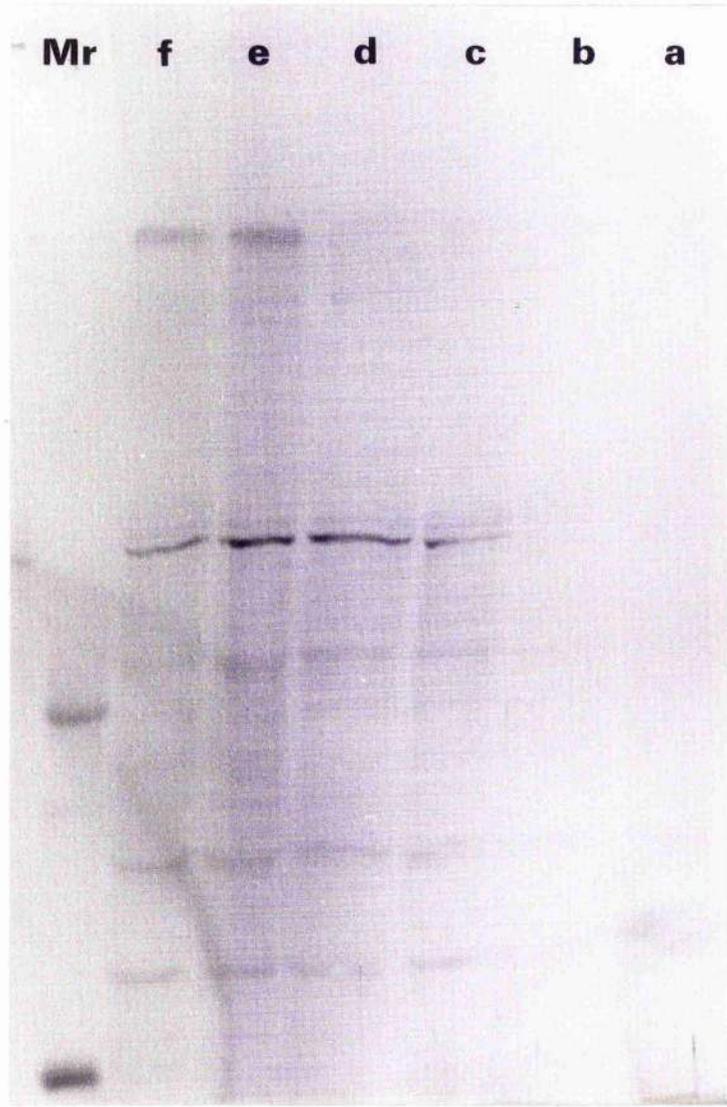
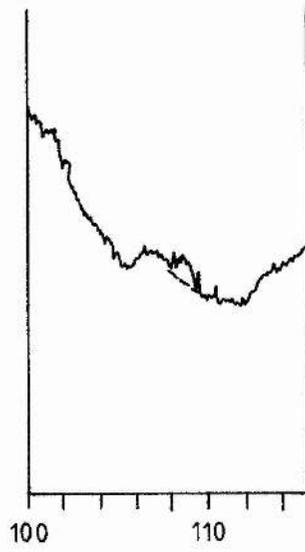


Figure 6.6

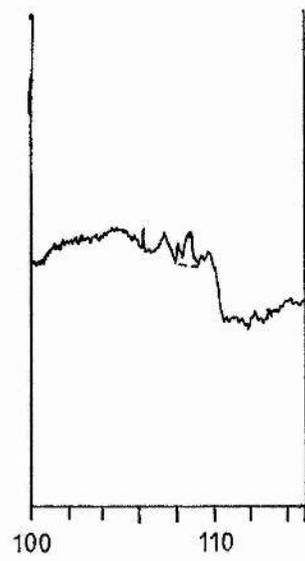
Figure 6.6 : Scanning densitometry analysis of a Western blot of fractionated cell proteins probed with anti-PS2 antibody. The figure for the area underneath the antigen peak was multiplied by the width of the band in the Western blot and divided by the amount of protein that was loaded onto the gel lane. The values for (a)-(f) correspond to the lanes on Figure 6.5.

a.)



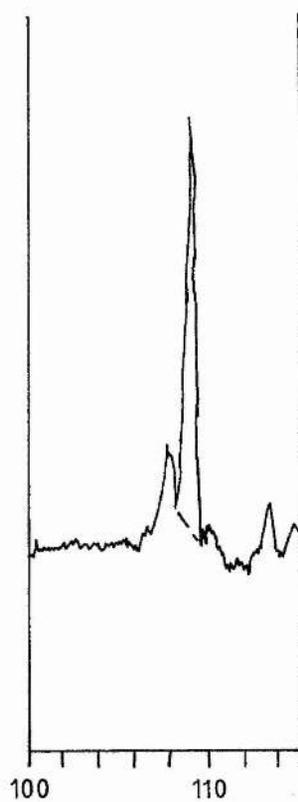
Area = 57.75

b.)



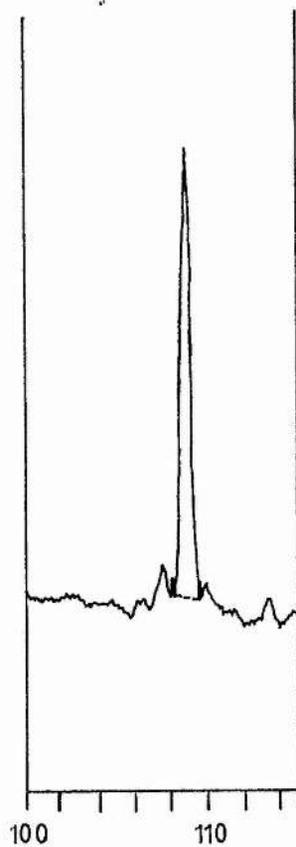
Area = 59.15

c.)



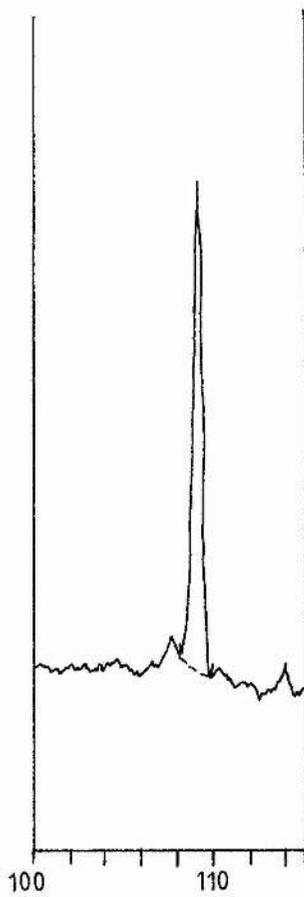
Area = 1322.426

d.)



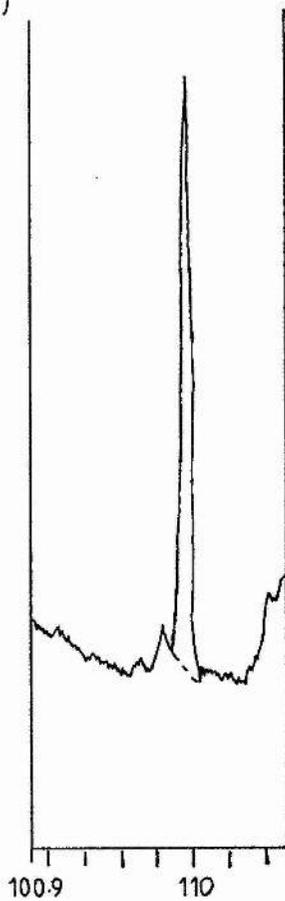
Area = 3174.473

e.)



Area = 3937.68

f.)



Area = 2147.01

Figure 6.7

Figure 6.7 : Graph of values obtained from scanning densitometry analysis of Western blots probed with anti-PS2 antibodies. Values are expressed as the area of the antigen peak multiplied by the width of the band on the blot and divided by the amount of protein that was loaded onto the gel lane. These values are then compared with the seeding densities of the cells as a measure of the cell density achieved.

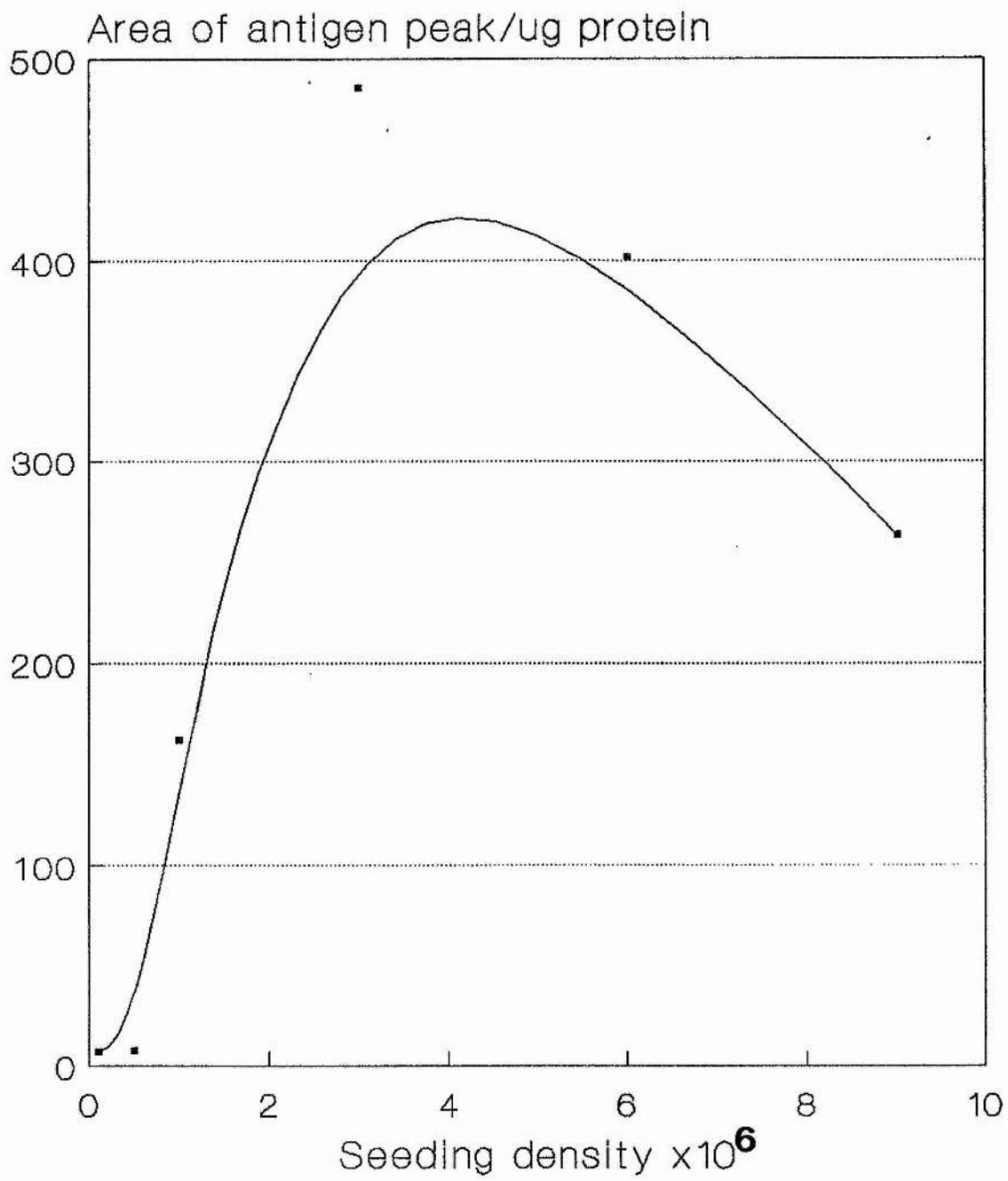


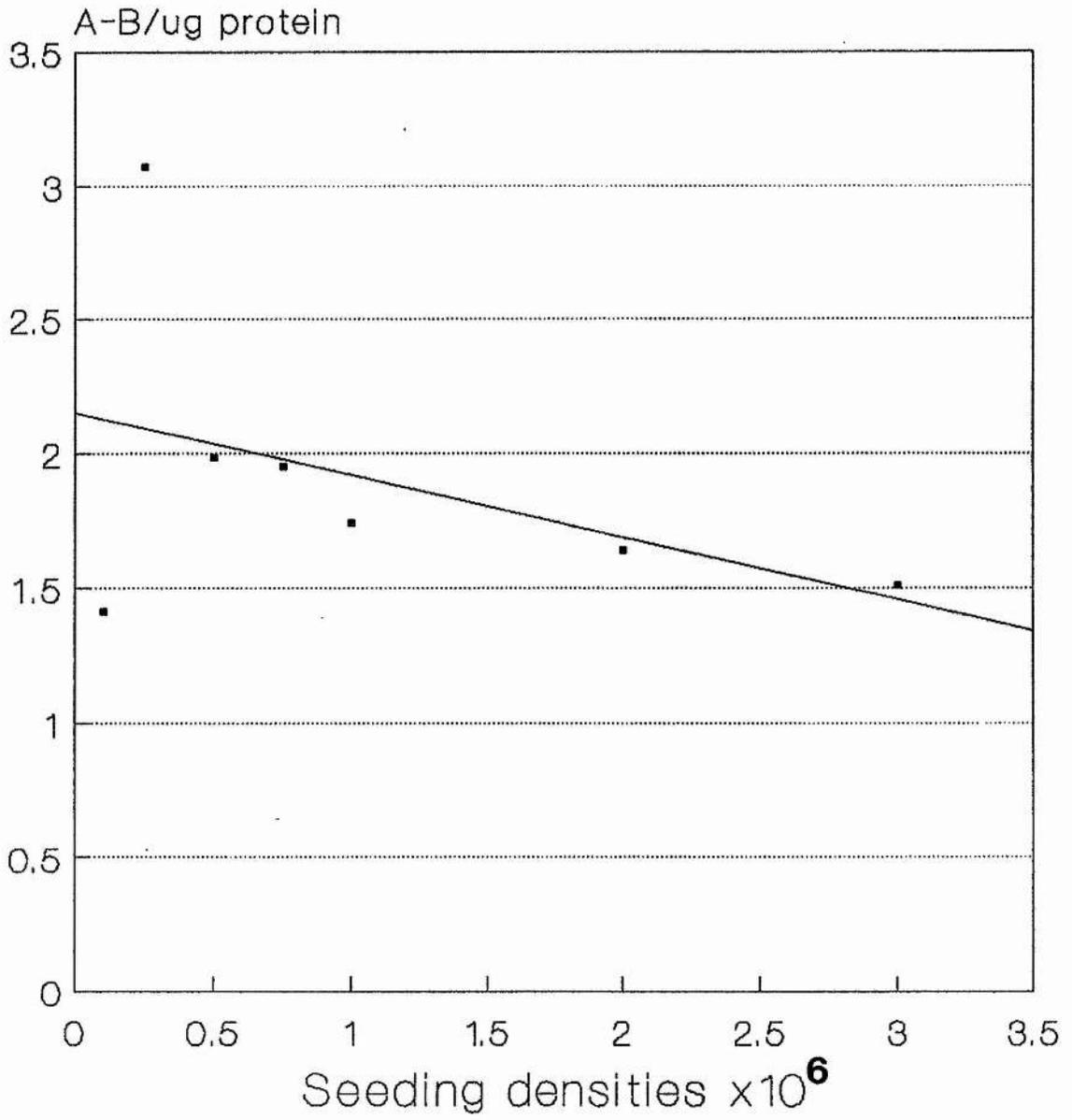
Figure 6.8

Figure 6.8 : Graph of the results from an ELISA analysis of the relationship between levels of PS2 antigen and cell density.

Fig. 6.8.1 : Graph using the level of seeding of the cells at the start of a culture as a measure of the cell density of the culture.

Fig. 6.8.2 : As above but using the cell density at harvest as a measure of the cell density of the culture and giving a figure for the absorbance achieved per cell.

1



2

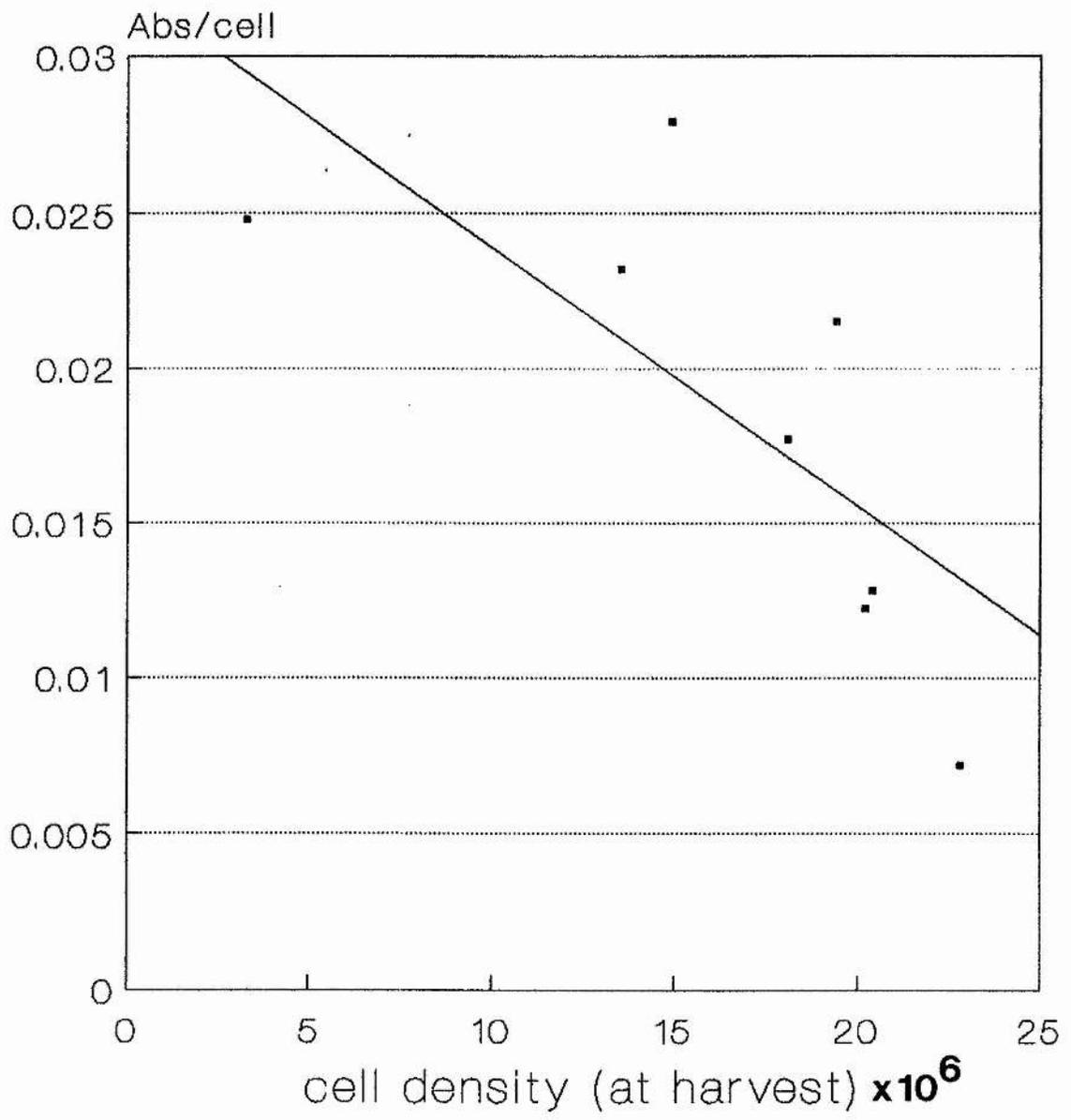


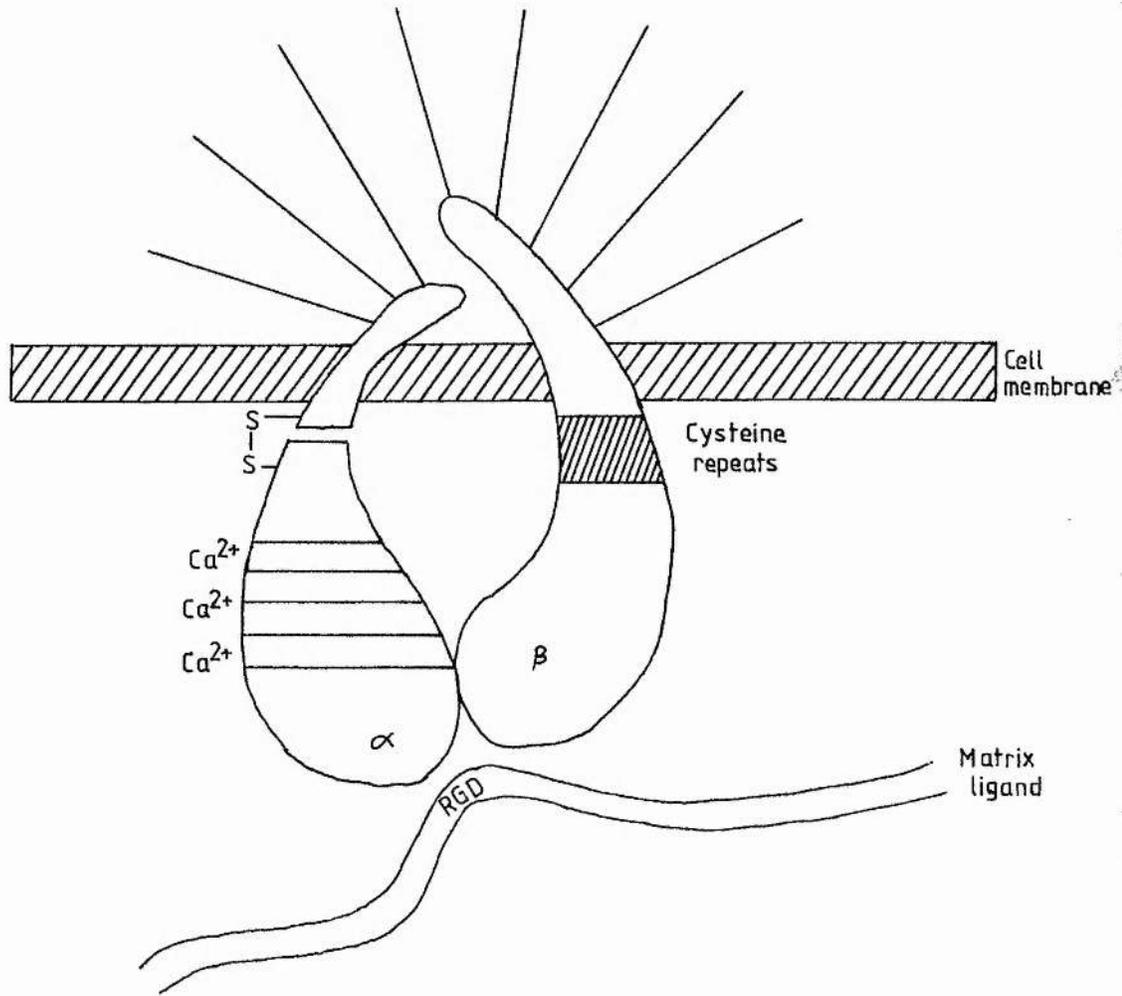
Figure 6.9

Figure 6.9 : Structural model for the integrin subunits.

The model shows an idealised integrin receptor with α and β subunits. Some α subunits interact with Ca^{2+} . The cytoplasmic part of the integrin receptor interacts with components of the cytoskeleton. Some but not all integrins react with the tripeptide RGD found in the matrix ligands such as fibronectin. After Dedhar (1990).

Cytoskeleton

(Talin, Vinculin, α Actinin, Actin)



Chapter 7

Reaggregation and sorting out in imaginal disc cell lines

Reaggregation and sorting out in imaginal disc cell lines

Introduction

When vertebrate embryonic cells are dissociated and allowed to reaggregate it is found that similar cells preferentially adhere to each other, that is they sort out, suggesting that differently determined cells have specific recognition differences (Moscona 1962; Steinberg 1970). Suggestions have been made that cells from imaginal discs that are determined to form different structures (e.g. wing and leg) also sort out (Nöthiger 1964; García-Bellido 1966). This led to the suggestion that differences in the state of determination of imaginal disc cells from different discs are reflected in different cell-cell recognition specificities (García-Bellido 1966).

In the experiments examining sorting out in imaginal disc cells, discs are dissociated either by using enzymes or mechanical shear or more commonly a mixture of both. Combinations of differently determined cells, some carrying cuticular marker mutations are then mixed, pelleted and implanted into a larva about to undergo metamorphosis. Implants can also be placed into adult females to enable a period of growth to occur *in vivo* before recovery and re-implantation into a metamorphosing larva. Under the influence of the moulting hormones *in vivo*, the implants differentiate into cuticular structures which can then be scored to determine their origin using the cuticular marker mutations. This results either in a mosaic of cells which is interpreted

as evidence that no sorting out has occurred or as patches of homotypic cells indicating that sorting out has taken place.

However the results from such experiments have been criticised for a number of reasons (Bryant 1978; Fehon et al 1987). One of the first, results from the fact that imaginal disc tissue is extremely difficult to dissociate. Thus in order to obtain a single cell suspension rather drastic dissociation procedures have to be employed. Indeed there is often a compromise to be struck between complete dissociation and viability of the dissociated single cells. The reason that a single cell suspension is so important for these sorting out experiments is due to the way that sorting out is scored, patches of like cells are interpreted as evidence of sorting out having occurred, where these might have just been the result of undissociated clumps of cells. This problem is exacerbated by the fact that the cell implants have to be cultured *in vivo* in order to allow differentiation to occur so that the cuticular markers can be scored. This results in a period of culture where nothing is known about what is happening in the cell implant. The technique of *in vivo* culture could also play a part in producing an appearance of sorting out. Poodry et al (1971) found that after culturing in adult abdomens for 3 to 5 days and transferring to a metamorphosing larva an island containing up to several hundred cells of homotypic tissue could result from a cell clump of 6 or less. As already mentioned dissociation procedures are often drastic and cell yield is low. Poodry et al (1971) found that many of the resulting single cells were dead based on uptake of dyes, also evidence from setting up

imaginal disc cell cultures shows that single cells produced during dissociation often die 1 or 2 days after culture initiation (Currie *et al* 1988; Cullen and Milner 1991). Thus these culturing techniques may well favour clumps of cells which survive to produce most of the final cuticular product which is interpreted as evidence of sorting out; indicating that cell division may play a part in any observed sorting out. Cell yields from these procedures are often very low so that the final product may only represent a small subset from the total population of the imaginal disc.

Cell aggregates producing an organised pattern which would be interpreted as evidence of sorting out could also be due to a process of pattern respecification caused by interactions between cells, brought together in aggregates (Ursprung and Hadorn 1962; Bryant 1978). Thus the fact that sorting out has been observed in disc cell aggregates has to be tempered with the fact that incompleteness of dissociation and cell division could have resulted in the appearance of sorting out.

The inadequacies of these experimental procedures led some authors to use other methods that did not require a long period of *in vivo* culture to study sorting out. Fehon and Schubiger (1985) used a cytological marker for undifferentiated cells and a relatively short period of *in vivo* culture. Fausto-Sterling and Hsieh (1987) dispensed with the need for any period of *in vivo* culture by testing for sorting out by allowing reaggregation in short term roller culture. These studies found that sorting out did occur in certain combinations of disc cells, notably between wing and

leg. These methods have gone a long way to remedy some of the previous deficiencies in experimental methods that were used to study sorting out in imaginal disc cells. However there are some problems involved in these new methods. The main difficulty is that imaginal disc cells are connected by tight intercellular junctions which are very difficult to disrupt to produce a single cell suspension without drastic dissociation procedures which lead to reduced cell viability. Fehon and Schubiger (1985) used collagenase followed by exposure to citric acid at a pH below 3 with pipetting to dissociate the cells, they reported a 65% cell yield with 90% single viable cells. Fausto-Sterling and Hsieh (1987) used collagenase followed by a brief exposure to trypsin, they also found that even a very brief exposure to citric acid resulted in a failure of the cells to reaggregate *in vitro*. They failed to give any figures for percentage of cell yields, viability and degree of dissociation using this method although I would suspect that the cells must have had to be subjected to quite vigorous mechanical shear to achieve any sort of single cell suspension.

Clearly the dissociation procedures for disrupting disc cells are often quite drastic. These procedures may well leave the cells in a viable state but it is likely that the cell surface is often severely damaged. It has already been mentioned that citric acid treatment prevents disc cells from reaggregating (Fausto-Sterling and Hsieh 1987), obviously affecting cell-cell adhesion molecules on the cell surface. These are the very molecules that are thought to be involved in mediating selective cell adhesion or sorting out (Takeichi

1990). Therefore although these studies have produced fairly consistent results suggesting that sorting out does occur, the experimental methods used mean that there still need to be some reservations about the results.

The ideal method would enable a single cell suspension of disc cells to be produced without extensive enzymatic dissociation and hence destruction of the cell surface. However this is really impractical using imaginal discs *in vivo*, for reasons already mentioned. Clearly the best method would be to use imaginal disc cells grown *in vitro* where a single cell suspension can be produced without too much destruction being wrought and the process of sorting out directly analysed. The aim of the experiments carried out in this chapter is to use the versatility of disc cells *in vitro* to examine this question. As a preliminary, the conditions of reaggregation of a dissociated cell suspension *in vitro* are analysed to optimise the conditions for sorting out assays. Apart from their use in setting up sorting out assays, reaggregation experiments can be used to compare the reaggregating capabilities of imaginal disc cell lines with those of established embryonic cell lines and directly dissociated imaginal disc and embryonic cells.

Methods

For both the reaggregation experiments and the sorting out assays disc cells were allowed to associate in roller culture according to Fausto-Sterling and Hsieh (1983). Cells were placed in a 10 ml siliconised centrifuge tube and rotated

at 100 rpm at an angle just off the horizontal. This allowed free association of the cells and prevented any cells adhering to the sides of the centrifuge tube.

Reaggregation

Cells were grown for 3 or 4 days so that they were in active growth and had not formed the large multicellular aggregates that are common in later stages. These cells were harvested by pipetting, washed in D=, centrifuged down and resuspended in 1 ml of D=. At this point a haemocytometer count was taken and the viability of the cells measured by virtue of the ability of the cells to exclude the vital dye trypan blue. An aliquot of 4×10^6 cells was taken and added to 1 ml of various treatment solutions, the cells centrifuged and resuspended in 1 ml of their treatment solutions. The tubes were then incubated on a roller and aliquots taken at 30 mins, 1 hour and 2 hour intervals and the presence of single cells counted with a haemocytometer. Results were expressed as percentages of single cells at various time periods compared to the numbers of single cells present at the start.

The amount of dissociation and degree of viability of the dissociated cells varied according to which cell lines were used and at which stage of culture the cells were. In general, cloned cell lines were easily dissociated and produced a single cell suspension with a very high degree of viability. For example, from one experiment a wing cell clone (Cl.7) produced a 90% dissociation into single cells, the rest being mostly 2 or 3 cell aggregates and on the basis of trypan

blue dye exclusion were 97% viable. Cell lines that had lower passage numbers, that is they had only recently been established were somewhat harder to dissociate. In one example an uncloned leg line L1 27D6, produced a 69 % dissociation into single cells and of these 72 % proved to be viable.

Cell sorting assays

Cell sorting assays using freshly dissociated imaginal disc cells have often relied on cuticular markers to differentiate groups of cells in any given combinations (García-Bellido 1966). These markers needed a period of *in vivo* culture in a metamorphosing larva to enable them to be scored. Other studies (Fehon and Schubiger 1985; Fausto-Sterling and Hsieh 1987) have used cytological markers so that different cells can be differentiated. These have often involved null mutations in common enzymes which can be stained histochemically e.g. acid phosphatase (AcpH) and succinate dehydrogenase (sdh). This approach was tried with disc cell lines by trying to initiate a cell line from discs of the AcpH null mutant AcpHⁿ⁻¹¹. However none of the cultures proved successful and so other procedures had to be used to mark the cells.

The most obvious markers to use for these sort of experiments with cell lines were fluorescent markers as used in one study of the adhesion of *Drosophila* embryonic cells (Gratecos et al 1990). A large number of fluorescent markers were tried to test for their effects on the cells' viability and also to test whether or not they were retained by the cell

during incubation and did not leach out. In the end the fluorochrome Rhodamine 123 (R.123) was used which had been used previously for marking *Drosophila* cells in another adhesion assay (Gauger et al 1985).

Cells were treated as per the reaggregation experiments. Cells were harvested by pipetting, washed in D= and incubated for 1 hour at room temperature on the roller in R.123 at a concentration of 100 $\mu\text{g/ml}$ in D= in a siliconised centrifuge tube which was covered in aluminium foil. After incubation the cells were washed 3 or 4 times with fresh D=, resuspended and a cell count taken with a haemocytometer. These labelled cells were then taken and mixed in various combinations at a cell concentration of 4×10^6 cells/ml in 2 mls of fresh medium. The incubation mixtures of cells were put in a siliconised centrifuge tube, surrounded by foil and placed on the roller. Aliquots were taken at 2 hours and 18 hours. The resulting cell clumps were assayed by taking an aliquot from the incubation mixture, which was then placed under a coverslip and viewed with a Leitz fluorescent microscope. The numbers of labelled and unlabelled cells were counted.

Results

Reaggregation

The cells were allowed to reaggregate in fresh MM3 medium in roller culture. Reaggregation occurred very quickly as can be seen from Figure 7.1, which illustrates the process of reaggregation over a 2 hour period. At the end of this time, most of the cells have aggregated into very large

agglomerations. As well as allowing the cells to reaggregate in fresh MM3 medium, cells were also allowed to reaggregate in a number of other solutions. These included PBS, D= and D= with 2 mM EDTA. These solutions were chosen to test to what extent these cells were dependent on Ca^{2+} for adhesion. PBS contains both Ca^{2+} and Mg^{2+} ions whereas D= which is similar to PBS is devoid of both of these, EDTA is a chelating agent which absorbs divalent ions such as Ca^{2+} and Mg^{2+} . The amount of reaggregation in these various solutions was compared by counting the numbers of single cells at different time intervals and expressing the results as a percentage of the numbers of single cells present at the beginning.

To begin with the degree of reaggregation was assessed by using cells that were dissociated by both vigorous pipetting and also trypsinisation. Figures 7.2.1 and 7.2.2 show the results from 2 such reaggregation experiments both using a cloned wing cell line Cl.7, one of which had been trypsinised (Figure 7.2.1). One of the most striking features of both these graphs is the rapid reduction in the numbers of single cells even after only 30 minutes in roller culture. This is more pronounced in the non-trypsinised cell experiment where in fresh medium over 80% of the cells have aggregated within 30 minutes. In both experiments in Figures 7.2.1 and 7.2.2 100% was taken as the number of cells added at the beginning of the incubation period. Later experiments took a separate cell count at time zero. The trypsinised cells never achieved 80% aggregation. Both the graphs show that there appears to be an approximate two way split between the four treatments. It appears that the cells which are allowed to

reaggregate in fresh medium and PBS are the ones which aggregate to the greatest extent and that cells in D= and D= with EDTA aggregate the least. This gives an indication that the presence of Ca^{2+} appears to have some effect on aggregation and that this dependence is not affected dramatically by trypsinisation. The difference between the trypsinised and non-trypsinised cells seems to be in the extent of aggregation and also in producing a somewhat more variable response in the cells that were trypsinised. These factors suggest that trypsinisation has quite an affect on the cells ability to aggregate.

Figures 7.2.3 and 7.2.4 give the results for two reaggregation experiments in different cell lines both of which had been dissociated by pipetting. In these experiments 100% was taken as the numbers of cells present at a separate cell count taken at time zero. Figure 7.2.3 gives the results of a reaggregation assay using L1 27D6, an early passage (p.6) leg cell line. Figure 7.2.4 gives the results from an assay using a cloned wing cell line Cl.8+ at a passage number of 30(20), see appendix 1 for explanation of cell line and passage number terminology. Hence these two results give a picture of two representatives from opposite ends of the spectrum of the existing cell lines, one uncloned early passage and the other cloned and late passage. Again one of the first things that can be seen is the rapid decrease in the numbers of single cells, approximately a 50% reduction in just 30 minutes. As with the last set of results the same two treatments are coming in the bottom two and the top two as before. This consistently suggests that those solutions

containing Ca^{2+} and Mg^{2+} ions lead to the greatest aggregation and those without the lowest. From all the numerical results it appears that PBS is almost as good a media in promoting aggregation as is complete MM3 medium. This is true but when the cell suspension is observed under a microscope a somewhat different picture is seen. Figure 7.1 shows the process of aggregation of cells in fresh complete MM3 medium, the end result after 2 hours in roller culture is the formation of large agglomerations of cells with relatively few single cells or small aggregates. However in PBS the cells do not form such large aggregates but rather a greater number of smaller sized aggregates are formed (Figure 7.3). Thus there is some difference between the two treatments which in the assay procedures used appear quite similar. Obviously there is some factor present in complete MM3 medium that aids the formation of such large aggregates, very probably the presence of 2% FCS and of a 2.5% fly extract.

Imaginal disc cells growing as established cell lines rapidly reaggregate once dissociated. These results give the first indication of this capacity using disc cells that have not been damaged by enzymatic or chemical treatment. The reaggregation that is seen also seems to be influenced by the presence of Ca^{2+} and Mg^{2+} ions. The results from these preliminary experiments gave an indication as to the best conditions for the sorting out experiments that are described below.

Sorting out

Labelled and unlabelled cells were mixed and aliquots of the cell suspension taken after 2 hours reaggregation in roller culture. A drop of this suspension was placed on a microscope slide and a coverslip added. The weight of the coverslip pressed down on the cell aggregates and helped to spread them out so that it was easier to count the individual cells within the aggregates. To prevent the preparations drying out too much and causing the aggregates to break up the slides were sealed with nail varnish.

Aliquots were also taken at 18 hours but by this time the fluorochrome labelling was not so distinctive and so the data which were collected after 18 hours were regarded as less reliable. The choice of cell lines was quite important due to the way sorting out was assayed. The results from the reaggregation experiments indicate that trypsinisation of disc cell lines whilst resulting in 100% single cell dissociation degrades the cell surface and the ability of the cells to reaggregate. So cell lines were chosen that were able to be dissociated by pipetting without leaving any clumps of undissociated cells which in this assay would be assayed as a positive sorting out result. From the reaggregation results it was found that cloned disc cell lines were the easiest to dissociate, uncloned early passage lines were more difficult to dissociate. Therefore it was decided to do a comparative study using just a few cloned cell lines. One of the most interesting ideas about the proposition that imaginal disc cells sort out is the ability for wing and leg cells to recognise each other and separate out. This question was

specifically asked by using the cloned leg line L1A and the cloned wing line Cl.8+. These cells were mixed in different combinations with one or other of the cell lines being labelled with R.123. In order to compare the results from this experiment, two different cloned wing cell lines were also assayed to see if there was any sorting out. This was done to see whether or not any observed sorting out between leg and wing cells was directly as a result of disc specific cell surface properties or whether it was due to general cell adhesion variations of different cell lines. Two cloned wing cell lines were used as a comparison in order to try and decipher if differences in adhesive properties of different cell lines played a part in any observed sorting out.

Table 1 gives the results from sorting out assays using Cl.8+/L1A and Cl.7/C9. Individual observations were taken as a group or clump of cells which were directly in contact and obviously in an aggregate. Then the total number of cells in that aggregate was noted and also the number which were labelled with R.123. Presumably sorting out would manifest itself as aggregates that are predominantly of one cell line or the other, giving largely unlabelled or labelled cells. Obviously some sort of statistical analysis would have to be applied to sift through the mass of data collected on numbers of unlabelled vs. labelled cells. This problem was taken to a lecturer responsible for biological problems at the university statistics department (Mr. Constable). He suggested that the best thing to do would be to use Chi squared (X^2) analysis to test for random association of the cells in the aggregates. One problem that this would not cater for is aggregates that

were homotypic for labelled or unlabelled cells. Such homotypic aggregates would suggest extensive sorting out but one labelled homotypic and one unlabelled homotypic aggregate would cancel each other out. However this did not seem to be the case, generally cells were more mixed, hardly any aggregates were homotypic for labelled or unlabelled cells.

χ^2 test example

Sorting assay between C9 and Cl.7

Total number of cells counted 262

Labelled 101

Unlabelled 161

	+ve	-ve	
Observed (O)	101	161	
Expected (E)	131	131	
O-E	-30	30	
(O-E) ²	900	900	
(O-E) ² /E	6.8	6.8	= χ^2 = 13.6

χ^2 values are given in table 1, averaged out from a number of different individual experiments, the figures in brackets give the standard error for the average. χ^2 values test for random association, values above 1 all suggest non-random association.

Figure 7.4 show aggregates of LIA/Cl.8+ cell lines, one of which had been labelled with R.123. These figures each show two images of the cell clumps, one in phase contrast and the

other of the same clump under fluorescent illumination. In all of the figures one of the cell lines has been labelled with R.123 (see figure legends). All the fluorescent images of the cells were viewed with a red filter block (no.4). These results clearly show labelled and unlabelled cells indicating that at least in the early stages of reaggregation the fluorescent dye does not leach into neighbouring cells. From the photographs, it sometimes appears difficult to discern exactly how many cells are present in a particular clump but by scanning the plane of focus in and out, the numbers of cells were fairly easy to discern. From such images the numbers of labelled and unlabelled cells were counted and the results calculated. Looking at the results from table 1 clearly all the χ^2 values suggest that there is non random association of the cells. This would seem to suggest that all the cell lines were sorting out sometimes even among themselves. The probable reason for this is the fast reaggregation time that the cells have *in vitro*, this can clearly be seen from the reaggregation experiments done earlier. Cells, once dissociated reaggregate very rapidly, from earlier results approximately 50 % of the single cells have aggregated in 30 minutes. Therefore, although every care was taken to prevent cells aggregating before the lines were mixed, for instance cells were pipetted after incubation with fluorochrome and after the various washing procedures and also kept on ice, nevertheless it is very probable that some cells formed small aggregates before mixing was complete. This would have eschewed the results somewhat and lead to a false analysis of the degree of random association. This obviously

makes the analysis less reliable, although every step was taken to try and prevent this problem as mentioned above. In other techniques for measuring sorting out using freshly dissociated disc cells, the method of dissociation clearly disrupted the cell surface and resulted in abnormal reaggregation as mentioned above. With these methods the chance of rapid reaggregation disrupting the results, using an analysis looking at random association would be less, but then again the reaggregation seen in these experiments is abnormal so that any results achieved with such methods must be subject to some doubt. In the analysis used here, it is perhaps better to look at the χ^2 values and compare them between the different combinations, this helps to cut out any background reaggregation that occurs before the cell lines are mixed properly. If this is done there seems to be some differences between χ^2 values obtained for Cl.8+/LIA combinations (average 58) and those obtained between Cl.8+/Cl.8+ (average 12) and C9/Cl.7 values (average 13). This does seem to suggest that there is something going on in Cl.8+/LIA combinations that is not going on in Cl.8+/Cl.8+ or C9/Cl.7 combinations. The suggestion would be that this is due to sorting out taking place between wing and leg cells in these particular cell lines, and not in control combinations using the same cell line or in combinations between different wing cell lines.

Discussion

Reaggregation

From the reaggregation experiments three separate conclusions can be drawn. Firstly the most striking fact is the speed with which the cells aggregate. Within 2 hours nearly all the cells are in very large aggregations. This is in marked contrast to results obtained using embryonic cell lines. Fausto-Sterling *et al* (1985) studied the ability of Schneider line 2 (S2) cells, an embryonic cell line to reaggregate in roller culture, in conditions very similar to those used here. In these assays after 2 hours, the greatest degree of aggregation observed was a 40% reduction in the numbers of single cells, whereas with imaginal disc cell lines about 70% was the equivalent figure. Also, no large aggregates were formed as with disc cells. The degree of aggregation and the formation of large aggregates seen in disc cell lines is similar to the aggregation of freshly dissociated imaginal disc cells *in vitro* (Fausto-Sterling and Hsieh 1983). Freshly dissociated embryonic cells also show a similar pattern of reaggregation as do disc cell lines with the formation of large aggregates of cells even after only 2-3 hours (Gratecos *et al* 1990).

Therefore it seems that imaginal disc cell lines show different aggregating properties from other established *Drosophila* cell lines, and that they are similar to the reaggregating properties of freshly dissociated *Drosophila* cells. This suggests that disc cell lines have similar adhesive properties to freshly dissociated *Drosophila* cells

and that some of the already established embryonic cell lines show different aggregation and adhesive properties (Gratecos et al 1990). This may have important implications given the fact that some cell lines particularly S2 are extensively used to study adhesion either by transfection of putative adhesion molecules or through 20-HE induced modulation of adhesion (Galewsky and Rickoll 1989; Barthalay et al 1990 and Elkins et al 1990).

One of the other facts that emerges from these reaggregation studies is the effect that trypsinisation has on reaggregating ability. From the results using the cloned wing cell line Cl.7, dissociated by both trypsinisation and pipetting it appears that trypsinisation has quite an effect on the degree of reaggregation. Trypsinised cells seem to aggregate to a lesser extent than non-trypsinised cells. Therefore trypsinisation directly affects a cells ability to adhere, presumably by degrading cell surface adhesion molecules. This may have quite an important effect on selective cell-cell adhesion as well as the observed quantitative effect on the degree of adhesion. Therefore sorting out experiments on trypsinised disc cells may well be subject to some error due to the effect of enzymatic treatments on the cell surface.

The final inference from these results is that divalent ions such as Ca^{2+} and Mg^{2+} do appear to be important in disc cell adhesion. The significance of this fact stems from studies of adhesion in vertebrate cells. Cell-cell adhesion in vertebrates can be functionally divided into Ca^{2+} dependent and independent mechanisms (Takeichi 1990). Of the families of

cell adhesion molecules known, it appears that cadherins are the family primarily involved in Ca^{2+} dependent adhesion and indeed were initially identified on that basis (Takeichi 1990). Therefore the observation that Ca^{2+} ions have an effect on the reaggregation potential of dissociated disc cells may imply that this is due to some effect on cadherin molecules present in *Drosophila* cells. Compared to the data gathered about adhesion in vertebrates, comparative studies in invertebrates including *Drosophila* are in their infancy. As already mentioned in a previous chapter the *l(2)gl* gene product has been shown to have some homology to vertebrate cadherins (Klambt et al 1989). However the lack of a transmembrane domain characteristic of cadherins and also the failure of antibodies against *l(2)gl* to stain disc cell lines, given that the gene product appears to be necessary for the proper development of imaginal discs (Bryant 1987) casts some doubt on its role as a cadherin molecule. The gene product of the *fat* locus has been shown to be necessary for the development of imaginal discs, controlling cell proliferation rates and epithelial morphogenesis (Bryant et al 1988). The predicted gene product of *fat* has been identified as having some homology to vertebrate cadherins, possessing 34 domains homologous to the extracellular domains found in vertebrate cadherins (P. Bryant pers. comm.).

Thus two molecules with possible homology to vertebrate cadherins have been identified. Mutations in the genes coding for these molecules cause a degeneration in the normal structure of the disc. Results from the reaggregation assays suggests that Ca^{2+} dependent molecules are important in disc

cell adhesion, suggesting that these molecules are *Drosophila* cadherins. However in *Drosophila* other cell adhesion molecules not belonging to the cadherin family have been found to be Ca^{2+} dependent. The products of two neurogenic loci, *Notch* and *Delta* have been found to promote cell-cell adhesion and specific aggregation in transfected S2 cells and are also Ca^{2+} dependent (Fehon et al 1990). Indeed, the trypsinised cells used in the reaggregation assay still show some dependence on Ca^{2+} for adhesion even though trypsinisation in the absence of Ca^{2+} ions is supposed to degrade cadherin activity (Takeichi 1988).

Therefore it seems that the Ca^{2+} dependency of adhesion in these reaggregation experiments whilst possibly attributable to *Drosophila* cadherin molecules may also be due to other non cadherin cell-cell adhesion molecules. These reaggregation experiments show that disc cell lines still possess a great deal of cell-cell adhesive capability, much greater than other *Drosophila* embryonic cell lines and more reminiscent of freshly dissociated *Drosophila* tissue. This underlines the importance of a defined cell type when studying cell adhesion *in vitro*.

Sorting out

The use of imaginal disc cell lines in sorting out experiments holds out the promise of providing the definitive experiment. Imaginal disc cells are difficult to manipulate *in vivo*, they are held together by tight intercellular junctions and surrounded by a basement membrane. Therefore in order to provide a single cell suspension to conduct mixing and sorting

out experiments rather drastic dissociation procedures have to be performed. The cells are partially digested with trypsin and other enzymes, subjected to acid pH's below 3, all treatments likely to severely damage the cell surface. It is this cell surface that is so important in any cell-cell recognition differences. Therefore the use of imaginal disc cells *in vitro* with their ease of dissociation should provide the ideal starting material for any study of sorting out between differently determined disc cells.

However several problems were encountered when this question was first asked. Chief among these was the problem of how to label cells so that they could be recognised later after any sorting out had taken place. The best method would have been to use one of the several cytological marker mutations that are available, generally null mutations of common cellular enzymes, lack of which can be distinguished histochemically. This was tried with the cell marker mutation $\text{Acp}^{\text{N-11}}$ which was used in a previous sorting out experiment using freshly dissociated disc cells (Fausto-Sterling and Hsieh 1987). However despite a couple of near misses this proved difficult and no cell lines were finally obtained. No other cytological marker mutants were obtained, although markers such as succinate dehydrogenase (sdh) might prove useful. The experience with the Acp mutant strain suggests that the culture initiation techniques still need refinement in order to get mutant cell lines which appear to be more difficult to initiate than wild type cells.

One of the other alternatives would have been to use cuticular markers but again this would have required the

initiation of a new cell line from a mutant strain which had proved difficult before. As well as this, any cuticular marker would have required a period of *in vivo* culture for differentiation to occur. This would have brought back some of the problems encountered in earlier studies of sorting out where nothing is known about what is happening *in vivo*, also no injection set up was running in the lab, which might have been difficult to get started.

In the absence of any genetically marked cells, other methods had to be used. Fluorescent labelling of cells has been used extensively to study sorting out phenomenon (Elkins *et al* 1990; Gratecos *et al* 1990). R.123 was eventually chosen after testing a number of fluorochromes. R.123 had been used previously to label cells in a study of segment specific recognition (Gauger *et al* 1985). The major limitation with this method was the short time that it appeared to specifically label cells. After a while it appeared that some of the fluorochrome was leaching out of the labelled cells and being taken up by the unlabelled cells making it more difficult to discern labelled/unlabelled cells. Cell counts were taken after 18 hours reaggregation *in vitro* but in some of these assays staining had become less distinct so the data obtained was not regarded as so reliable as that obtained earlier. In contrast counts taken after 2 hours reaggregation *in vitro* had very distinct labelled/unlabelled cells. This produced a problem in that sorting out was being measured in a short time scale. What was being measured was initial adhesive and recognition differences between cells and not prolonged sorting out. In one of the nearest comparable studies to that

performed here, Fausto-Sterling and Hsieh (1987) found sorting out in their aggregates only after 18 hours *in vitro* and not after 5 hours. Whilst a short time period for reaggregation is one criticism for this study it must be borne in mind that freshly dissociated disc cells were used in the other study which had been subjected to drastic dissociation procedures, so it might be expected that disc cells would sort out rather quicker.

The results from the reaggregation assays suggest that there is some sorting out occurring in the wing and leg combinations, but not in the control combinations or in wing to wing combinations. These results concur with sorting out experiments carried out with freshly dissociated disc cells which show sorting out between wing and leg disc cells (Fehon and Schubiger 1985; Fausto-Sterling and Hsieh 1987). These results adds to the evidence showing that cells from some discs such as wing and leg have different recognition properties and can sort out from each other. These recognition properties may well be important in the pattern formation interactions that occur in the developing imaginal disc or during pupation when the imaginal discs meet and fuse.

Table 1 Accumulated results from cell sorting assays.

<u>Cell line</u>	<u>T</u>	<u>N</u>	<u>n</u>	<u>x</u>	<u>+ve</u>	<u>-ve</u>	<u>Total</u>	<u>χ^2</u>
LIA ^r /8+	2	4	144	11	456	1308	1645	102 (24)
8 ^r /8+	2	2	84	10	364	506	876	12 (1.5)
8 ^r /LIA	2	2	70	11	254	525	779	48 (1.5)
LIA ^r /8+	18	2	42	9	170	312	482	24 (1.5)
C9 ^r /C1.7	2	2	75	11	371	498	870	8 (2)
C1.7/C9	2	1	25	10	82	182	264	37
C1.7 ^r /C1.7	2	1	60	12	312	408	720	10 (3)
C9 ^r /C1.7	18	1	50	12	282	318	600	2
C1.7 ^r /C9	18	2	92	9	336	426	762	8 (5.9)

T Time of incubation

N Number of experiments

n number of clumps counted

x Average number of cells per clump

+ve Numbers of labelled cells

-ve Numbers of unlabelled cells

Total Total number of cells assayed

χ^2 Chi squared figure. Value below 1 = random association

r Indicates which line was labelled with R.123

Value in bracket represents the standard error of the accumulated χ^2 values.

Figure 7.1

Figure 7.1 : Reaggregation in complete medium using the leg
line L1 26D7. All scale bars = 100 μm .

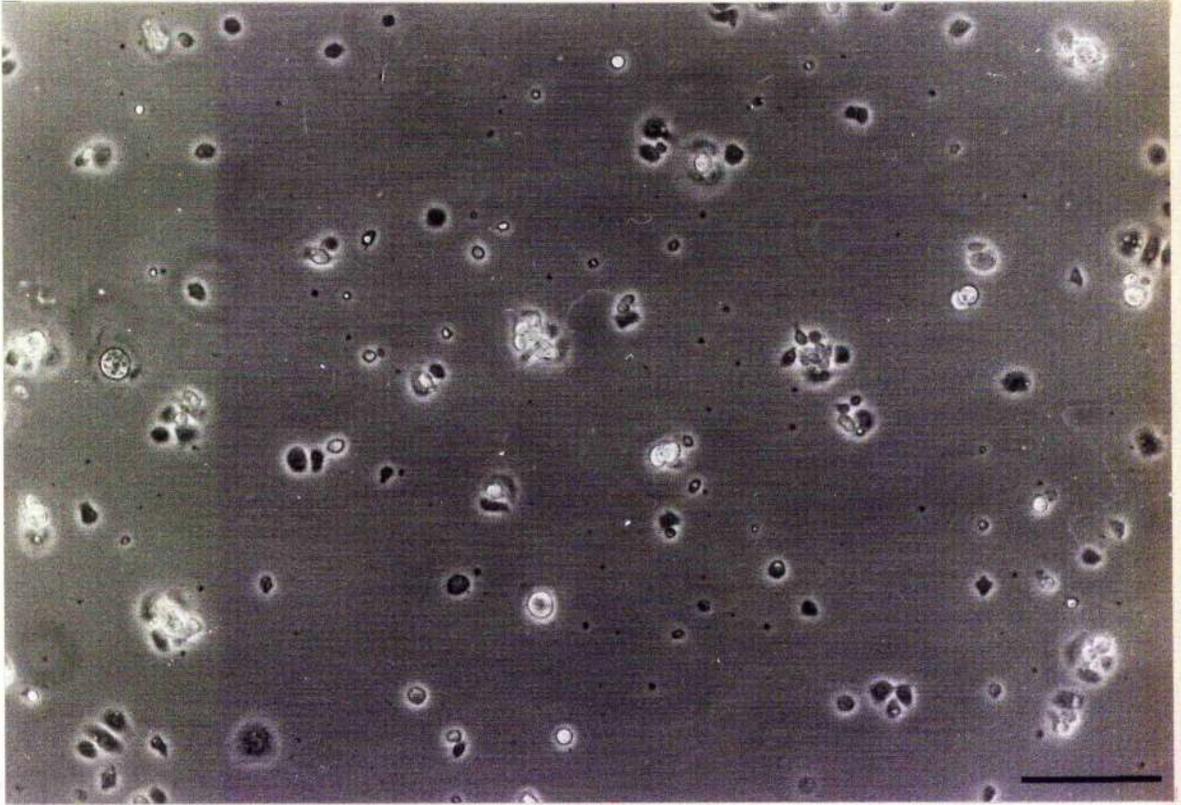
Fig. 7.1.1 : Time zero.

Fig. 7.1.2 : 30 minutes after dissociation.

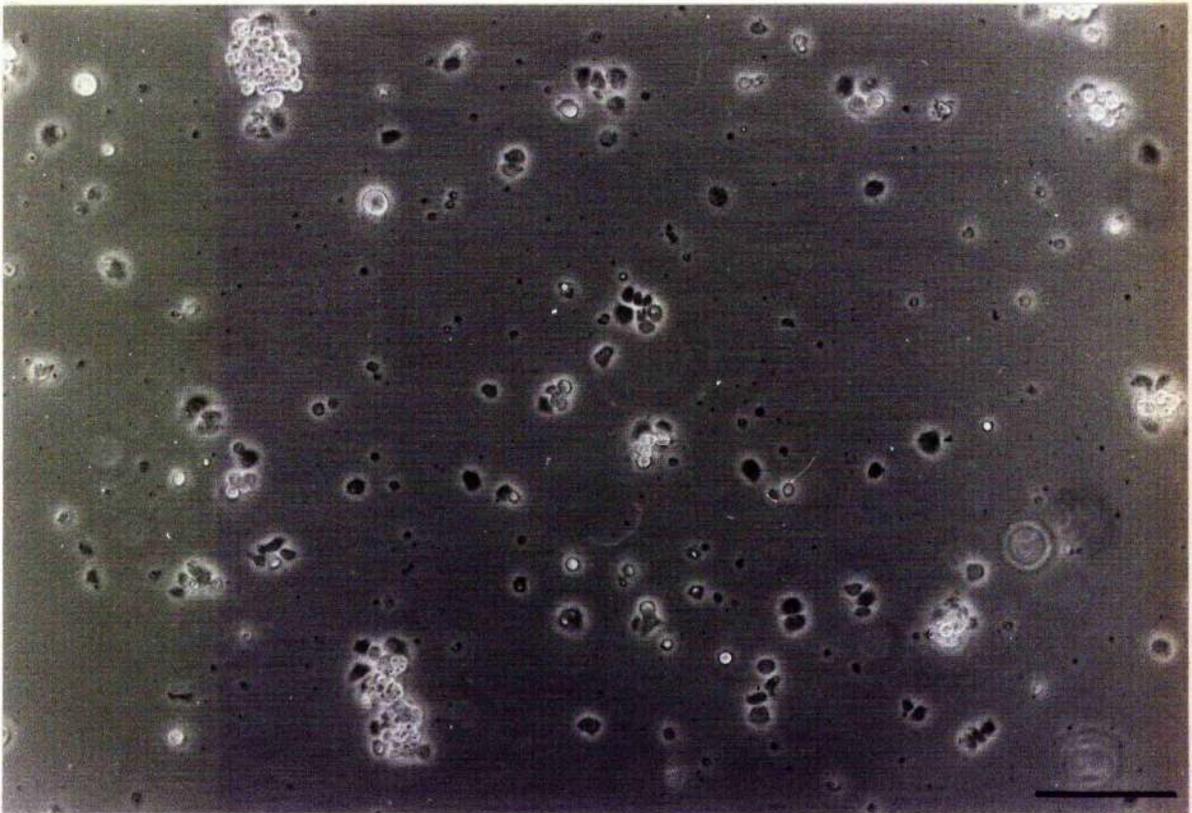
Fig. 7.1.3 : 1 hour after dissociation.

Fig. 7.1.4 : 2 hours after dissociation.

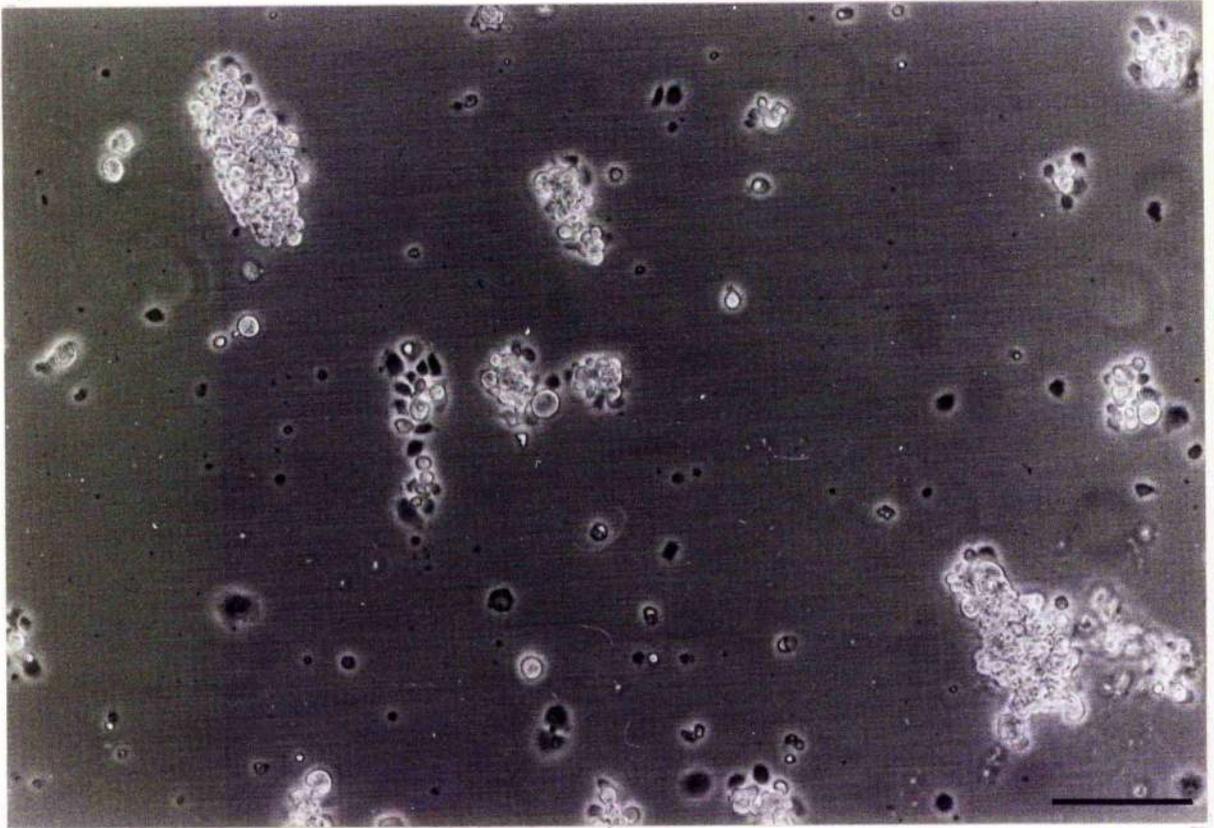
1



2



3



4

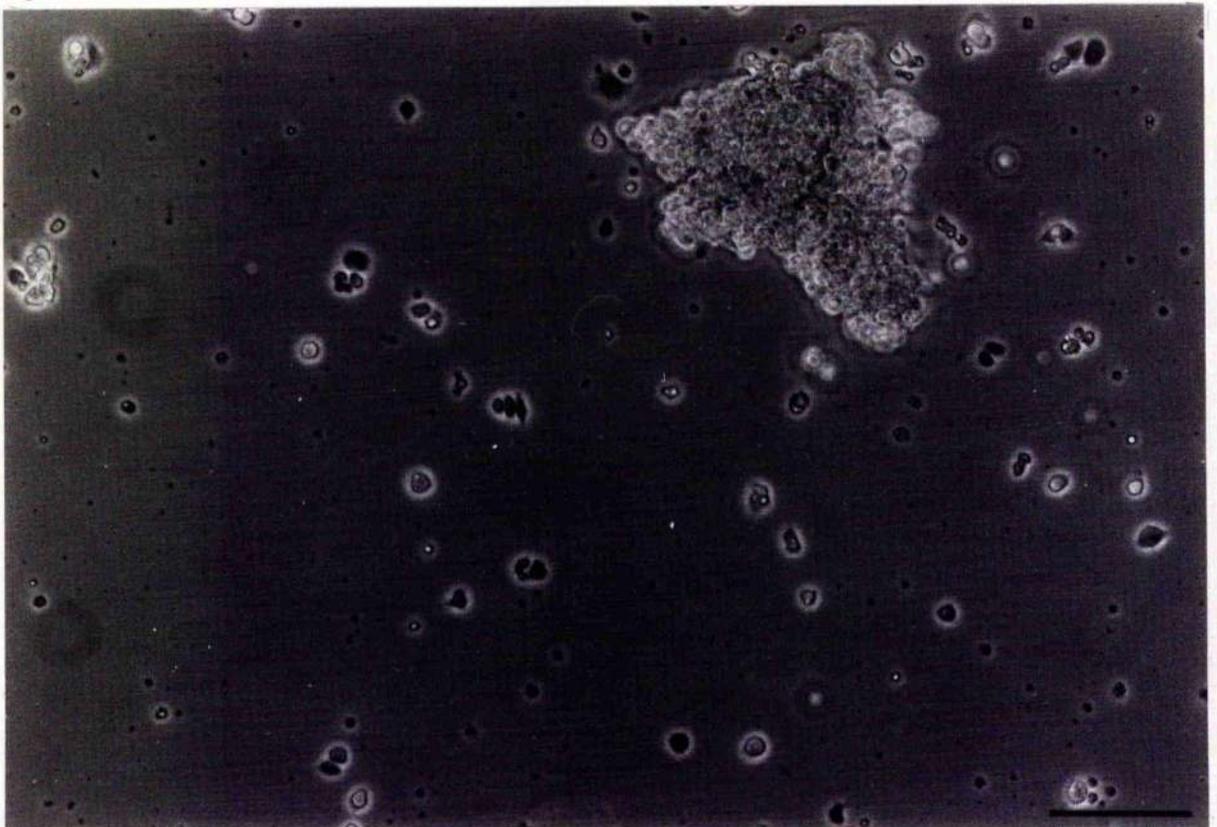


Figure 7.2

Figure 7.2 : Reaggregation experiments with cells in roller culture. Cells were dissociated by pipetting. All error bars represent \pm S.E.

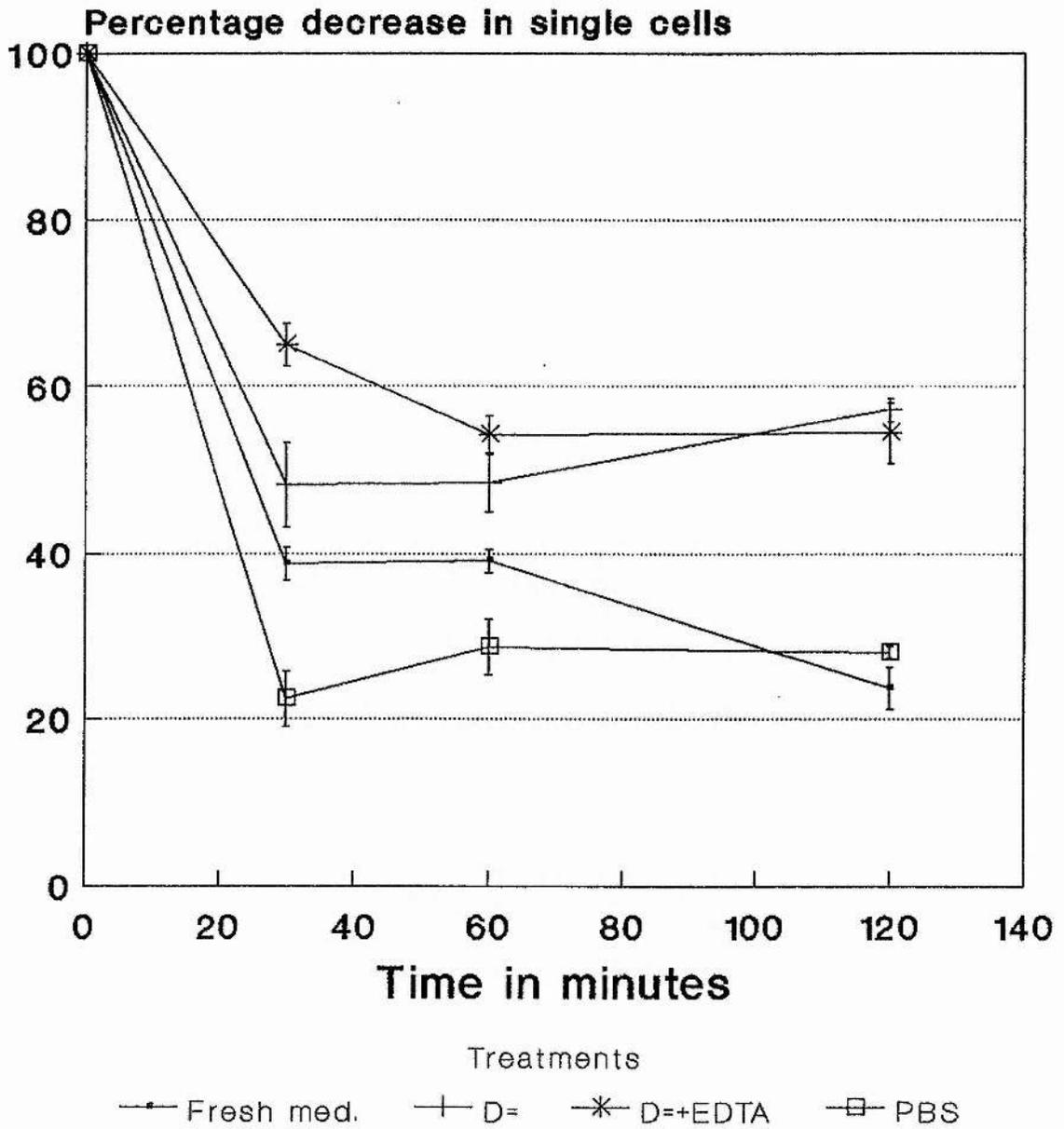
Fig. 7.2.1 : Reaggregation experiment with the cloned wing cell line Cl.7 which had been trypsinised.

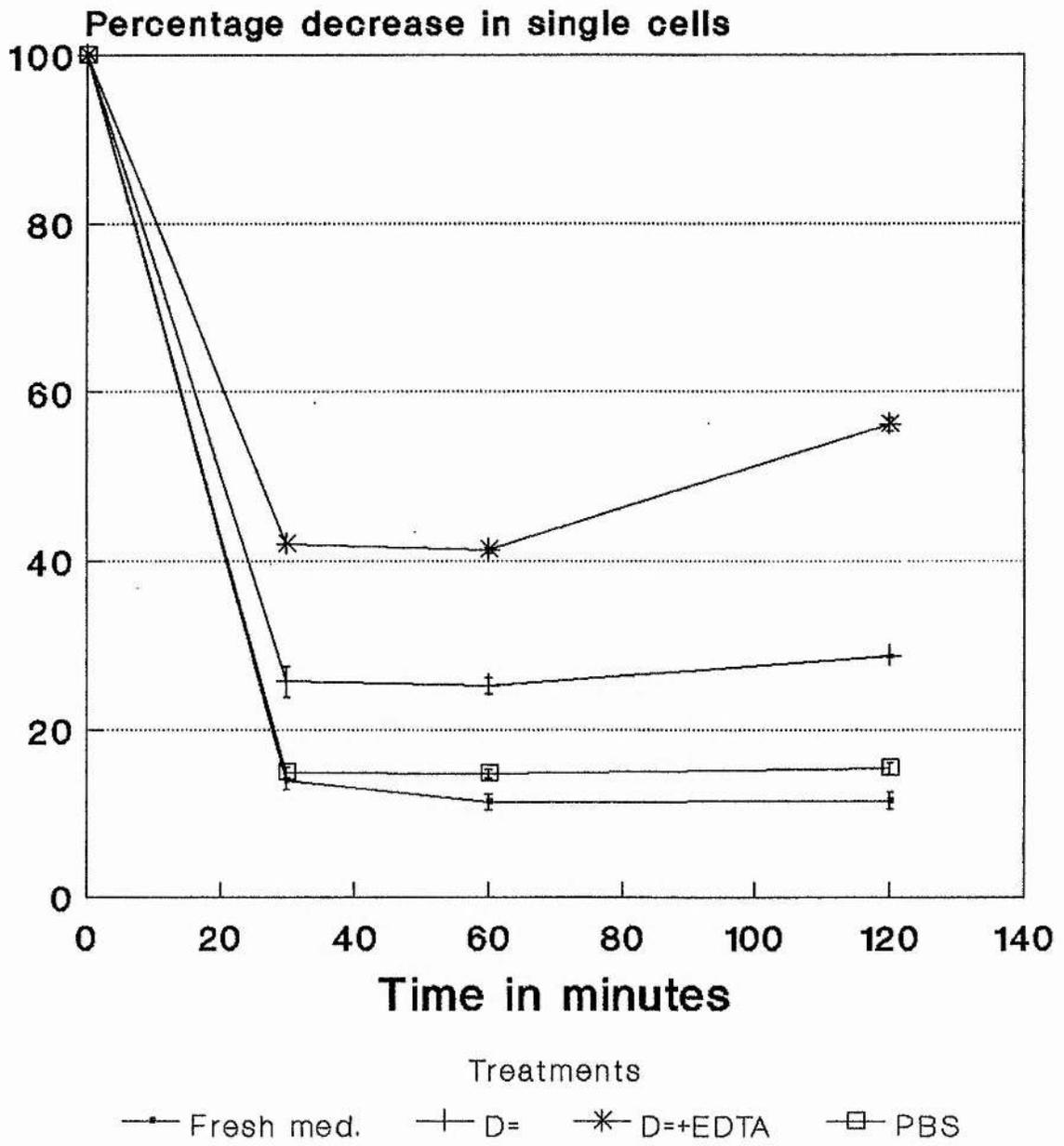
Fig. 7.2.2 : The same cell line as in 7.2.1 which had been dissociated by pipetting.

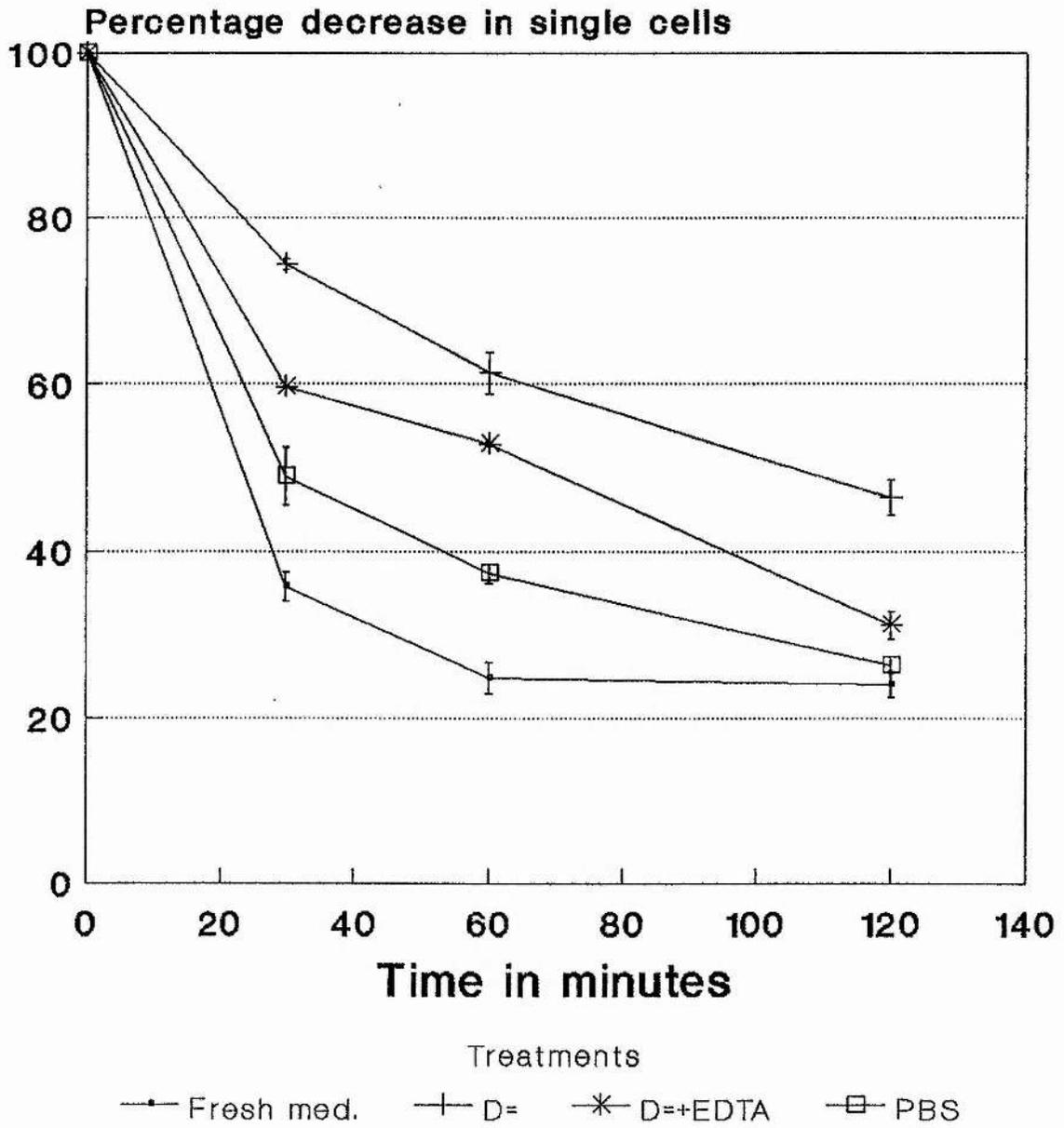
Fig. 7.2.3 : The uncloned leg cell line L1 27D6 dissociated by pipetting.

Fig. 7.2.4 : The cloned wing cell line Cl.8+ dissociated by pipetting.

1







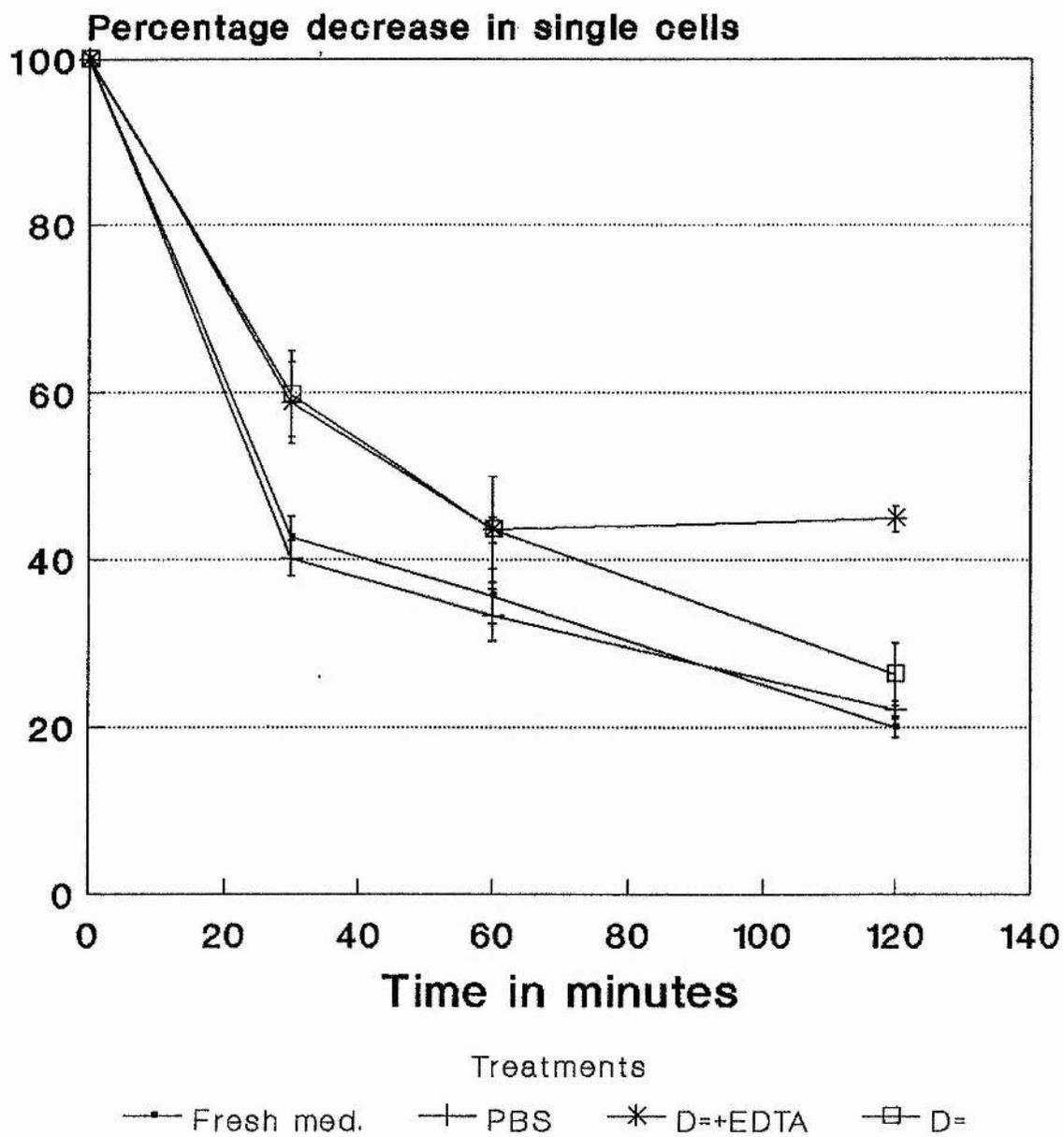


Figure 7.3

Figure 7.3 : Reaggregation in the leg line L1 26D7 in PBS.

Note the small size of the resulting aggregates (A).

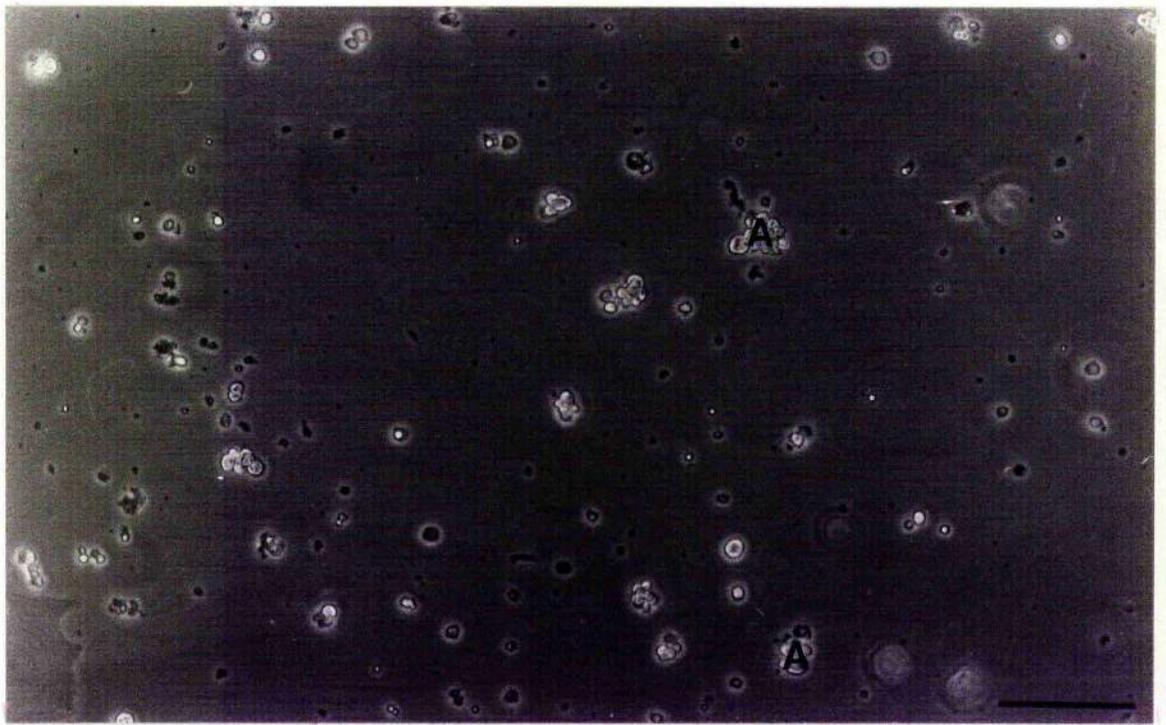


Figure 7.4

Figure 7.4 : Sorting out between the cloned wing cell line Cl.8+ and the cloned leg cell line LIA. The LIA line was labelled with R.123. The (a) series shows the phase contrast view of the cell clump whilst the (b) series shows the corresponding fluorescent image. All scale bars = 40 μm .

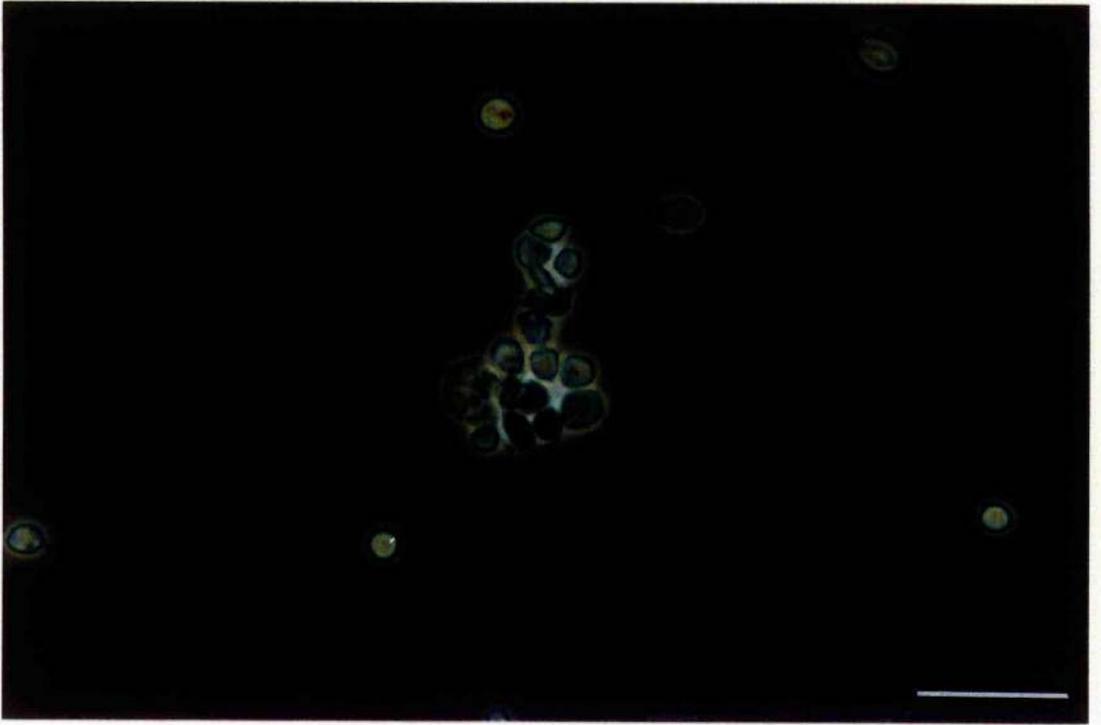
Fig. 7.4.1 : Cell clump showing a predominance of labelled cells.

Fig. 7.4.2 : Cell clump with an approximately equal predominance labelled, unlabelled cells.

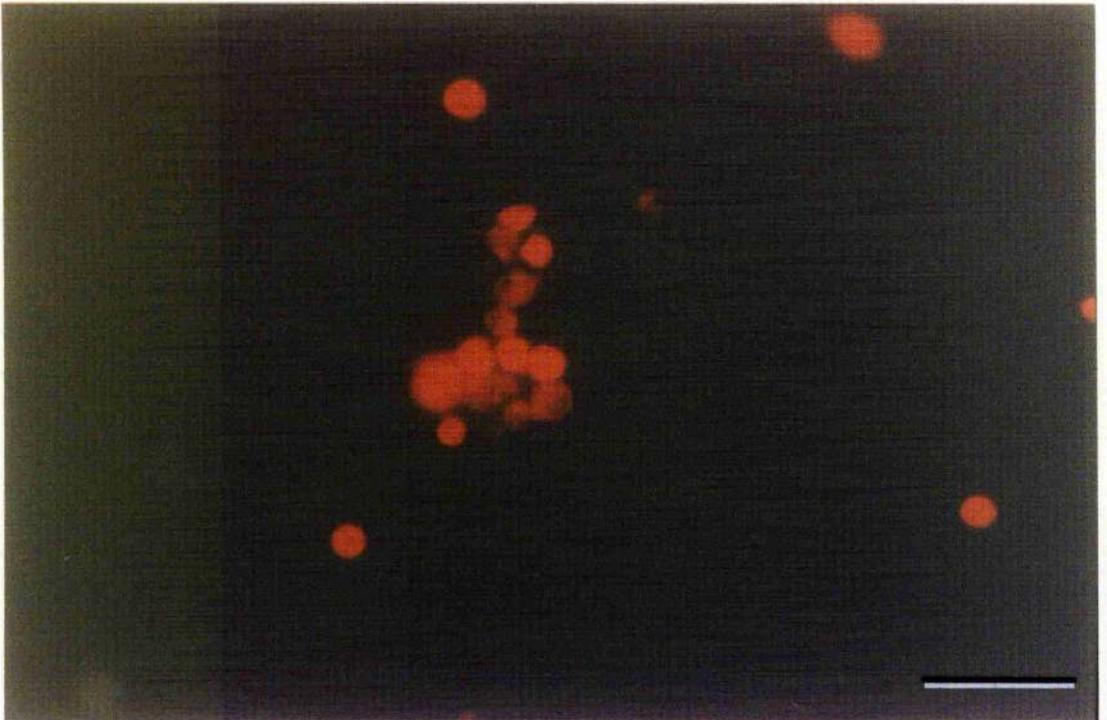
Fig. 7.4.3 : Cell clump with a predominance of unlabelled cells.

1

a



b

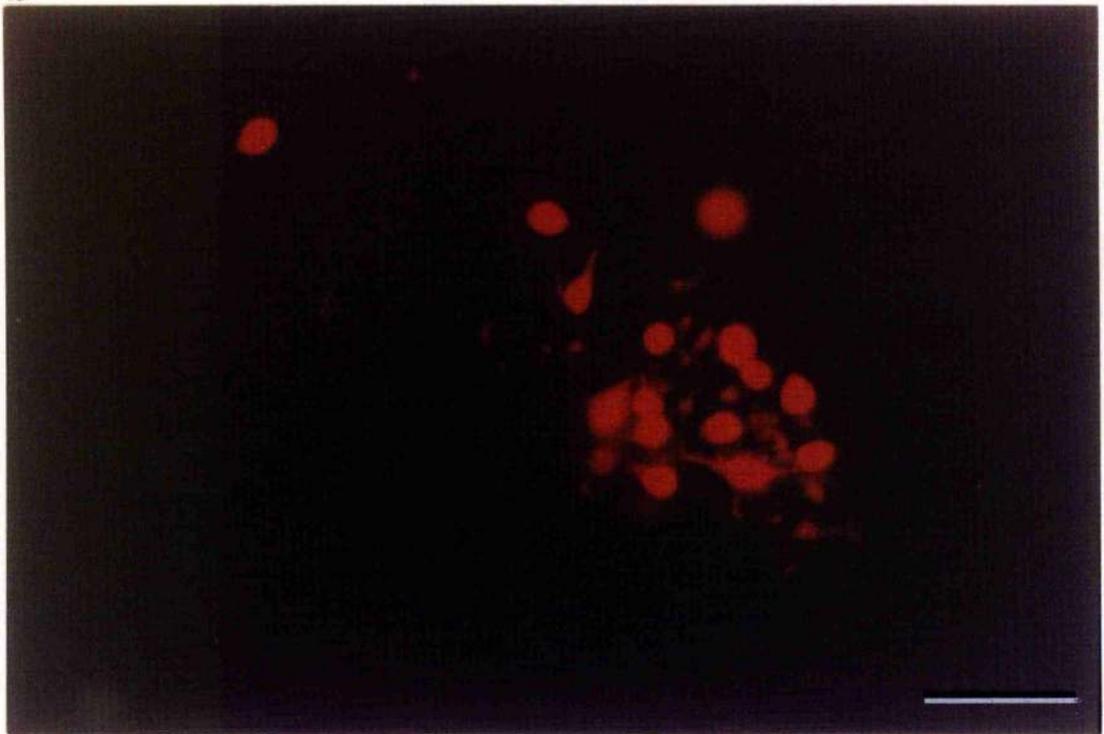


2

a

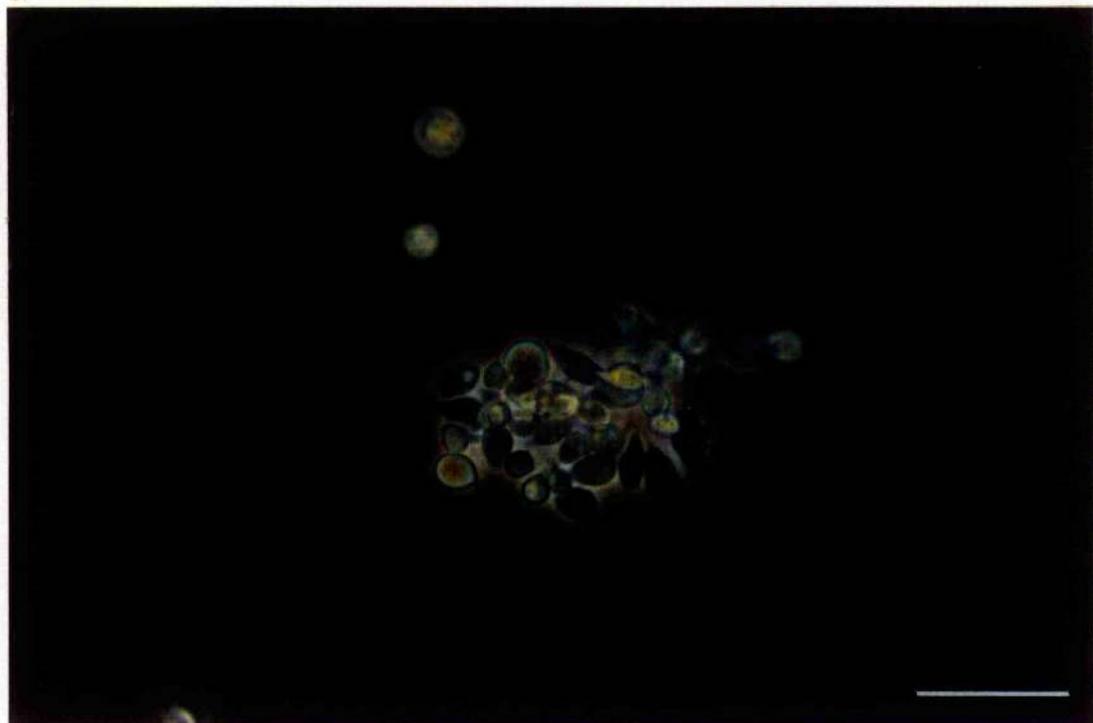


b

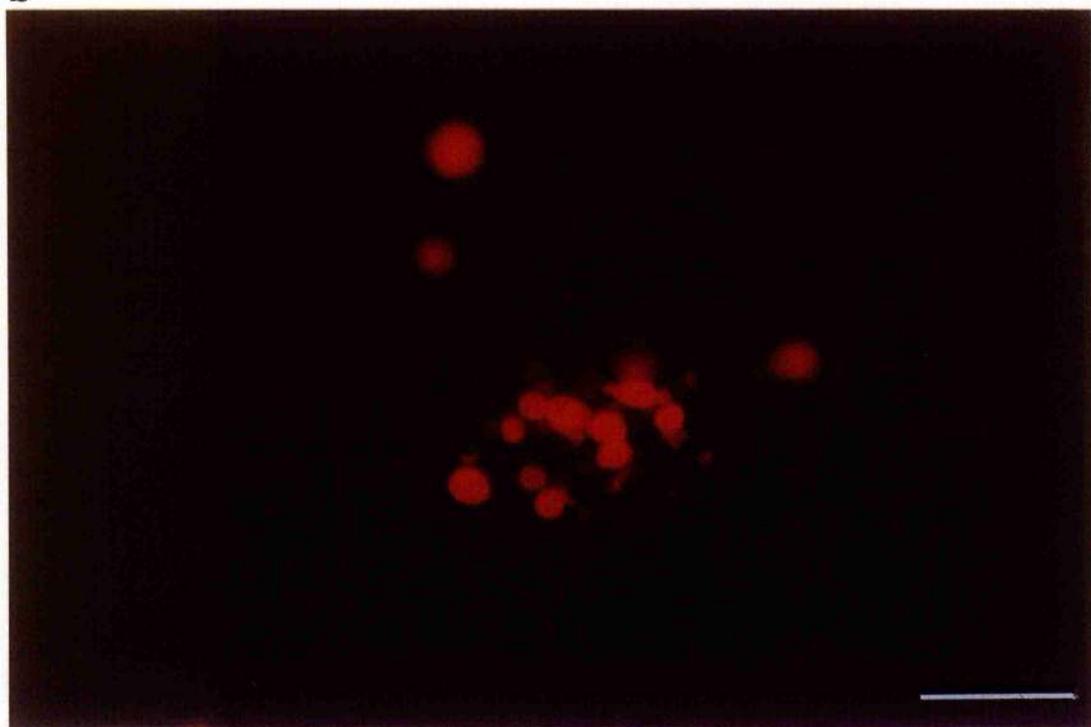


3

a



b



Chapter 8

Discussion

Discussion

This discussion draws general conclusions from the experiments that have been carried out. The aim is to try to bring together the many different aspects of the biology of imaginal disc cell lines that have been studied and to try and establish the present and future relevance of imaginal disc cell lines. More specific comments about particular experiments are included at the end of each chapter.

Imaginal disc cell lines are relatively new, being established only 4 years ago in my present laboratory by Douglas Currie, Martin Milner and Clive Evans. I joined the laboratory just under 3 and a half years ago with the basic remit to try and study the biology of imaginal disc cell lines. Imaginal discs *in vivo* have been studied extensively over the years and a great deal is known about their growth, pattern formation and genetics. However imaginal disc cells *in vivo* are rather difficult to manipulate in order to study aspects of their cell biology due to their tight intercellular junctions and basement membrane. The promise of imaginal disc cell lines is the ease of manipulation of this defined imaginal disc epithelial cell population, enabling a coming together of the vast range of genetic data known about *Drosophila* with an increasing sophistication in the techniques of cell biology.

However the major problem with any cell line is its relevance to the *in vivo* situation. As has been mentioned more specifically in Chapter 1 there is quite a big difference between the way disc cells grow *in vivo* to that *in vitro*. The polarised epithelium is lost and cells take on a more

fibroblastic type of growth. This change in growth morphology is not surprising given the conditions of culture. *In vivo* the growth of imaginal discs is limited, discs grow to a characteristic size and stop (Bryant 1987). Therefore in primary culture, conditions bring about a change in this property of the disc tissue, cell division is once again initiated, cell growth commences and a cell line is developed. Cells adapt to the conditions of culture by growing as loose cells rather than as a polarised epithelium. It is perhaps not surprising that mutations in genes causing a neoplastic transformation of imaginal disc tissue *in vivo*, such as *l(2)gl* (Gateff and Schneiderman 1974) and *lethal(1)discs-large-1* (Woods and Bryant 1989), result in a loss of epithelial polarity as well as a loss of growth control. The cells in primary culture that go on to form the cell line must down-regulate or lose proteins involved in maintaining the polarised phenotype. This could be the result of the loss of certain molecules rather than by a general dedifferentiation of the cells. The gene *crumbs*, encoding an EGF-like protein seems to be required for the proper organisation of epithelia, lack of the gene product leading to the formation of a disorganised mass of cells in epithelial tissues (Tepass et al 1990). Other molecules have also been shown to re-impose a polarised phenotype. Transfection of fibroblasts with the gene coding for the CAM uvomorulin leads to the redistribution of $\text{Na}^+ \text{K}^+$ ATPase, an indication of a conversion to a polarised epithelial phenotype (McNeill et al 1990).

Thus imaginal disc cells growing *in vitro*, whilst obviously showing different growth and morphological

characteristics to their counterparts *in vivo*, may retain most of their imaginal characteristics. The differences that are seen could result from only a few changes, necessary to make growth possible *in vitro*. The results from the cloning experiments in Chapter 1 make the point that dramatic differences in morphology, that at first sight appear to be related to differences in origin are actually just reflections of different states of the descendants of a single cell. Thus morphological properties are not always the best guide to a cells origin or state of determination in this situation. Perhaps a better criterion is to look at the properties that the cells exhibit *in vitro* and compare them with the known properties of imaginal disc cells *in vivo*. This is what has been attempted throughout this thesis. However one caveat should be added to this general hypothesis of comparing *in vitro* with *in vivo*. This is that cells *in vitro* find themselves in a very different situation to their counterparts *in vivo*. This may sound fairly obvious but it should always be borne in mind when trying to compare imaginal disc cell properties. An example is the response of imaginal disc cells to 20-HE. *In vivo* the response to 20-HE is elongation and eversion of the disc. Traditionally this has been regarded as due to cell flattening and rearrangement (Fristrom and Fristrom 1975; Fristrom 1976; Natzle et al 1988), although more recently cell shape changes have also been invoked as a major morphogenetic mechanism (Condic et al 1991). *In vitro* the situation is somewhat different, as the cells are not constrained by closely packed neighbouring cells held together by tight intercellular junctions, nor are they bordered by a

basement membrane. Instead cells find themselves in loose association with their neighbours and are not as constrained as they would be *in vivo*. An analogy can be drawn to a coiled spring, the spring being able to expand in a coordinated and ordered fashion as all the coils are connected. If the coils in the spring were cut, releasing the spring would result in pieces of metal going everywhere. Disc cells *in vitro* respond to 20-HE by elongating and throwing out cell processes in all directions, as they are not constrained by their neighbours.

Thus what might appear at first to be a deranged response of the cells to 20-HE, the throwing out of cell processes, may actually approximate a normal response but in an abnormal situation. However any such argument should be balanced with the fact that cell process extension is only one of several responses to 20-HE and that embryonic cell lines also show such an extension, though to a lesser degree. So when examining the properties of imaginal disc cell lines these facts should be borne in mind.

Imaginal disc cell lines show a dramatic morphological response to 20-HE, cells aggregate, elongate and throw out processes, also many cells die off. These dramatic morphological changes may, as mentioned above, be due to the *in vitro* culture conditions rather than as a purely deranged reaction to 20-HE. Along with these morphological changes there are many biochemical adaptations as well. There is an elevation in levels of chitin synthesis and AchE activity. The observation that the synthesis of chitin is stimulated by 20-HE is not surprising given that chitin is a major component of the cuticle which imaginal disc cells will go on to form under

the influence of 20-HE. An increase in AchE activity is perhaps less obvious. However, this may be part of a general increase in proteolytic activity associated with tissues that are actively rearranging.

Imaginal disc cells *in vitro* show a dramatic morphological transformation on addition of 20-HE, with concomitant biochemical changes. The cells have been shown to have a specific ecdysteroid receptor and the morphological and biochemical changes have been demonstrated to be directly receptor mediated through the use of a specially selected receptor negative cell line. Therefore from the insect physiology point of view there now exist defined epithelial cell lines from a system about which a great deal of genetic information is known. So the use of this cell line could well prove useful for looking at ecdysteroid action.

The cells show an interesting pattern of aggregation in culture, and this has been shown to be correlated with the expression of a *Drosophila* integrin (Chapter 6). Aggregation could well result from the extension of cell processes, bringing cells together which are then stabilised by increased cell-cell adhesion. The relationship between aggregation and the expression of *Drosophila* PS integrins shows how the use of a defined epithelial cell line can help elucidate the way a molecule works between cells. Further biochemical analysis of such molecules would be greatly enhanced by utilising these cell lines, providing large amounts of homogeneous cellular material of a known cell type. Use of such a defined cell line should help in understanding the function of the increasing number of molecules and genes being isolated that are thought

to be important in cell adhesion and cell-cell interactions in *Drosophila*. The development of a transfection procedure for disc cell lines will complement these studies, increasing the understanding of their function and allow an analysis of the structure versus function relationships of the genes.

Theoretically, imaginal disc cell lines provide the ideal starting material for a study of sorting out. Where else could you find two easily amenable cell populations, which in most other respects are identical but are clearly destined to form such different structures as wing and leg. The results from the *in vitro* experiments, although somewhat open to error, suggest that wing and leg cells can recognise each other (see Chapter 7), and confirm studies done with freshly dissociated disc cells. Thus, from these experiments, different cell lines from different discs do appear to retain some of their imaginal disc specific characteristics. As to whether the individual cells of a particular cell line retain any of their intra-disc spatial characteristics little is known. Staining with the integrins PS1 and PS2 which are found on the dorsal and ventral aspects of the disc epithelium respectively, suggest that this particular spatial distinction is lost (see Chapter 6). Experiments on regeneration of imaginal disc fragments suggest an intimate relationship between cell proliferation and positional information (Bryant 1987). Where epithelial polarity and structure is lost, the interactions between neighbouring cells that control cell proliferation may be deranged or impossible, therefore epithelial structure may also be very important in spatial patterning. In the cell lines where epithelial polarity has

been lost and cell proliferation is unbridled, it seems likely that most of the spatial identity of individual cells will have been lost. However, this has to be further tested, several gene products show a spatially restricted pattern of expression in the imaginal disc (Whittle 1990), and these may well help to analyse the extent to which any positional information is retained by cells in a cell line. The use of these spatially restricted markers in conjunction with the new technique of producing cloned cell lines which was developed in this study, could prove helpful in trying to analyse the interactions that govern spatial patterning.

The imaginal disc cell lines described here are not the only cell lines in existence that have been derived from imaginal disc tissue. Ui et al (1987) developed imaginal disc cell lines which appear to be similar to those described here. These lines were developed from the mutant *Drosophila* stock *y v f mal*, they are predominantly diploid and are capable of differentiation into adult cuticular structures when injected into a metamorphosing larvae. Recovery of these differentiated aggregates suggests that transdetermination is occurring in these cultures (Ui et al 1987 a). Cell lines have been established from several different discs, such as haltere and eye-antennal besides the wing and leg cell lines that have been established in this laboratory. In one description of the lines (Ui et al 1987 b), it is mentioned that 20-HE enhanced cell aggregation but did not show much effect on cell proliferation, however few data are given on the exact effect of 20-HE on the cells. Clearly the aggregating effect of 20-HE on disc cell lines is similar to that described here,

but the effects of 20-HE on cell proliferation appear to be quite different. The cell lines appear to be similar to our disc cell lines in many respects, but as little has been published since their initiation a fuller comparison of exact properties is not yet possible.

The use of imaginal disc cell lines is most likely to be as a defined epithelial cell population for which the original cell type is well known. This study has helped to classify these cell lines as defined epithelial cells and provides a necessary preliminary to any further experiments. However further studies are needed to more fully analyse the exact state of determination of these cells in culture.

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Appendix I
Cell line nomenclature

Cell line nomenclature

The cell lines which were the basis of this study were the uncloned wing and leg lines which were established before as per Currie, Milner and Evans (1988). From these I developed the cloned wing and leg lines which have been predominantly used in this study. These cell lines as described in Chapter 3 are more stable in culture, exhibit a higher growth rate and are more easily amenable to different culture techniques. These cell lines have been named according to two different systems.

Wing cloned cell lines

Five different cloned lines were established, one of which (Cl.8) was to yield a further 20-HE resistant subline (Cl.8R). These were called Clone 7,8,9,13 and C9 and are referred to generally as Cl.8 etc., Cl. being short for Clone. These numbers were derived purely from numbers given to cloned colonies as they were observed to grow, the only exception being C9, the name being derived from the well on a 96 well plate that the line was grown up in. As mentioned in the text the Cl.8 line was subdivided into 20-HE resistant and susceptible sublines and given the names Cl.8+ (denoting the positive action of 20-HE) and Cl.8R (denoting the resistance of this line). These notations were originally proposed by Andreas Turberg during my stay in Düsseldorf.

Leg cell lines

Only two cloned leg lines were isolated and these were named after fields on my fathers farm on the Isle of Wight. hence the names LI Astrew (LIA) and LI Prussacks (LIP). The LI prefix denotes the parent uncloned line that these cells were obtained from.

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Appendix II

Published papers and those submitted for publication

*Short communications***The diversity of cell morphology
in cloned cell lines derived from *Drosophila* imaginal discs**

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Summary. We have devised a protocol for the cloning of our cell lines, and have demonstrated that a cloned line may contain cells of widely differing morphology – epithelial, fibroblast-like, and lamellocyte-like. These different morphologies must therefore represent diversity in the microenvironment of the culture rather than diversity in the cellular origin of the line.

Key words: *Drosophila* – Imaginal disc – Cell culture, wing – In vitro

Introduction

We have recently been successful in developing a culture system in which partly dissociated *Drosophila* imaginal discs can grow and give rise to cell lines (Currie et al. 1988). This has been achieved by supplementing a widely-used medium – Shields and Sang's M3 (Shields and Sang 1977) – by the addition of 2% foetal bovine serum, 1 ng/ml 20-hydroxy ecdysone, 0.125 IU/ml insulin, and 50 µl/ml of a fly extract. The latter was prepared by the homogenisation of 200 flies in 1.5 ml of medium without additives, followed by heat treatment and centrifugation. Sterile discs were partly dissociated by exposure to EDTA and trypsin in calcium and magnesium free saline, and cultured in 96 well plates (see Currie et al. 1988).

This culture system enabled us to establish first primary cultures and then continuously growing cell lines. Single cells and cell clumps adhered to the plastic, and then spread across the substrate as sheets of epithelial cells, networks of fibroblast-like cells and as individual, flattened lamellocyte-like cells. After a few weeks, cell division started, giving rise to densely packed areas of single cells which could be subcultured. These quickly gave rise to cell lines which were maintained by weekly passaging.

Offprint requests to: M.J. Milner

We wished to investigate whether this range of cell types indicated a diversity of origin for our cell lines, or whether a single cell type is able to exhibit a range of distinct morphologies in culture. If the latter were found to be the case, this would have implications for the interpretation of cell morphology in a range of culture systems.

Materials and methods

The technique we have devised for cloning our cell lines utilised a special cloning plate, which was made by sticking an inverted 3 cm Petri dish base to the underside of the lid of a 5 cm Petri dish (Fig. 1). The two were stuck together after dipping the rim of the smaller dish into propylene oxide, which partly dissolved the plastic. A feeder layer of disc cells was grown in the 5 cm dish, and single cells were plated out on to the bottom of the 3 cm dish. This arrangement permitted the diffusion of conditioning factors from the feeder layer to the single cells, but prevented any contamination by the cells of the feeder layer, which adhered firmly to the plastic of the 5 cm dish. This layer was plated out at a concentration of 3×10^6 cells and left for 2 to 3 days before use to ensure that the cells were in active growth and were conditioning the medium. The feeder layer was changed at intervals of approximately 4–5 days to ensure that the cells were in active growth and were adherent to the plastic rather than piling up in large aggregates as is common during later stages of culture (Currie et al. 1988). Regular visual checks were also made to eliminate

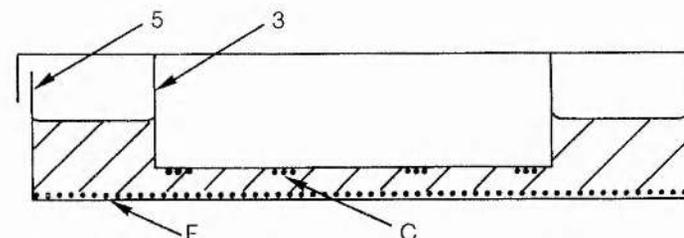


Fig. 1. The plate devised for cloning our cell lines. The hatching represents culture medium. The cloning plate consists of the base of a 3 cm Petri dish (3) which was stuck to the underside of a 5 cm Petri dish lid (5). The feeder layer (F) and clones (C) are indicated

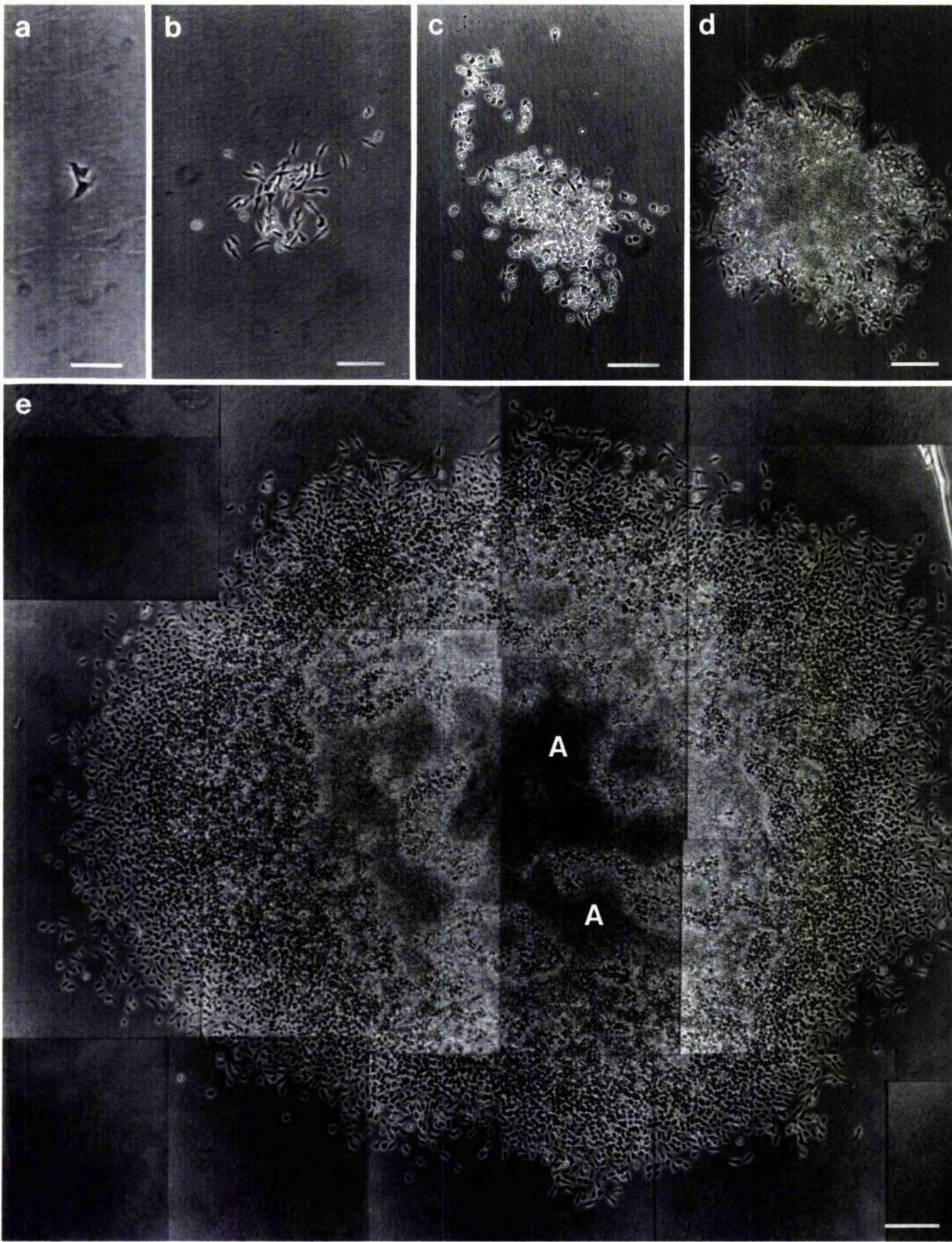


Fig. 2a–e. The establishment of cloned cell lines from the wing line CME W1. **a** Clone at the two cell stage 13 days after culture initiation. **b** Another clone from the same dish as **a**, 13 days after culture initiation, at a more advanced stage of growth. **c** and **d**

The clone illustrated in **b** at 18 and 22 days after culture initiation respectively. **e** A clone ready for subculture 29 days after culture initiation. The cells in the centre are forming cell aggregates. Bars represent 50 μm in **a** and **b**, and 100 μm in **c**, **d** and **e**

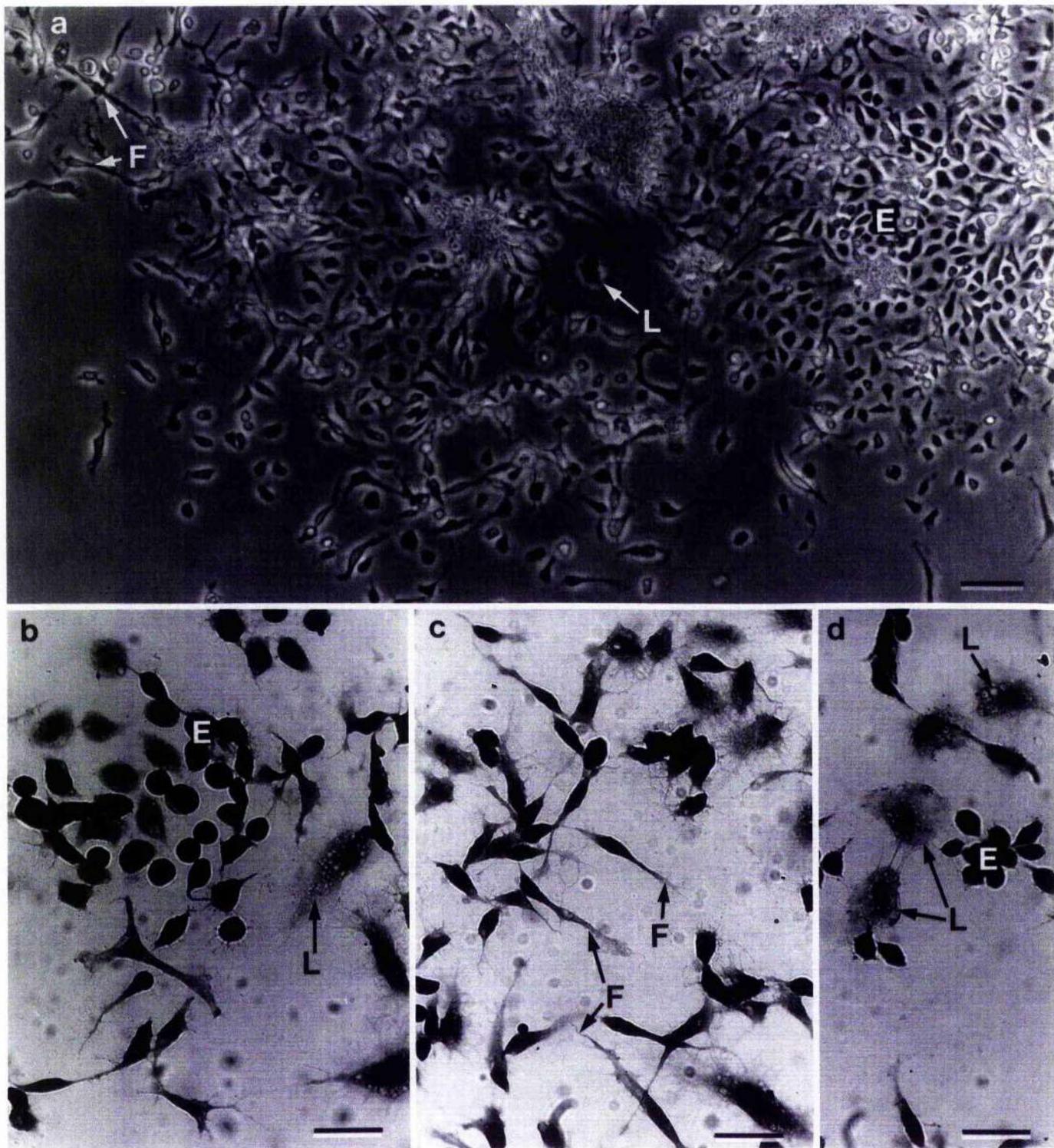


Fig. 3a-d. Cell morphology in cloned lines. **a** The edge of a leg clone 35 days after clone initiation, which includes epithelial-like (*E*), bipolar fibroblast-like (*F*) and flattened lamellocyte-like (*L*) cells. **b, c, d** Cells of wing clone 13, 11 passages after clone initiation,

showing the three cell types referred to above. Cells were fixed in 4% paraformaldehyde for 10 min, and stained in Mayers haematoxylin. Bars represent 50 μm in **a**, and 20 μm in **b, c** and **d**

this possibility, which might have resulted in the contamination of the cloned cells with those of the feeder layer.

A suspension of cells to be cloned was produced using standard dissociation procedures; a concentration of 100 cells/ml which would give rise to 10–12 clones per 5 cm dish being routinely used.

This suspension was plated out onto the upturned surface of the 3 cm lid and left overnight to adhere to its surface. The medium was then pipetted off, the lid inverted and gently laid onto a 5 cm dish containing the feeder layer so that the surface of the 3 cm dish was immersed in medium (Fig. 1). The positions of the single

cells were mapped out and they were carefully followed during growth to ensure that no contamination occurred from the feeder layer. The design of the cloning plate enabled the growing clones to be closely monitored with an inverted microscope. Once the clones were growing rapidly, the lid of the cloning dish was removed and transferred to a fresh dish without a feeder layer for further growth. Eventually clones could be transferred to the wells of a 96 well plate and progressively moved up to larger culture dishes. Cultured cells were examined with a Leitz Diavert microscope, and photographed with a Wild MPS45 photoautomat.

Results and discussion

The method used to produce clones from our cell lines was established after a number of alternatives had been tried without success, including limiting dilution in conditioned medium and using *X*-irradiated and mitomycin treated feeder layers in soft agar. After culture initiation, the fate of single cells was followed and photographed. The pattern of division was very erratic, but usually after about a week some of the single cells began to divide and the growth of the colony could be followed (Fig. 2a–e). In mature colonies, cells began to pile up at the centre in a similar fashion to that observed in the original lines (Currie et al. 1988), while the periphery steadily expanded. After about four weeks, the colonies were large enough to be transferred to 96 well plates and grown up as lines (Fig. 2e).

During earlier work on this system we had observed a range of cell types in our cultures. Primary cultures exhibited epithelial cells growing both as vesicles and as sheets adhering to the substrate, fibroblast-like cells, and flattened lamellocyte-like cells. A similar diversity was found in established cell lines, where epithelial and fibroblast-like cells predominated, and some lamellocyte-like cells and sickle-shaped cells were found (Currie et al. 1988). We were concerned that this diversity of cell morphologies indicated that our cultures originated from a number of different cell types, rather than solely from imaginal epithelial cells as we had hoped. Candidates for the origin of contaminants included the adipothelial cells which give rise to adult muscles and are found between the basal lamina and the imaginal epithelium of the disc (Poodry and Schneiderman 1970), and blood cells which adhere to the exterior of the disc (Milner and Muir 1987). If our lines originated from a number of cell types we would expect that cloned lines would exhibit less than the full range of cell morphologies observed in the original line. However, we found that a single cloned colony could contain cells with epithelial-like, fibroblast-like, and lamellocyte-like morphologies (Fig. 3a). Established cloned lines also exhibited a range of cell morphologies (Fig. 3b, c, d) although the cells of established cloned lines tend to have a more uniform cell morphology than uncloned lines (Peel and Milner, unpublished observations). Thus, these differences in morphology must be the result of different local-

ised conditions of substrate, cell contact or medium composition rather than indicating that the lines originate from a number of different cell types. The examination of cell morphology has always played an important role in the investigation of cells in culture (Shields and Sang 1970), and in particular in the analysis of the response of cells to insect hormones (Courgeon 1972, 1975; Berger et al. 1978; Cherbas et al. 1980; Kislev et al. 1984). Our results suggest that correlations between cell morphology and tissue origin and/or developmental state are not always to be relied upon.

We feel that the development of a technique by which our imaginal disc cell lines can be cloned represents an important advance in *Drosophila* cell biology. At large quantities of a defined cell type can be produced for a range of cellular and molecular studies. We are currently investigating interactions between cultured imaginal disc cells using this system. Selective pressure can be applied to these lines. For example, we have recently developed ecdysone-resistant cell lines by gradually raising the titre of 20-hydroxy ecdysone in the medium (Milner and Muir, unpublished observations).

Acknowledgements. This work was supported by grants from the Wellcome Foundation and from the Science and Engineering Research Council. We wish to thank Dr. Corrado D'Arrigo for helpful suggestions, particularly on the methodology of cloning, and Harry Hodge for skilled technical assistance. D.J.P. is in receipt of a SERC postgraduate studentship.

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D. J. PEEL, S. A. JOHNSON and M. J. MILNER*

THE ULTRASTRUCTURE OF IMAGINAL DISC CELLS IN PRIMARY CULTURES AND DURING CELL AGGREGATION IN CONTINUOUS CELL LINES

Keywords: *Drosophila*, imaginal disc, *in vitro* wing, cell aggregation, insect epidermal feet, cytoskeleton

ABSTRACT. We have examined the ultrastructure of cellular vesicles in primary cultures of wing imaginal disc cells of *Drosophila melanogaster*. These cells maintain the apico-basal polarity characteristic of epithelial cells. The apical surfaces secrete extracellular material into the lumen of the vesicle from plasma membrane plaques at the tip of microvilli.

During the course of one passage, cells from the established cell lines grow to confluence and then aggregate into discrete condensations joined by aligned bridges of cells. Cells in these aggregates are tightly packed, and there appears to be a loss of the epithelial polarity characteristic of the vesicle cells. Elongated cell extensions containing numerous microtubules are found in aggregates, and we suggest that these may be epithelial feet involved in the aggregation process. Virus particles are commonly found both within the nucleus and the cytoplasm of cells in the aggregates.

Introduction

We have established cell lines from leg and wing imaginal discs of *Drosophila melanogaster* (Currie, Milner and Evans, 1988; Cullen and Milner, 1990). Cells both in the primary cultures and in the established cell lines show a range of morphologies, and we originally thought that both blood (lamellocyte-like) and muscle precursor (fibroblast-like) cells might be contaminating the epithelial cell cultures (Currie, Milner and Evans, 1988). However a similar range of morphologies has been found in cloned lines (Peel and Milner, 1990), indicating that a single cell type can adopt a range of morphologies in culture. The cells in our leg and wing lines are clearly derived from the

epithelial cells of the imaginal discs, as 10 ng/ml of the moulting hormone 20-hydroxy ecdysone stimulates chitin synthesis in both cloned and uncloned cell lines (Peel, Spindler and Milner, unpublished observations).

So far, our analysis of cell morphology in our cultures has been solely at the level of the light microscope. This paper represents a first step in the ultrastructural characterisation of these epithelial cells both in the primary cultures derived directly from imaginal discs and in continuously growing cell lines. We concentrate on whether the morphological characteristics of an epithelium are maintained in the vesicles which are characteristic of primary cultures (see previous paper), and on aggregation in cell lines. The presence of viruses is also discussed.

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Materials and Methods

Cultures were initiated and maintained as described in Currie, Milner and Evans,

(1988) and processed for electron microscopy as described by Milner and Muir (1987) for whole imaginal discs. Cells were prepared for transmission electron microscopy in one of two ways. The first of these involved carefully lifting vesicles or aggregates of cells off the culture dish surface with a Pasteur pipette after fixation. Embedding was then performed in a Petri dish as before (Milner and Muir, 1987). The alternative method involved fixation *in situ* in the culture dish, and then embedding the material in agar, after which the agar block could be removed from the culture dish and embedded in Araldite. Sections were cut on a LKB ultratome 111, and examined using a Phillips 301 transmission electron microscope. Cells were prepared for scanning electron microscopy by fixation in a 2.5% solution of glutaraldehyde in 0.05 M phosphate buffer pH 7.57 for 15 min at room temperature. The cells were then washed in buffer, dehydrated in a graded series of alcohols, critical point dried and sputter coated before viewing in a Jeol 35CF SEM.

Immunofluorescence

Labtek culture chamber slides were used for immunofluorescence studies, as these allowed the cells to be processed with minimum disturbance. Cells were plated out at 7.5×10^5 /ml in 0.4 ml of medium per chamber, grown for 7 days and processed as follows. The cells were permeabilised for 10 min using a cytoskeletal buffer consisting of 100 mM NaCl, 300 mM sucrose, 10 mM PIPES, 3 mM MgCl₂, 0.5% TRITON $\times 100$ and 1.2 mM phenylmethylsulfonyl fluoride (PMSF) pH 6.8. The solution was then diluted 1:1 with 4% paraformaldehyde in PBS and fixed for 20 min in this mixture. After two five-min washes in PBS 1% BSA, the cells were incubated overnight at 4°C on a shaker in primary antibody (monoclonal anti alpha tubulin, Sigma T-9026), which was

diluted in PBS 1% BSA at 1:500. After washing as before the cells were incubated for 1 hr at room temperature in biotinylated anti-mouse IgG (Vector) diluted 1:200 in PBS 1% BSA. The cells were again washed as before and incubated for 1 hour at room temperature in Avidin FITC (Vector) diluted 1:250 in BPS 1% BSA.

Alternatively, cells were washed and fixed in 4% Paraformaldehyde in PBS for 10 min and then permeabilised in PBT buffer (PBS with 0.01% BSA, and 0.01% Triton X-100) to which was added 0.02% horse serum for 3 hr. The cells were then processed as above for antibody incubations except that PBT buffer was used instead of PBS 1% BSA. This method gave better visualisation of more isolated cells whereas the permeabilisation before fixation method was superior for the examination of processes within and between aggregates.

After washing in PBS the cells were mounted in 70% glycerol, 30% 0.1 M TRIS pH 9 with 2% n-propyl gallate to reduce bleaching. The samples were viewed using a Leitz Diavert fluorescent microscope and photographed with 1600 ASA film using a Wild photoautomat.

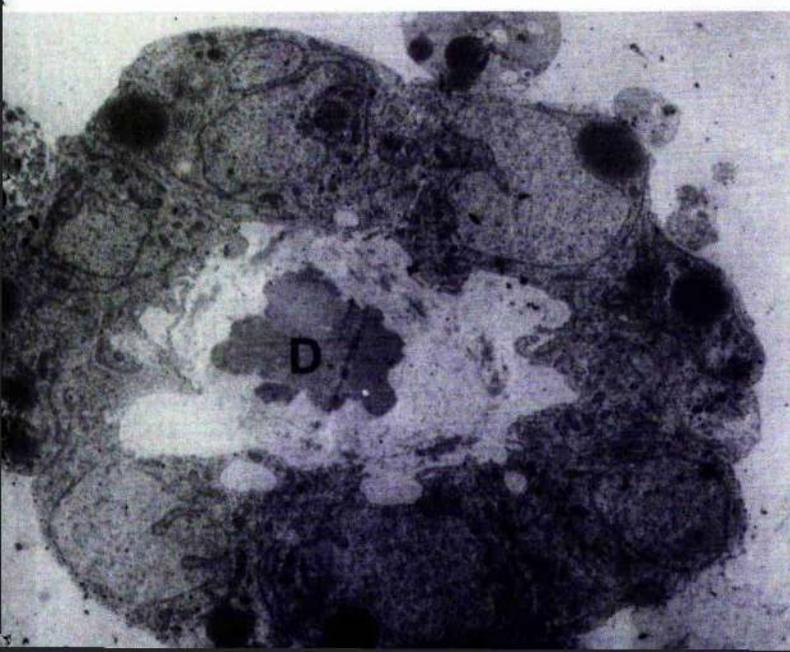
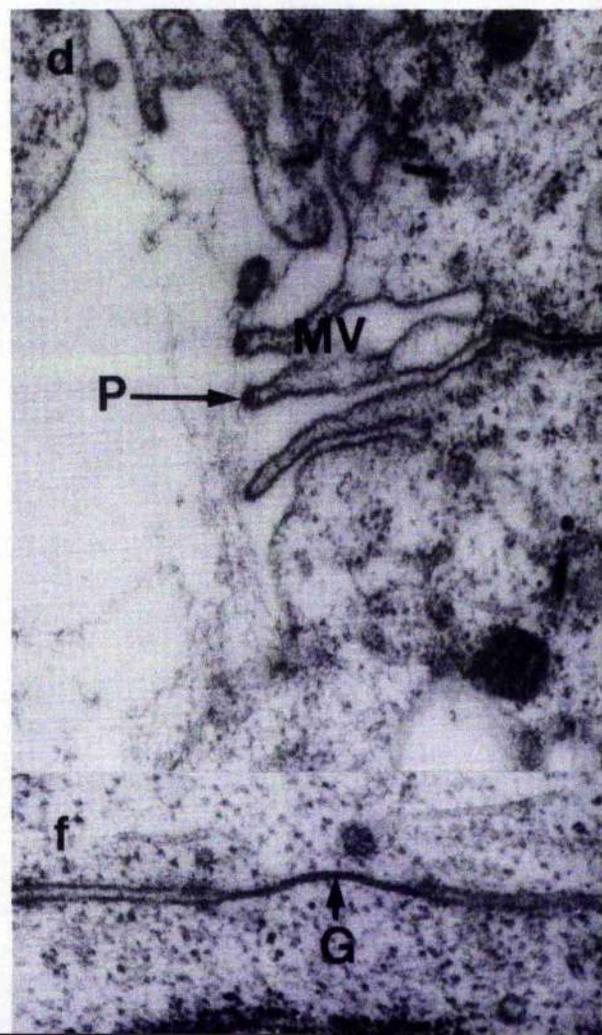
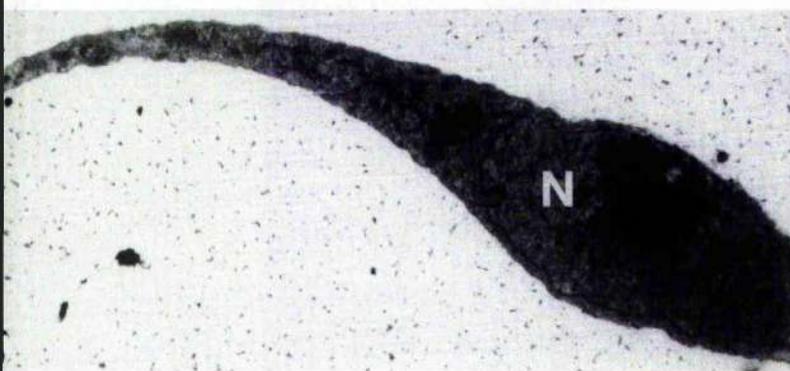
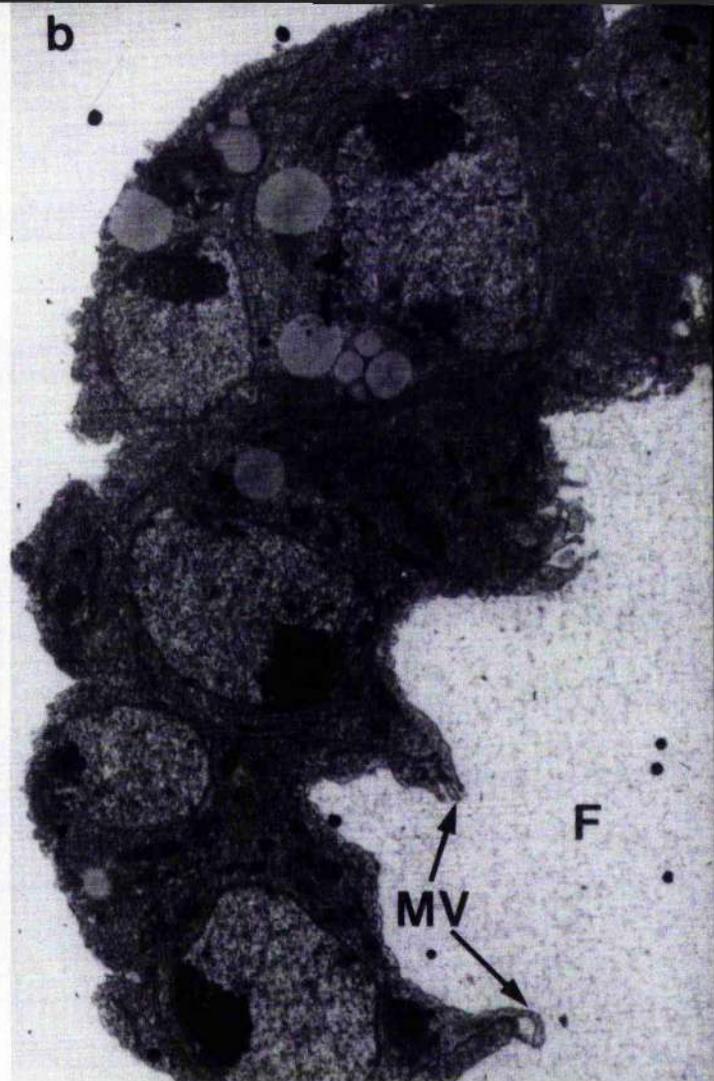
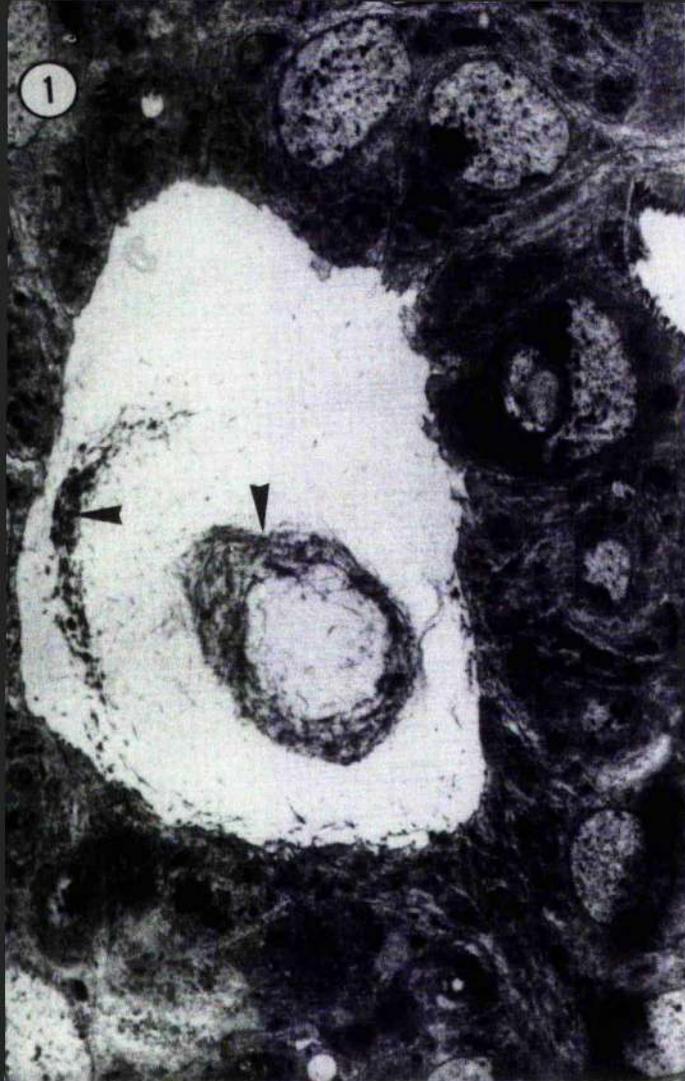
Results and Discussion

The ultrastructure of vesicles in primary cultures

The vesicles in primary cultures are of particular interest to us as cell growth is usually initiated in the vicinity of vesicles (Cullen and Milner, 1990), and we suspect that cells involved in the initiation of growth may originate from vesicles.

We have fixed and sectioned vesicles in wing primary cultures three and nine days after culture initiation. Vesicle cells retain many of the characteristics of cells in intact imaginal discs. Thick-walled vesicles (Fig. 1a, b) resemble the pseudostratified cells of

Fig. 1. The ultrastructure of vesicles in wing primary cultures. (a) A thick-walled vesicle 3 days after culture initiation, with loosely organised extracellular material in the lumen (arrowheads). $\times 4,460$. (b) A thick-walled vesicle 9 days after culture initiation, with apical microvilli (MV). Gloccular extracellular material (F) occupies the lumen. $\times 8,980$. (c) A thin-walled vesicle 9 days after culture initiation, showing the nucleus (N) of a cell occupying a bulge in the wall. $\times 7,500$. (d) Apical microvilli (MV) with dense plaques (P) secreting extracellular product. $\times 34,450$. (e) A thick-walled vesicle 9 days after culture initiation, with dense (D) extracellular material in the lumen. $\times 4,740$. (f) A gap junction (G) between two cells in a vesicle after 35 days in culture. $\times 48,000$.



the columnar epithelium (Poodry and Schneiderman, 1970), while the cells of thin-walled vesicles (Fig. 1c) appear similar to the squamous epithelium of the peripodial membrane (Milner, Bleasby and Kelly, 1984).

Vesicle cells examined at both three and nine days after culture initiation were found to have maintained the apico-basal polarity characteristic of epithelial cells. Basal epithelial extensions are found, similar to those which have been implicated in the contraction of epithelia in *Calpodes* (Locke and Huie, 1981). However, little or no basal lamina was observed, unlike the vesicles of imaginal disc cells which grow in *Drosophila* primary embryonic cultures (Dübendorfer, Shields and Sang, 1975; Eichenberger-Glinz, 1979).

The apical surfaces of the cells, which face the fluid-filled lumen, exhibit numerous secretory microvilli with dense plaques (Fig. 1b, d). The extracellular product of these microvilli accumulates in considerable quantities in the lumen of the vesicles. In many cases this appears floccular and disperse (Fig. 1b), while in others the material is concentrated into loosely organised (Fig. 1a) or dense (Fig. 1e) layers within the lumen. Presumably this material corresponds to the pupal cuticle-like material observed in vesicles at the light microscope level (see previous paper). It would, indeed, not be surprising if vesicles in our primary cultures secreted chitin, as a continuous cockroach cell line UMBGE 4 has been shown to do so (Ward, Newman, Klosterman and Marks 1988). This line grows in the form of vesicles, the cells of which exhibit secretory microvilli. Here, chitin production appears to be under the control of ecdysteroid(s) released into the medium by the vesicles (Ward, Kelly, Woods and Marks, 1987). It may also be the case in our system that the cells in primary

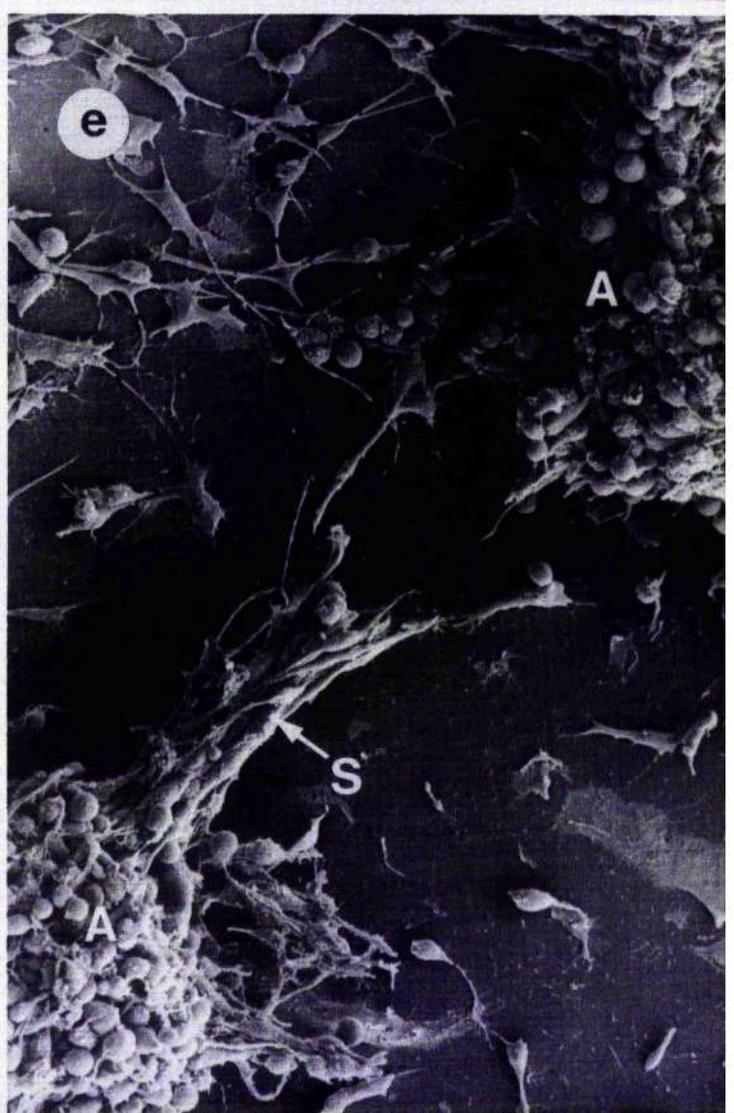
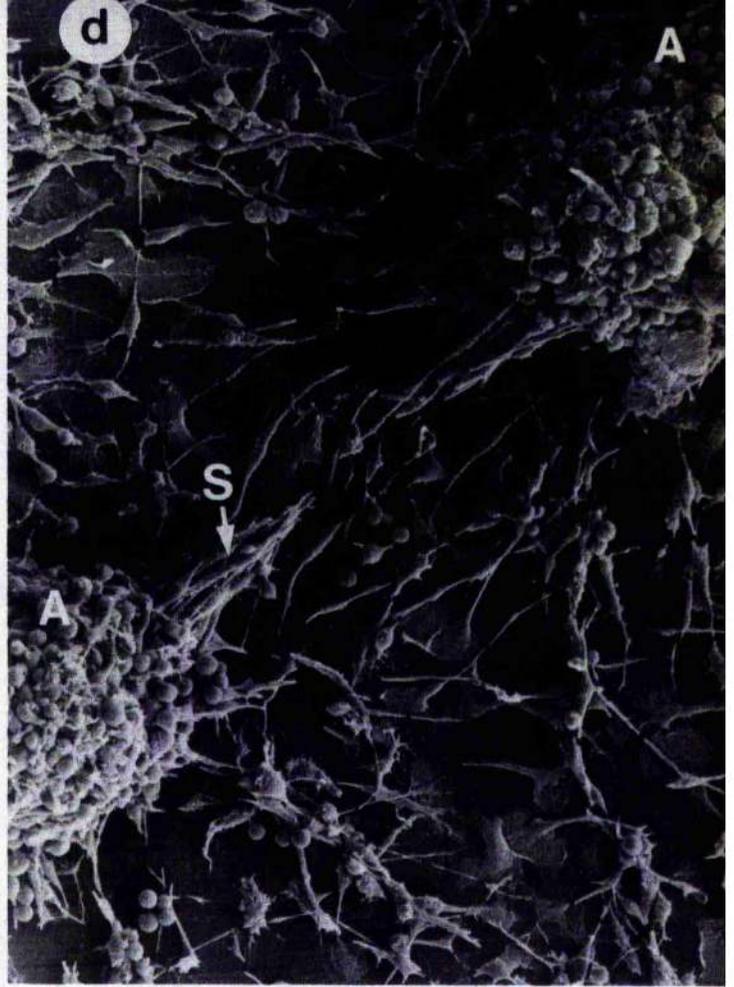
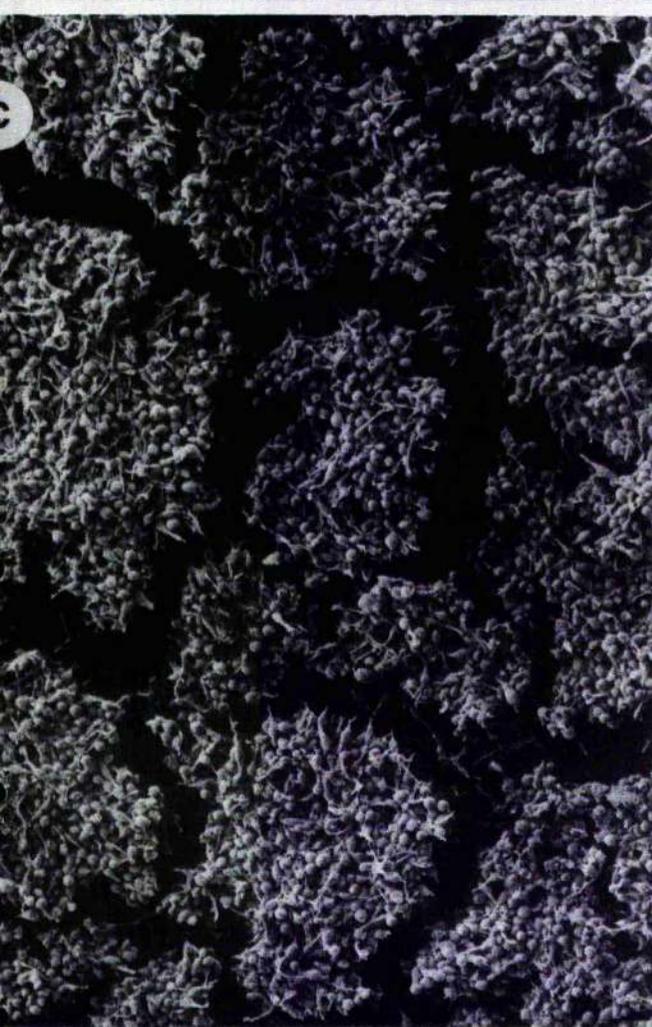
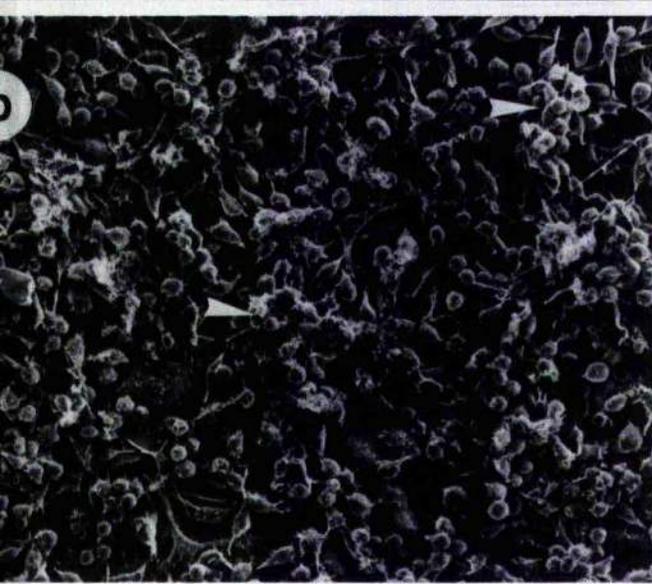
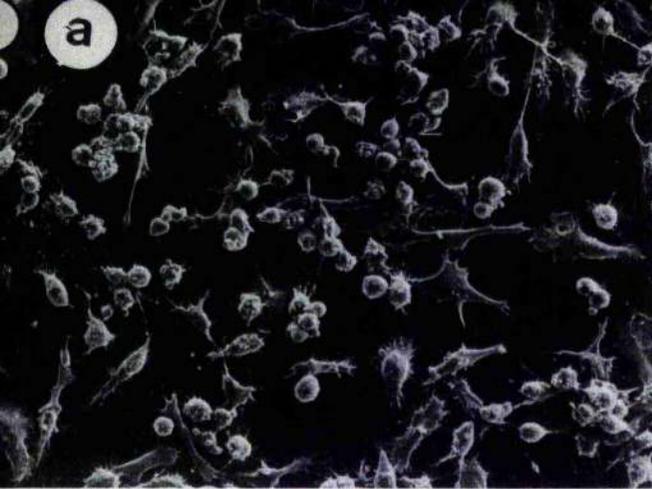
cultures synthesise ecdysteroids, but the quantities of medium required to examine this possibility are too great to be obtained. A very low level of 20-hydroxy ecdysone would be supplied with the fly extract (60 pg/ml for FE1, see Cullen and Milner, 1990). Imaginal disc cells do possess apical microvilli and secrete extracellular matrix material from dense plaques at their tips during the third instar, before the rise in ecdysteroid titre (Fristrom and Rickoll, 1982), and this may account in particular for the more floccular material.

The usual range of junctions found in imaginal discs—zonulae adherentes, septate junctions and gap junctions (Fig. 1f), have all been found in vesicles. However, the frequency and length of these junctions seem to be less than would be seen in intact imaginal discs cultured *in vitro* (Johnson, unpublished observations). This contrasts with the situation in imaginal vesicles derived from embryonic material, where in particular extensive septate junctions are found (Eichenberger-Glinz, 1979).

The aggregation of cells in established cell lines

Established leg and wing cell lines, both cloned and uncloned, showed a very characteristic mode of growth during the course of one passage. Cells were plated out at a density of approximately 3×10^6 per 5 cm. Petri dish (Fig. 2a). After two days of culture, the cells had reached confluency (Fig. 2b). Division did not stop at this point, and cells began to pile up one on top of another—early stages of this process are indicated by arrowheads in Figure 2b. Shortly afterwards, the cells separate into discrete blocks, with cell-free areas of plastic separating them (Fig. 2c). This is the beginning of a process of aggregation which after about a week of culture results in the formation of large, discrete

Fig. 2. A SEM study of aggregation in established, cloned wing cell lines. (a) Cells of line W1, fixed one day after the start of a passage. $\times 400$. (b) W1 cells fixed 2 days after passage initiation, having reached confluency. Arrowheads indicate areas of the culture in which cells are beginning to pile one on top of another. $\times 400$. (c) After 4 days, cells are separating into discrete blocks, separated by largely cell-free areas of plastic. $\times 230$. (d, e) After 6 days, aggregates (A) composed of large numbers of cells have formed, and these are separated by areas of plastic which have been cleared of cells to a greater (e) or lesser (d) extent. Connecting strands of aligned cells (S) can be seen forming in both cases. d $\times 400$, e $\times 500$.



aggregates containing many hundreds of cells, which are separated by substantial areas of plastic on which only a few (Fig. 2d), or almost no cells (Fig. 2e) are to be found. Often large aggregates form connecting strands of aligned cells between themselves, these usually being suspended above the bottom of the dish (Fig. 2e).

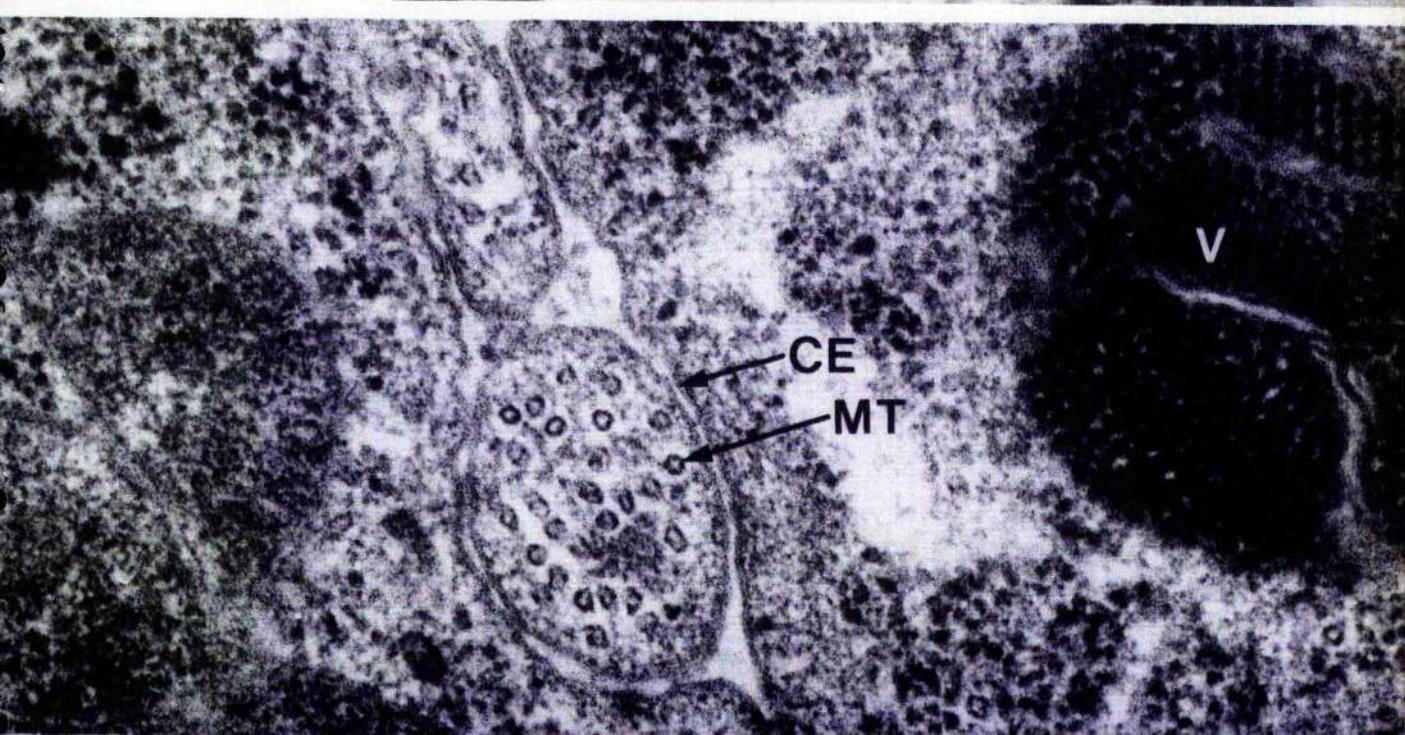
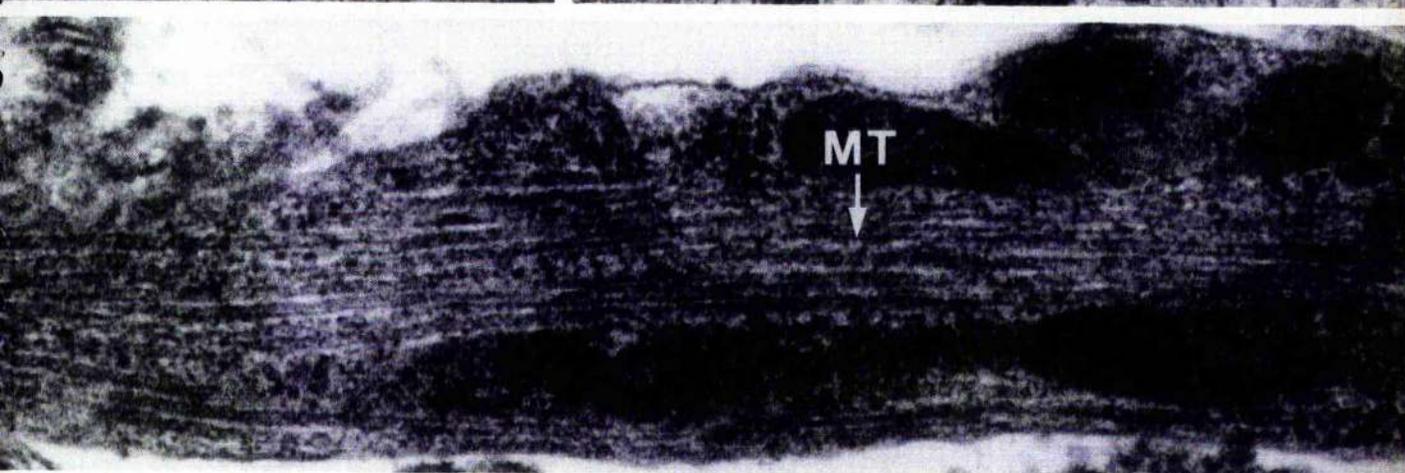
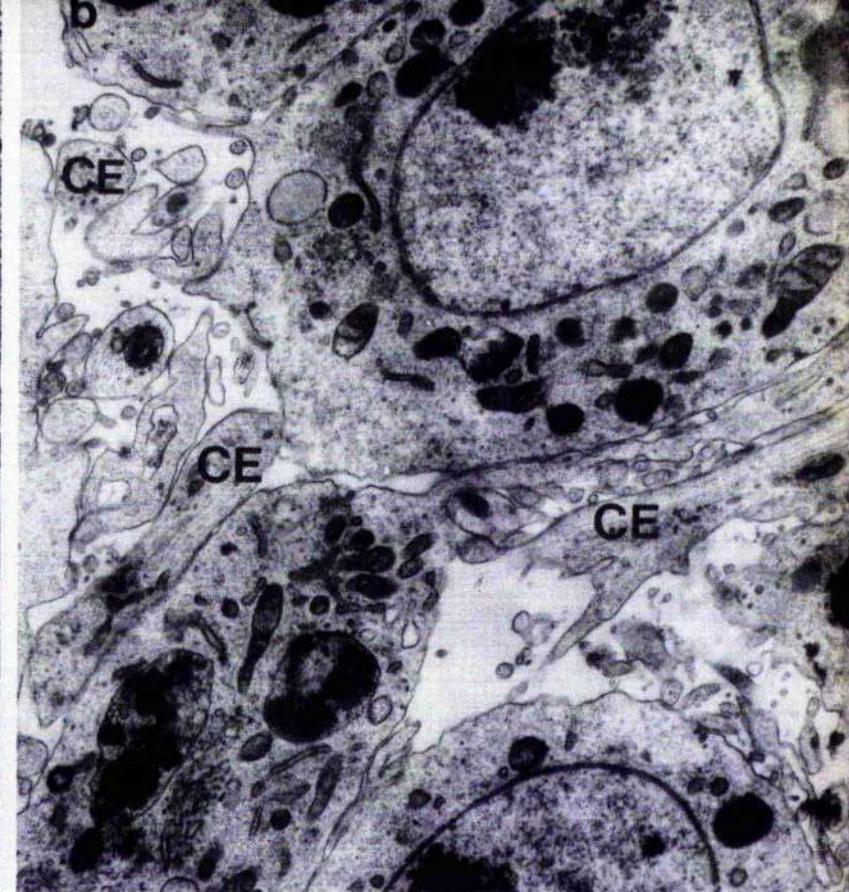
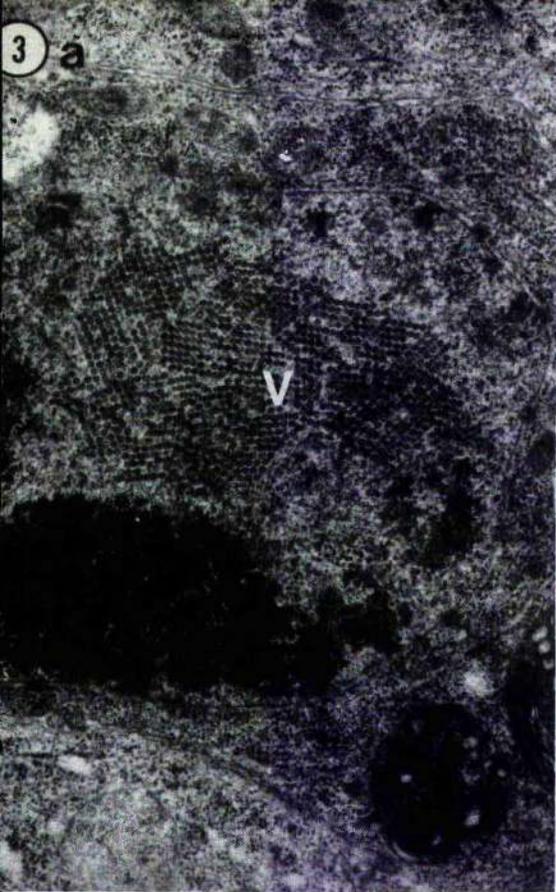
An ultrastructural examination of aggregates reveals that in many instances, cells are tightly packed, with no intercellular spaces (Fig. 3a). There appears to be a loss of the epithelial polarity so characteristic of vesicle cells. However, elongated cell extensions are found which contain numerous microtubules (Fig. 3b, c, d), and their frequency is particularly apparent in loosely-packed aggregates (Fig. 3b). These extensions may be analogous to the basal cell processes or epidermal feet involved in the contraction of *Calpodes* epidermis (Locke and Huie, 1981; Delhanty and Locke 1989). Junctions have not been seen between cells in aggregates, and so the process of aggregation must involve mechanisms which do not involve junction formation. A significant amount of cell death is seen in aggregates, and this is probably because the tight packaging of cells within aggregates would preclude nutrients from the culture medium from reaching the cells in the final stages of a passage.

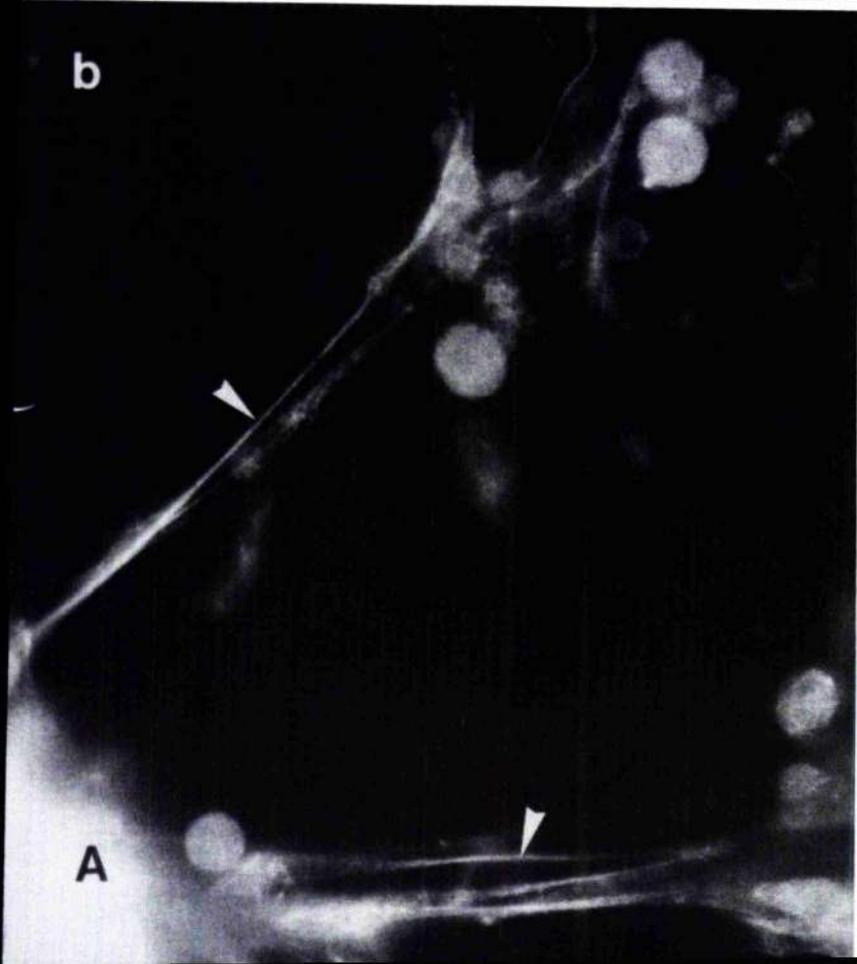
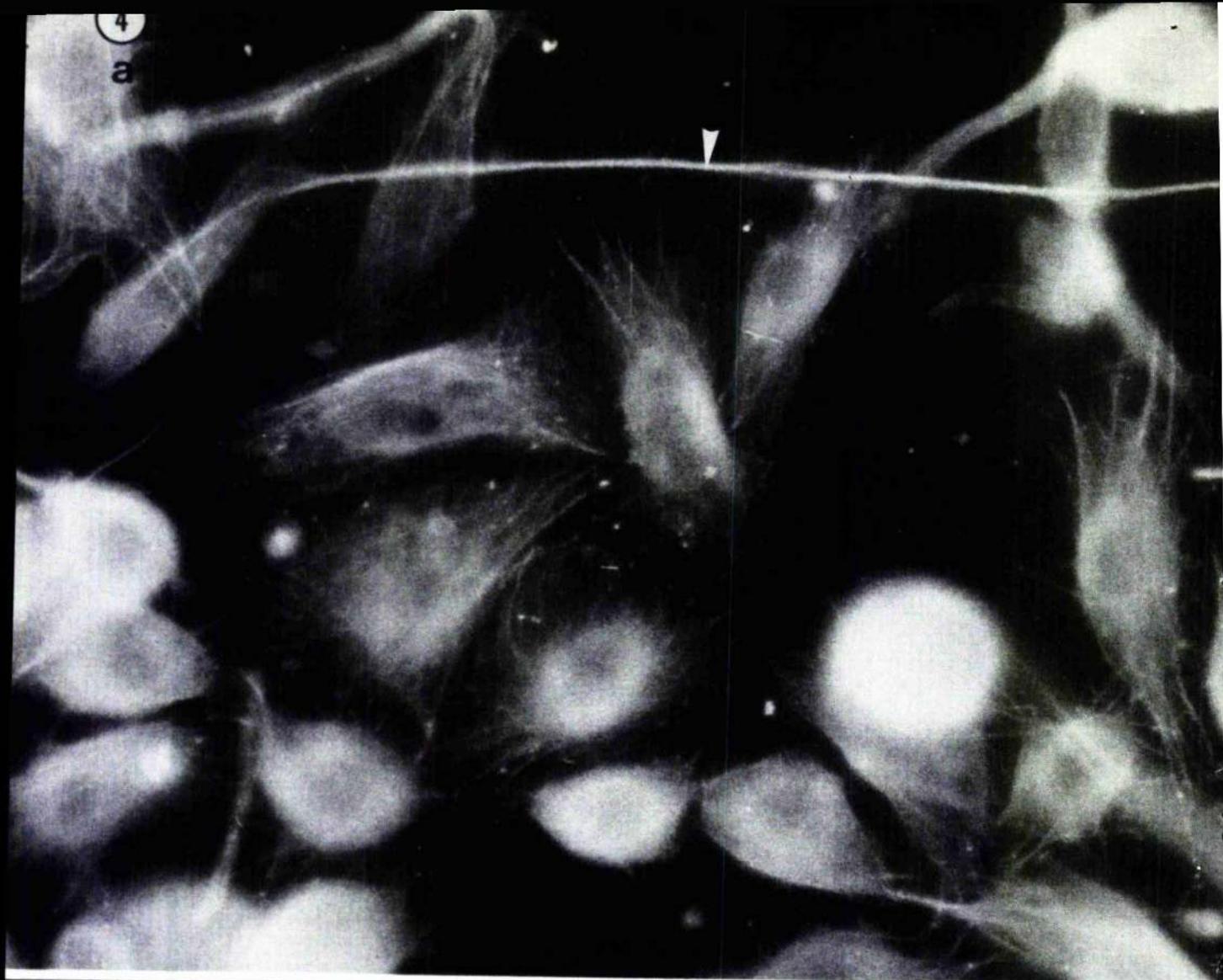
We have found that cell extensions can best be visualized by immunofluorescence after staining with monoclonal anti alpha tubulin. Before confluence is reached, a few cells may be found which have extensions extending over many cell diameters (Fig. 4a). In aggregating cultures, small groups of cells are found to be connected to larger aggregates via cell processes (Fig. 4b), and two adjacent aggregates are frequently connected by cellular bridges containing many cell extensions (Fig. 4c). In both these cases, further aggregation would be expected to occur.

One possibility for the causal mechanism for the aggregation process may be that found in the case of fibroblasts cultured on a collagen gel (Harris, Stopak and Warner, 1984). Here, traction brings neighbouring cells into contact, where they adhere to one another. This process results in the formation of periodic condensations connected by bridges of stretched and aligned cells very similar in appearance to disc cell aggregates (see Fig. 4b, Harris, Stopak and Warner, 1984). In our system, we find that cells grown on tissue culture plastic aggregate, whereas in the fibroblast system a collagen gel was required for condensation to occur. Traction is now well understood *in vitro*, and has been proposed as a causal mechanism in a number of developmental systems, such as chondrogenesis, feather morphogenesis, and somitogenesis. However, the link between traction and such morphogenetic systems remains tenuous (for discussion see Bard 1990 chapter 5).

The ability of mesenchymal cells to aggregate *in vitro* depends on the cells applying a force that can deform their substratum (Bard, 1990). If traction is to operate in our culture system, we might suppose that our disc cells laid down some extracellular material on the surface of the plastic on which cells could exert a tractile force. If this was so, then cells plated onto a used culture surface from which the cells had been removed by pipetting might be expected to aggregate more rapidly than cells plated onto a surface lacking any extracellular material. This has been tested by growing cells for one week in six-well Nunc multiwell dishes, and then flushing the cells off the plastic by vigorous pipetting, and removing all but a trace of medium. Immediately after this, fresh cells and medium were plated into the wells. These cells showed an initial difference in binding to the tissue culture plastic but as the cells grew up, confluence and aggregation

Fig. 3. The ultrastructure of cells in aggregates. (a) Tight packing of cells in aggregates of line CME W1 (passage 28). An array of virus-like particles (V) may be seen in a nucleus. $\times 18,000$. (b) A loosely packed region of an aggregate in line CME L1 (passage 28). Dozens of cell extensions (CE) may be seen. $\times 18,200$. (c) LS and (d) TS of cell extensions in aggregates of CME W1 (passage 28), showing numerous microtubules (MT) within them. In the latter micrograph, virus-like particles (V) may be seen in the cytoplasm of the cell. c $\times 65,000$; d $\times 63,000$.





occurred over the same time-scale and in a similar fashion in experimental and control wells. This suggests that a cell-cell phenomenon rather than a cell-substrate phenomenon was involved. We have also noticed that cells appear to become more adhesive to each other and less to the culture dish as time in culture and cell density progresses.

An alternative, and in our view more plausible possibility for the aggregation mechanism in our culture system involves the contraction of epithelial processes. The elongated, microtubule-containing cell extensions found in aggregates are very similar to the basal processes or feet which are involved in the contraction of the larval epidermis of *Calpodes* (Locke and Huie, 1981; Delhanty and Locke, 1989), in the contraction of the peripodial membrane in *Drosophila* (Milner, Bleasby and Pyott, 1983), and in epithelial cell rearrangements (Fristrom, 1988). Epidermal feet elongate and then contract as the cell sheet shortens, effecting a contraction in the surface area of the epidermis as the cells become columnar (Delhanty and Locke, 1989). Such a mechanism would be substrate independent, and we plan to investigate this possibility further.

Viruses in the cell lines

Virus particles were commonly found both

within the nucleus and the cytoplasm of cells in aggregates, often in paracrystalline arrays (Fig. 3a, d). This was expected, as Plus (1978) has screened 47 different *Drosophila* cell lines, and has found 32 of these to be infected with viruses. Indeed, most strains of flies carry viruses, and where they are endemic they are not pathogenic. More viruses are found where the conditions are suboptimal, for example in old flies and in malnourished adults (Schneider, 1975). It is therefore not surprising that virus-like particles are much more common in aggregates of cells at the end of a passage which, as discussed above, will probably be starved of nutrients, than in vesicle cells in primary embryonic cultures (Johnson, personal communication).

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Fig. 4. The visualisation of cell processes in cloned wing cell lines (a—clone C9; b, c—clone 13) by immunofluorescence. (a) A long cell process (arrowed) 6 days after culture initiation, before confluence is reached. $\times 1,470$. (b) Small, outlying groups of cells radially connected by cell processes (arrowheads) to a large aggregate (A) 8 days after culture initiation. $\times 978$. (c) Two aggregates (A) joined by a cellular bridge containing a number of cell processes (arrowheads) 8 days after culture initiation. $\times 1,115$.

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The expression of PS integrins in Drosophila melanogaster imaginal
disc cell lines

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Running title: PS integrins in disc cell lines.

Abstract

Drosophila imaginal disc cell lines show a characteristic pattern of aggregation in culture, which appears to be due to cell-cell rather than cell-substrate interactions. We have examined the distribution of PS integrins in wing and leg cell lines, and find that these integrin homologues are expressed preferentially in aggregates. Cell sheets, small cell clumps and chains of cells express antigen at points of cell-cell contact only.

Introduction

Imaginal disc cells growing as continuous cell lines show a very characteristic pattern of aggregation between passages to form large, multicellular aggregates. (Peel, Johnson and Milner, 1990). The process of aggregation in culture, and the internal structure of the aggregates, have been studied using scanning and transmission electron microscopy. In particular, the role of cell processes was examined by looking at patterns of microtubule deployment in aggregating cells with anti-tubulin immunofluorescence.

The present paper is a first attempt to examine cell-cell interactions during the process of aggregation by looking at the expression of cell surface molecules known to be important in adhesive mechanisms. In this study, the expression of Position Specific (PS) integrins was examined. These were originally identified in a monoclonal antibody screen as cell surface glycoproteins which showed spatially restricted distributions in the mature wing imaginal disc. (Wilcox et al., 1981; Brower et al., 1984). PS1 and PS2 antigens were defined by their preferential binding to cells of the dorsal and ventral compartments respectively. PS antigens have been found to be the invertebrate equivalents of vertebrate integrins (Bogaert et al., 1987; Leptin et al., 1987,1989), these being cell surface molecules which bind to components of the extracellular matrix and interact with the cortical cytoskeleton. The PS2 integrin has been found to be analogous to the vertebrate fibronectin receptor (Bogaert et al., 1987) and this molecule was selected for this study. The expression of PS integrins in our cell lines was investigated using immunocytochemistry, to see if there was any correlation between its expression or localisation and aggregation.

Materials and Methods

The cell lines used were initiated as described in Currie, Milner and Evans (1988) and Cullen and Milner (1991), and cloned lines were set up as discussed in Peel and Milner (1990). Labtek culture chamber slides were used for immunocytochemistry, cells being plated out at differing densities based around the figure of 7.5×10^5 cells/ml, which was found to be the optimal cell concentration for these slides. The cells were washed with phosphate buffered saline (PBS) and then fixed for 10-15 minutes with 4% paraformaldehyde in PBS. After two five minute washes in PBS 1% BSA the cells were incubated for 2 hours at room temperature in primary antibody (anti-PS2 mouse IgG CF2C7) which was diluted in PBS 1% BSA at 1:250. The cells were washed extensively in PBS 1% BSA as previously and then incubated for 1 hour at room temperature in diluted biotinylated anti-mouse IgG (Vector). After 1 hour the cells were washed twice for 10 minutes each time in PBS 1% BSA. Then the cells were incubated for 1 hour at room temperature in ABC reagent (Vector). The cells were then washed as before in PBS 1% BSA and then in PBS alone.

The cells were stained for horse radish peroxidase (HRP) as follows. The cells were incubated for 2-5 minutes in a substrate solution consisting of 3.8 mls 0.05 M acetate buffer pH 5.0, 0.2 ml stock 3-amino-9-ethylcarbazole (AEC) made up as 20 mg AEC in 2.5 mls dimethylformamide and finally 20 ul of 3% H₂O₂ (freshly diluted from a stock solution of 30% H₂O₂ with double distilled water) was added to the substrate solution. The reaction with HRP, which was terminated by washing with double distilled water, produced a red reaction product. The cells were then counterstained in Mayers' haematoxylin (BDH), and mounted in an aqueous mounting medium

(Aquamount).

Results and Discussion

During aggregation in culture, disc cells appear to become less adherent to the tissue culture plastic and more adherent to each other. This change in the growth pattern of the cells could either be a cell-cell or a cell-substrate phenomenon. Deposition of extra-cellular matrix (ECM) by the cells during early stages of culture could result in the changes that are seen during aggregation. Alternatively, these changes may be the result of a purely cell-cell process. In order to test the hypothesis that changes in the substrate brought about the pattern of aggregation, cells have been plated out both onto fresh culture dishes and also onto dishes in which cells had previously been grown for one week and hence had acquired any ECM material that might have been secreted during that period (Peel, 1990). If substrate influences were involved in aggregation, cells plated onto a used culture surface would be expected to aggregate at a different rate, compared to cells plated onto a surface lacking any ECM material. Used culture dishes were prepared by growing cells for one week, and then vigorously pipetting the cells off the plastic and removing all the medium whilst leaving the surface damp. Fresh medium and cells were then added.

The cells that were plated out onto used culture dishes showed some initial differences in binding to the tissue culture surface. The cells initially did not bind as evenly as control cells plated onto fresh tissue culture plastic. However, as the cultures progressed, no differences were seen in the way that cells grew, reached confluence and started to aggregate (Peel, 1990). This suggests that aggregation is more likely to be dependent on a cell-

cell process then on a cell-substrate mechanism.

In an attempt to characterise cell-cell interactions in culture, the expression of the PS2 integrin was analysed during aggregation. Fig 1 shows the staining pattern seen in aggregating wing cells of a cloned line, Cl.9. Aggregates of cells show heavy staining with PS antibodies. Indeed, staining appears to correlate with aggregation. Single cells appear to show little or no staining whereas heavy staining occurs where cells are aggregating (fig. 1A). Fig. 1B shows a close up of the edge of an aggregate, the cells of which show diffuse staining all over their surface whereas two cells nearby which have no firm connections with the aggregate do not show any staining.

In more isolated groups of cells, staining with PS2 integrins can clearly be seen in areas of cell-cell contact (fig. 1C,D). Fig. 1C shows a sheet of cells all showing staining at contact points between neighbours. This type of staining can be seen particularly clearly in fig. 1E, where a chain of cells all show staining at their points of contact. Similar PS2 expression patterns were found for five other cloned wing lines (Cl.C9, Cl.7, Cl.13, Cl.8+, Cl.8R), and two cloned leg lines (L1A and L1P). PS1 and PS3 integrins showed similar patterns of expression.

PS integrins were originally identified as showing spatially restricted expression patterns on mature wing imaginal discs. Thus, not all the cells of a disc were stained by all the different antibodies. In the late third instar disc from which these cell lines were derived, the pattern of PS1 and PS2 integrin staining is related to the dorsoventral lineage restriction (Brower *et al.*, 1985). PS1 antibodies stain dorsal cells and PS2 antibodies ventral cells. Therefore one might expect that ordinary uncloned lines from

these discs would be heterogeneous in their PS integrin staining and that cloned lines would show a more homogeneous pattern, all cells either staining or not. However all the lines that were tested proved positive for both PS1 and PS2 suggesting that the cells had lost their identity as either dorsal or ventral. The dorsoventral lineage restriction pattern of PS integrin staining is not apparent throughout the whole development of the wing disc (Brower et al. 1985). This restriction only appears during the third instar and is approximately coincident with the appearance of a zone of reduced cell division along the wing margin separating the dorsal and ventral areas (O'Brochta and Bryant 1985). Perhaps in culture where no such area separates the dorsal and ventral cells such a distinction is negated.

Immunocytochemistry of aggregating disc cells suggests that increased cell-cell contact in aggregates results in a higher expression of PS integrins. Indeed it suggests that single cells do not express integrin but that only cells in aggregates do. This leads us to suspect that cell-cell contact induces the expression of integrin.

As already mentioned, aggregates of cells showed heavy diffuse staining with PS antibodies, whereas single cells lacked any staining. Besides single cells and large multicellular aggregates there were other groups of cells in culture. These consisted of small groups of relatively few cells which had joined together but had not yet got to the stage of an aggregation. These more isolated groups of cells showed staining at regions of cell-cell contact very reminiscent of the type of staining that is seen with cadherin adhesion molecules (Takeichi, 1988).

Not all integrins are restricted to cell-ECM interactions.

Integrins are also involved in cell-cell interactions especially in the immune system. These integrins, notably the LFA-1 lymphocyte adhesion receptor are involved in cell-cell adhesive mechanisms between lymphocytes (Hynes 1987; Springer 1990). Another lymphocyte integrin VLA-3 has been localised to cell-cell contact sites and so seems to be involved in cell-cell adhesion (Kaufman et al. 1989). Therefore integrins can participate in cell-cell adhesive interactions.

Recently, other integrin molecules have been found to be involved in mediating cell-cell adhesion in epithelial cells (Carter et al., 1990; Larjava et al., 1990). These studies revealed integrin staining at cell-cell contact sites very similar to that seen in imaginal disc cell lines. Either integrin molecules are interacting directly between adjacent cells, or there are as yet unidentified ligand molecules that can bridge cell-cell interactions. Information from lymphocyte integrins suggests that ligand molecules may be involved.

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Figure legend

Figure 1

The distribution of PS2 integrin in cells of the cloned wing line Cl. 9 (passage no.42) at 3 days post passage.

A Preferential staining of aggregates (A). Single cells interspersed between the aggregates show little staining.

B Two unstained cells (C) in close proximity to an aggregate which expresses PS2 uniformly.

C A sheet, D a clump and E a chain of cells expressing PS2 at points of cell-cell contact only.

Bar represents 100 um in A, 20 um in C and D, and 10 um in B and E.

