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The growth and differentiation *in vitro* of leg and wing imaginal disc cells from *Drosophila melanogaster*

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Summary

We have devised a new *in vitro* culture system in which cells from dissociated *Drosophila* leg and wing imaginal discs grow and differentiate. Primary cultures consist of epithelial and fibroblast-like cells, together with some lamellocyte-like cells. These cultures have given rise to continuously dividing leg and wing cell lines, in which epithelial, fibroblast-like, lamellocyte-like and distinct sickle-shaped cells are found. Vesicles composed of epithelial cells form in primary cultures and these differentiate imaginal cuticular structures under the influence of 20-hydroxyecdysone. Two types of cuticle-like material are formed spontaneously in established cultures. One type is present as thin, untanned sheets resembling apolysed pupal cuticle, while the other consists of thicker, tanned material similar to imaginal cuticle.

Key words: *Drosophila*, imaginal disc, tissue culture, growth, leg, wing, *in vitro*.

Introduction

Despite the high level of interest in the developmental biology of the fruitfly, progress in *Drosophila* tissue culture has been slow. Several culture media based on analyses of larval haemolymph have been devised principally for the culture of embryonic tissues (Echalier & Ohanessian, 1970; Shields & Sang, 1970). In primary embryonic cultures, a wide variety of cell types differentiate. These cell types include nerves, blood cells and both larval and adult muscle and fat cells (Shields *et al.* 1975; Dübendorfer *et al.* 1978) and imaginal disc cells (Dübendorfer *et al.* 1975). These disc cells grow in hollow spherical vesicles and, when challenged with the moulting hormone ecdysone, differentiate imaginal bristles and trichomes. Recognizable patterns of adult structures are not formed, however, unless the vesicles are allowed to differentiate *in vivo* (Dübendorfer *et al.* 1975). Similar vesicles have been obtained from whole embryos cut into two or three fragments (Schneider, 1972). Although these vesicles grow for a time, as yet it has not proven possible to establish a cell line from them (Sang, 1981).

A considerable number of cell lines has been obtained from primary embryonic cultures, over 30 being listed by Sang (1981). However, the cellular origin of these lines is unclear and this significantly limits their value for developmental studies. Little progress has been made in obtaining cell lines from specific larval or adult tissues, although short-term organ culture has been used, for example, to study imaginal disc morphogenesis and differentiation (Fristrom *et al.* 1973; Milner & Muir, 1987). Much effort has been put into devising a medium that would support the proliferation in culture of *Drosophila* imaginal disc cells and two studies have met with limited success. In the first of these, Davis & Shearn (1977) designed a complex, serum-free medium which supported regenerative growth from cut surfaces of imaginal discs, but not the proliferation of individual cells. Transdetermination occurred in such cultures (Shearn *et al.* 1978).

In a second study, Wyss (1982) developed a new defined medium supplemented with insulin, which has been shown to stimulate division in primary embryonic cultures (Seecof & Dewhurst, 1974), and

also with an extract of adult flies. Wyss (1982) dissociated imaginal discs with protease VIII and was able to demonstrate division to the point where isolated cells would form colonies containing up to 100 cells. Further proliferation was not observed, however, and no cell lines were established.

We have devised culture conditions that enable imaginal disc cells to proliferate continuously *in vitro*, and we have established cell lines from both leg and wing discs. This report details the primary culture of imaginal disc cells and the establishment of cell lines in our new culture system.

Materials and methods

Dissection and dissociation

Eggs from stock Oregon R were collected over a 3 h period, washed and placed on a filter paper in a scintered glass funnel connected to a vacuum line. The eggs were then surface sterilized by exposure for 5 min to a solution of sodium hypochlorite (BDH, diluted 50 %) and washed thoroughly with sterile distilled water. Surface-sterilized eggs were distributed on pieces of filter paper to sterile Petri dishes containing David's (1955) killed yeast medium, and the resulting larvae grown under axenic conditions. Groups of 30–40 discs were dissected from late third instar larvae taken from Petri dishes in which a number of animals had already pupariated, collected in a drop of culture medium and kept on ice. The medium used was Shields and Sang's M3 culture medium (Shields & Sang, 1977) modified for the addition of low serum levels (Edwards *et al.* 1978) and referred to below as MM3. To this, 2 % foetal bovine serum (FBS) had been added. Discs were then cut into three to five fragments to facilitate dissociation. These fragments were transferred to a 10 ml plastic centrifuge tube, as much of the medium as possible was removed, and 0·8 ml of calcium- and magnesium-free Dulbecco's phosphate-buffered saline (CMF-PBS, Flow) with 2 mg ml^{-1} dispase (Boehringer Mannheim) and 4 mM-EDTA was added. The tube was then rotated at about 1 rev. s^{-1} on a bottle roller at room temperature for 45 min. 80 μl of 1 % Bacto trypsin (Difco) in CMF-PBS was then added, giving a final concentration of 0·1 % and this was left for an additional 10 min. Fragments were further dissociated by brief shaking on a vortex mixer. The preparation was then centrifuged for 4 min at 150 *g* in a refrigerated centrifuge to pellet single cells and small clumps. The supernatant was then removed and replaced by 0·75 ml of MM3 supplemented with 2 % FBS, 0·125 i.u. ml^{-1} insulin, 1 ng ml^{-1} 20-hydroxyecdysone and 50 μl ml^{-1} of fly extract (see below). The cells were pipetted up and down 15 times to further dissociate remaining clumps, and aliquots of 150 μl were pipetted into wells of a 96-well plate (Nunc). These plates were then incubated unsealed at 25 ± 1 °C in a humidified environment of 5 % carbon dioxide in air. In a few cases, large fragments of discs were cultured in 96-well plates without dissociation.

Medium and cell culture

After many variations had been tried, it was discovered that a satisfactory medium for the survival and division of imaginal disc cells was MM3 together with four other additives. These were 2 % heat inactivated FBS, 1 ng ml^{-1} 20-hydroxyecdysone (Simes, Milan), 0·125 i.u. ml^{-1} insulin (Sigma, cat. no. 15500) and 50 μl ml^{-1} of a fly extract, which was prepared as follows. 200 well-nourished mature flies, of which at least 150 were female, were homogenized in a tissue homogenizer (15 ml capacity, 0·1 mm clearance) with 1·5 ml of MM3 without additives. This homogenate was centrifuged at 1500 *g* for 15 min in a refrigerated centrifuge. The supernatant and the oily film above it were removed and heat treated at 60 °C for 5 min. This preparation was then spun at 1500 *g* for 2 h in a refrigerated centrifuge and the supernatant (fly extract) was removed and stored at −20 °C prior to use. Medium was resterilized by filtration (0·22 μm pore size) after the addition of fly extract. Cells were passaged at regular intervals after division had started, first into 24-well plates (Sterilin) and then into 5 cm Petri dishes (Nunc). For passaging, cells were washed in CMF-PBS and then exposed to a solution of Bacto trypsin (0·1 %) and EDTA (4 mM) in CMF-PBS for 5 min, after which MM3 with 2 % serum was added to neutralize the trypsin. The medium was then sucked up and down to wash the cells off the plastic. Cells were centrifuged at 250 *g* for 6 min, the supernatant removed and the cell pellet resuspended in 1 ml of MM3 supplemented with 2 % FBS, 1 ng ml^{-1} 20-hydroxyecdysone, 0·125 i.u. ml^{-1} insulin, and fly extract at half the concentration used for culture initiation (25 μl ml^{-1}). Cells were then plated out at a concentration of 3×10^6 cells per 5 cm Petri dish.

Microscopy

Cells were viewed with an Olympus IMT inverted phase contrast microscope and photographed with an Olympus OM2 camera. For scanning electron microscopy, cells were fixed in phosphate-buffered 2·5 % glutaraldehyde (pH 7·6) and postfixed in 1 % osmium tetroxide (pH 7·6), both for 20 min (Tucker, 1967). Fixed material was dehydrated, dried in a Samdri 780 critical-point drier, coated in an Emscope sputter coater Sc500 and examined in a Jeol 35CF scanning electron microscope. Material for transmission electron microscopy was fixed as above, embedded in Araldite epoxy resin and 50–70 nm sections were cut on an LKB ultratome III. These were stained with uranyl acetate and lead citrate (Reynolds, 1963) for observation on a Philips 301 electron microscope.

Results

Primary cultures

Immediately after culture initiation, most cells were spherical and floated freely in the medium. However, after 24 h individual cells had adhered to the substrate and were beginning to flatten out (Fig. 1A). Over the next 2 days, many of these single cells died, and the surface of the plastic became populated by cells migrating out from undissociated clumps. It therefore

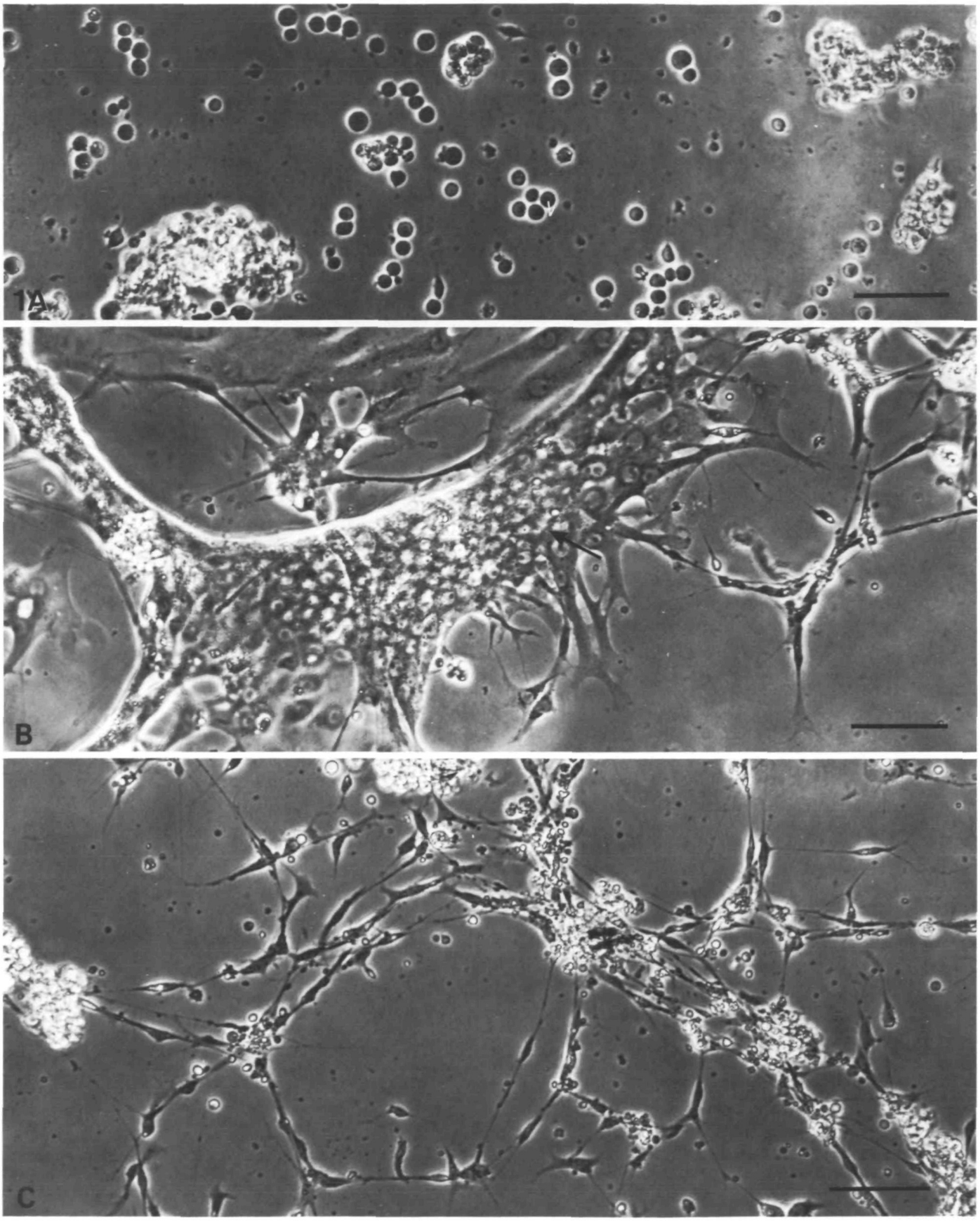

Fig. 1. The first week after culture initiation. (A) Individual cells and small clumps of cells from wing discs after 1 day of culture. (B) A sheet of leg epithelial cells (arrowed) 4 days after culture initiation. (C) Fibroblast-like cells from a leg culture 4 days after culture initiation. Bar, 50 μm.

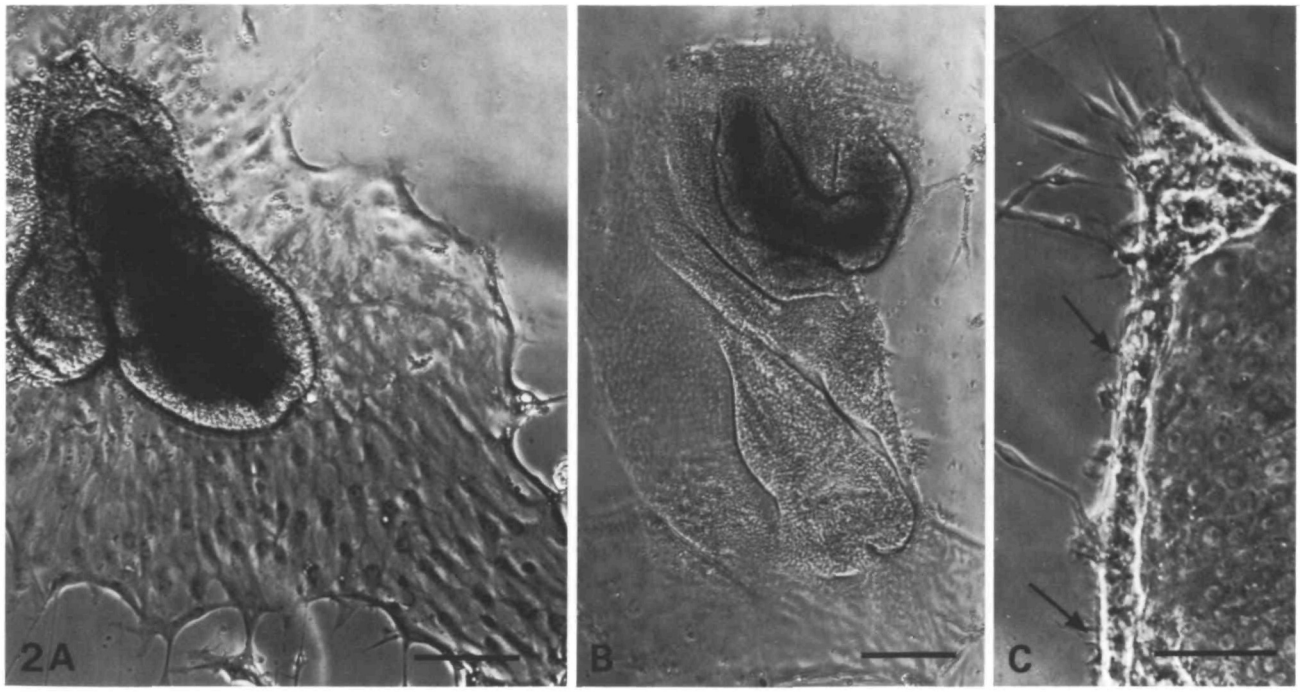

Fig. 2. Cells spreading from large untrypsinized fragments of wing discs. (A) Presumptive peripodial membrane cells spreading onto the plastic, after 7 days of culture. (B) Cells from the columnar epithelium spreading after 1 day of culture. Note the increasing separation of the disc folds during this process. (C) Epithelial sheet from a wing disc 12 days after culture initiation. The edge of the sheet appears folded back, and cells at the edge exhibit fibroblast-like processes (arrows). Bar, 100 μm in A,B, 50 μm in C.

seems important not to effect a complete dissociation of the discs.

Two main cell morphologies were already evident at this early stage. First, we found spreading sheets of epithelial cells, which were typically small (20 μm diameter), flat and polygonal, with a centrally placed nucleus and a dark, prominent nucleolus (Fig. 1B). The spreading of imaginal epithelial cells was particularly clear in cases where large fragments of discs were cultured without additional dissociation (Fig. 2A,B). It appeared in some of these cases that the spreading cells originated from the peripodial membrane (Fig. 2A), and in others from the columnar epithelium (Fig. 2B). The edges of such sheets were sometimes thickened in a manner reminiscent of the edge of the peripodial membrane during disc evagination (Fig. 2C; cf. Milner & Muir, 1987, fig. 1C). The second cell type evident was of fibroblast-like cells (Fig. 1C), many of which were bipolar, with a prominent nucleus and nucleolus. Cells at the edges of epithelial sheets could often be seen adopting a fibroblast-like morphology as they moved away from the sheet onto the plastic (Figs 1B, 2C).

Some of the clumps of undissociated tissue which did not adhere firmly to the substrate formed cellular vesicles after 4–7 days of culture (Fig. 3A). The production of such vesicles was greatly enhanced if the trypsinization step in the dissociation procedure was omitted and if the cells were placed in glass-bottomed wells. In a small number of cases, fibroblast-like cells were observed attached to both the vesicle surface and to the surface of the culture dish (Fig. 3D). These vesicles differentiated imaginal cuticular structures after treatment with 50 ng ml^{-1} of the moulting hormone 20-hydroxyecdysone for 6 days (Fig. 3B,C).

After one week of culture, many more cells had migrated out of the clumps, and continued to cover new areas of the wells. Scanning electron micrographs of leg cultures at this stage revealed the presence of large, oval, flattened cells ranging from 50 to 100 μm long (Fig. 4A) amongst the other cells. These were similar to a type of blood cell, the lamellocyte, previously identified in primary embryonic cultures (Dübendorfer & Eichenberger-Glinz, 1980). After three weeks in culture, fibroblast-like cells had begun to divide slowly and they most commonly produced random configurations (Fig. 4B), although occasional cases of cellular alignment have been noted (Fig. 4C), particularly in cultures derived from leg discs.

Establishment of cell lines

About 14 days after culture initiation, two types of cell started dividing rapidly. The first of these was the

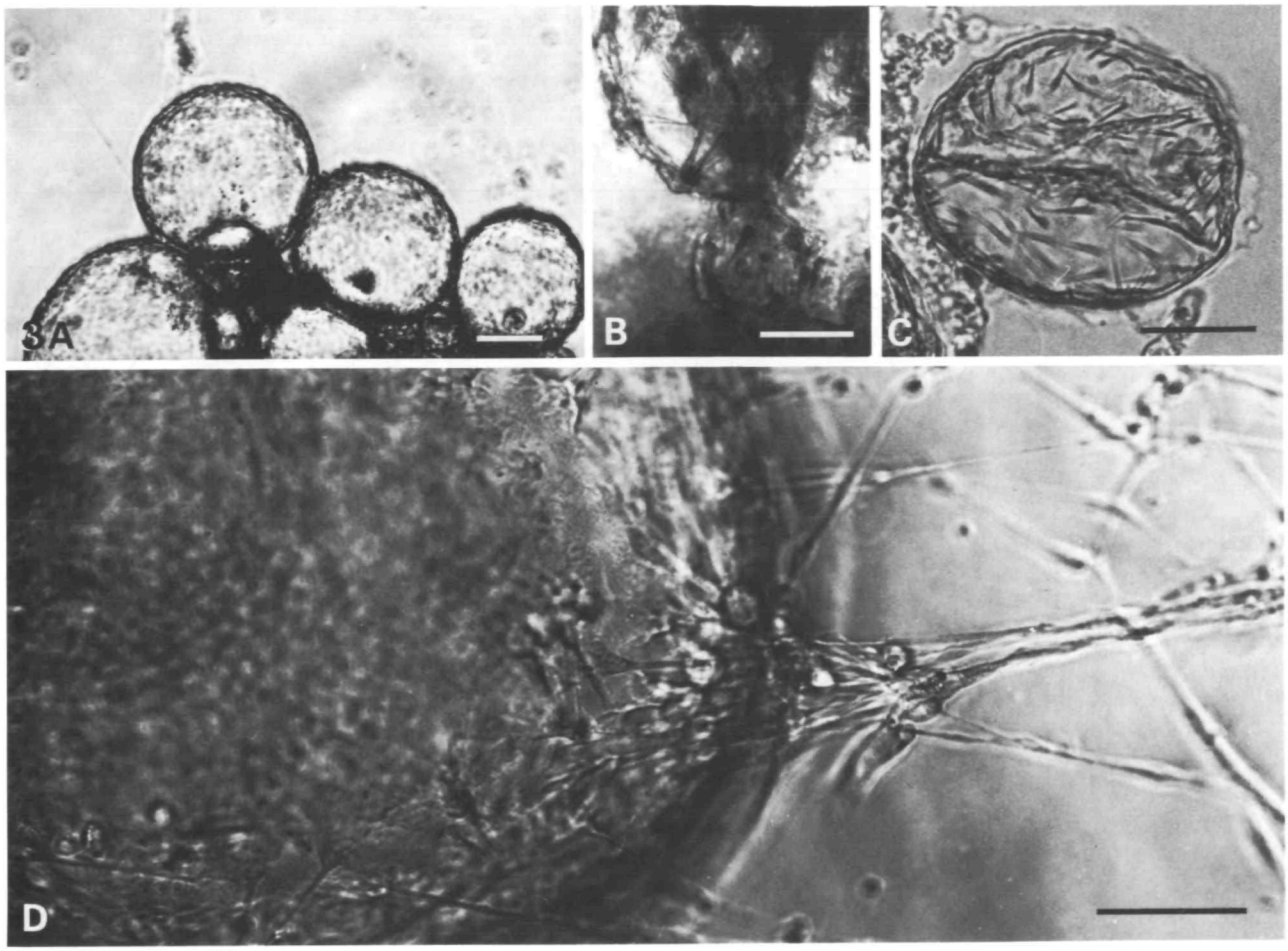

Fig. 3. The formation and differentiation of vesicles of epithelial cells cultured in glass-bottomed culture vessels. (A) Wing vesicles after 4 weeks of culture. (B) Bristles and (C) trichomes differentiated after treatment with 50 ng ml^{-1} of 20-hydroxyecdysone for 6 days. (D) Fibroblast cells attached both to the vesicle cells and to the plastic substrate after 53 days in culture. Bar, 50 μm in A,B & D, 100 μm in C.

small epithelial cell type, which began to divide much more rapidly than adjacent fibroblast-like cells, covering a large proportion of the base of the well (Fig. 4D). Second, spherical cells which originated on or near clumps of undissociated tissue began to divide rapidly to form clumps. Some of these cells flattened out and took on the bipolar morphology of the fibroblast-like cells. During the early stages of division, a new cell type became evident. These cells were sickle-shaped and were up to 100 μm long (Fig. 4E). The nucleus was not very prominent and there were numerous small dense vesicles mostly concentrated towards the centre of the cells. These sickle cells did not attach well to the plastic and were found floating freely in the medium.

After one month in culture, some of the rapidly dividing cells were washed off the plastic by gentle pipetting and passaged into the wells of a 24-well plate. One month later they were again passaged in a similar manner into 5 cm dishes, in which they were subsequently maintained. All subsequent passages followed the trypsinization procedure described in the Materials and methods. We have two wing cell lines, designated CME W1 and CME W2, which have now been maintained for 8 months, and two leg cell lines, CME L1 and CME L2, maintained for 6 months, all of which are passaged every 7–10 days. The percentage success obtained with this technique was quite high. Of 34 wells from six primary cultures, 15 produced rapidly dividing cells of the type from which we established our cell lines. The growth curve for cells from line CME W1, 5 months after culture initiation is shown in Fig. 5.

The established cell lines contained epithelial cells, sickle cells, fibroblast-like cells and lamellocyte-like cells (Fig. 6A,B,C). In many parts of the culture, small colonies of rapidly dividing cells became established and our observations suggest that these were derived from the fibroblast-like cells. Such colonies were often interlinked by distinct strands of cells

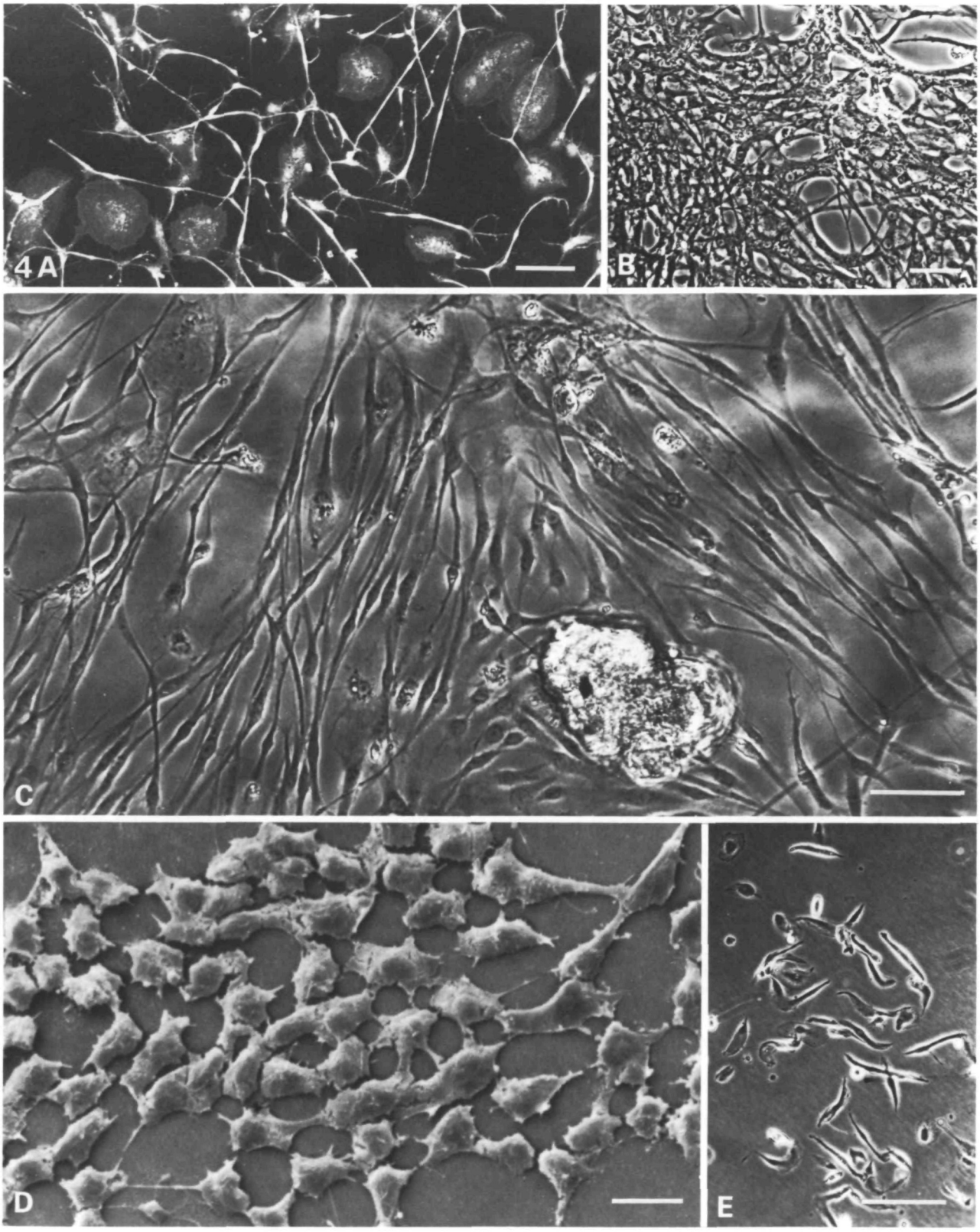

Fig. 4. Cell types present in cultures. (A) Flattened lamellocyte-like cells in a 1-week-old leg cell culture, together with fibroblast-like cells. (B) Random organization of dividing wing cells after 4 weeks of culture. (C) Cellular alignment in a 19-day leg cell culture. (D) Wing epithelial cells after 19 days in culture. (E) Sickle cells growing in cell line CME W2 after 6 months of culture. Bar, 50 μm in A & C, 100 μm in B & E, 10 μm in D.

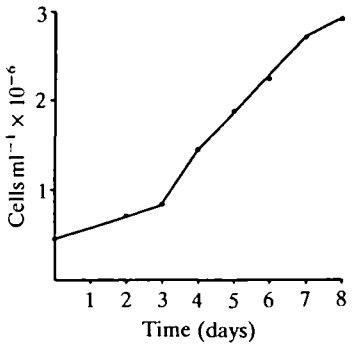

Fig. 5. Growth curve for the cell line CME W1 after 5 months of culture. Zero time is the time of initiation of a subculture.

(Fig. 6B). The fibroblast-like cells were frequently found overlying other cell types (Fig. 6A) and in such cases appeared to be attached to the substrate by their processes.

Cuticle secretion

After about 4 weeks of growth, a number of multilayered colonies were formed by dividing cells, and these subsequently appeared to exhibit two aspects of cuticle secretion. First, cells at the upper surface of some colonies acted in a coordinated manner during the second month of culture to produce sheets of untanned, cuticle-like material. Such sheets were then released from the surface of the colonies and could be found floating in the culture medium (Fig. 7A). Second, cells in some of the colonies were seen to melanize and produce thicker, tanned cuticular material (Fig. 7B). This was deposited around the outer surface of individual cells and in some cases totally encapsulated them (Fig. 7C). The extent of melanization and cuticle secretion decreased with time, although differentiation was still apparent in limited numbers of cells after 7 months of culture.

Discussion

We have developed a culture system in which imaginal disc cells will survive and proliferate to form a cell line. Previous studies fell substantially short of this goal. Davis and Shearn's medium supported growth of wounded imaginal discs (Davis & Shearn, 1977), but the discs did not adhere to the substrate and cells did not appear to spread onto the plastic. However, it seems that their medium was only marginally capable of supporting growth *in vitro*. Davis and Shearn's medium was supplemented with a very low level of insulin (0·0004 i.u. ml^{-1}), and was conditioned with fat body. In early work on insect cell culture, Grace (1962) found that the addition of insect haemolymph proved beneficial to cell survival *in vitro*. Wyss (1982) used a fly extract, which was extracted with diethyl ether, in conjunction with a high concentration of insulin (10 μg ml^{-1}) and a low level of 20-hydroxyecdysone (1–10 ng ml^{-1}). Although some cell division occurred in this medium, growth did not continue and no cell lines were established.

We have found three principal cell types in primary cultures, namely epithelial, fibroblast-like and lamellocyte-like cells. Cell lines are composed of epithelial cells, fibroblast-like cells, distinct sickle-shaped cells and less numerous lamellocyte-like cells. Epithelial cells in primary cultures grow either on the substrate or in spherical vesicles, depending on the nature of the substrate and degree of dissociation. Some cells at the edges of epithelial cell sheets send out processes and appear to assume a fibroblast-like morphology as they move across the substrate (Figs 1B, 2C), but as yet we are uncertain whether cells can convert between the two morphologies. A considerable number of blood cells adhere to the surface of discs *in vivo* (Milner & Muir, 1987). Some of these remain after dissection and are found in early stages of culture, and also in older cultures.

The vesicles of epithelial cells are very similar to vesicles of imaginal disc cells which grow in primary embryonic cultures (Dübendorfer *et al.* 1975) and to certain lepidopteran wing disc cell lines which also grow as fluid-filled epithelial vesicles (Lynn *et al.* 1982). We have shown that 20-hydroxyecdysone initiates the differentiation of imaginal cuticular structures in these vesicles and we have also found spontaneous cuticle secretion in our cultures, demonstrating that we are culturing imaginal epithelial cells. The thin, sheet-like material bears a striking resemblance to pupal cuticle (cf. Milner & Muir, fig. 2C), and its release from the surface of some colonies seems to be very similar to the process of apolysis which occurs *in vivo* and *in vitro* in intact imaginal discs. Similar material has been seen in primary cultures from whole embryos, although the precise origin of the cells that produced it was unknown (Shields *et al.* 1975). The thicker, tanned cuticular material (Fig. 7B,C) is strongly reminiscent of imaginal wing cuticle secreted *in vivo* (Johnson & Milner, 1987), although it is more amorphous and less-well structured than some adult cuticle. Imaginal cuticle is normally secreted from the apical surface of the cell only, and total encapsulation with cuticular material would appear to be deleterious to the cell (Fig. 7C). It is of interest that cuticle secretion can occur in response to a very low level (1 ng ml^{-1}) of 20-hydroxyecdysone, although the fly extract may contain some additional ecdysteroids.

It should be noted that imaginal discs contain one additional cell type, namely the adepithelial cells, which give rise to adult muscles during the pupal stage. Adepithelial cells have occasionally been seen to migrate out of intact discs cultured in unmodified M3, fuse to form myotubes and even contract

(M.J.M., unpublished observations). Fusion of fibroblast-like cells to form contractile myotubes has not been seen in our primary cultures and cell lines, and we consider it unlikely that adepithelial cells are contributing significantly to the dividing cell population. However, we cannot rule out the possibility

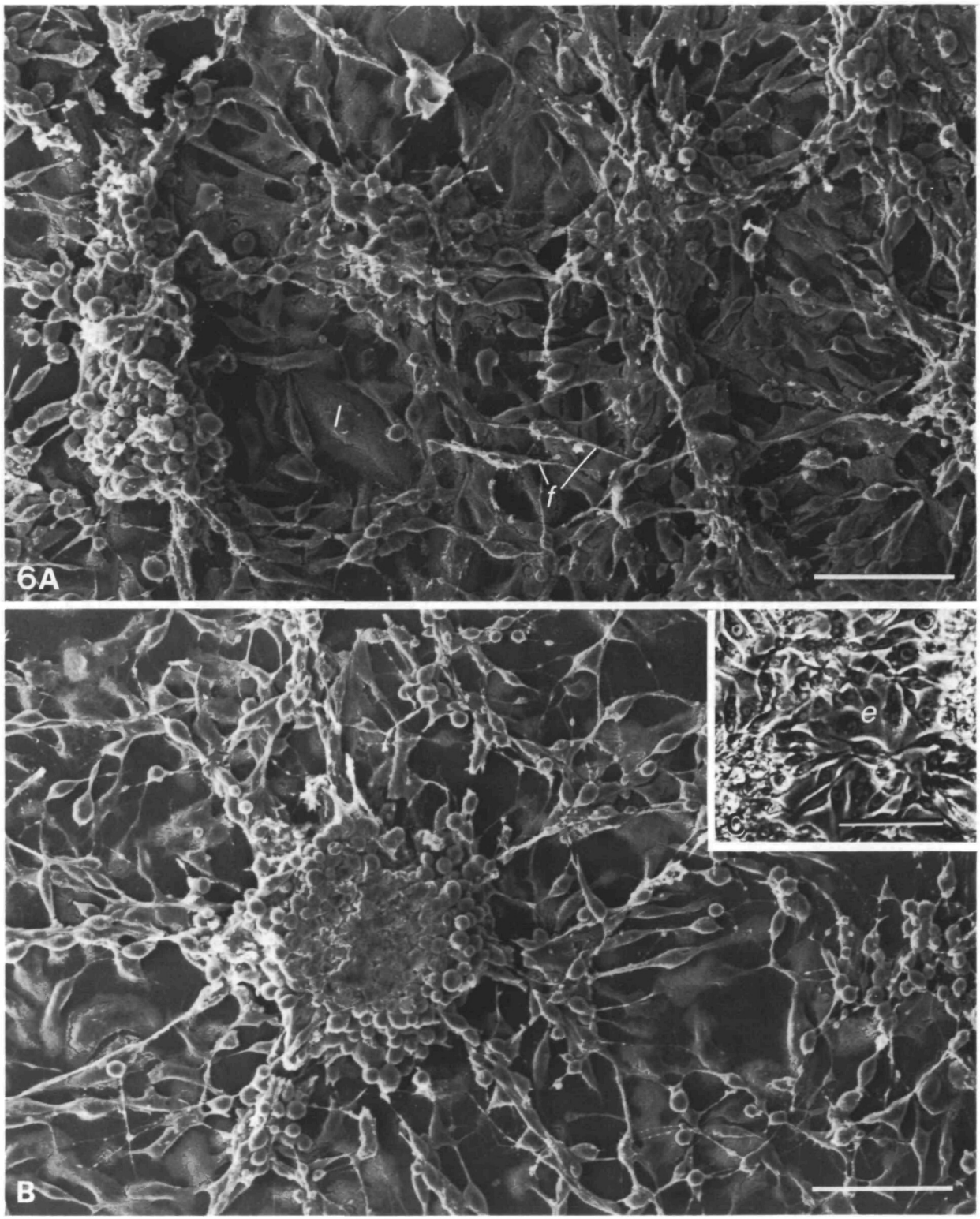

Fig. 6. Wing cells after several months of culture. (A) Lamellocyte-like (*l*) and fibroblast-like (*f*) cells after 7 months of culture. (B) Colonies of rapidly dividing cells, interlinked by distinct strands, after 7 months of culture. (C) Epithelial cells (*e*) after 5 months of culture. Bars, 50 μm.

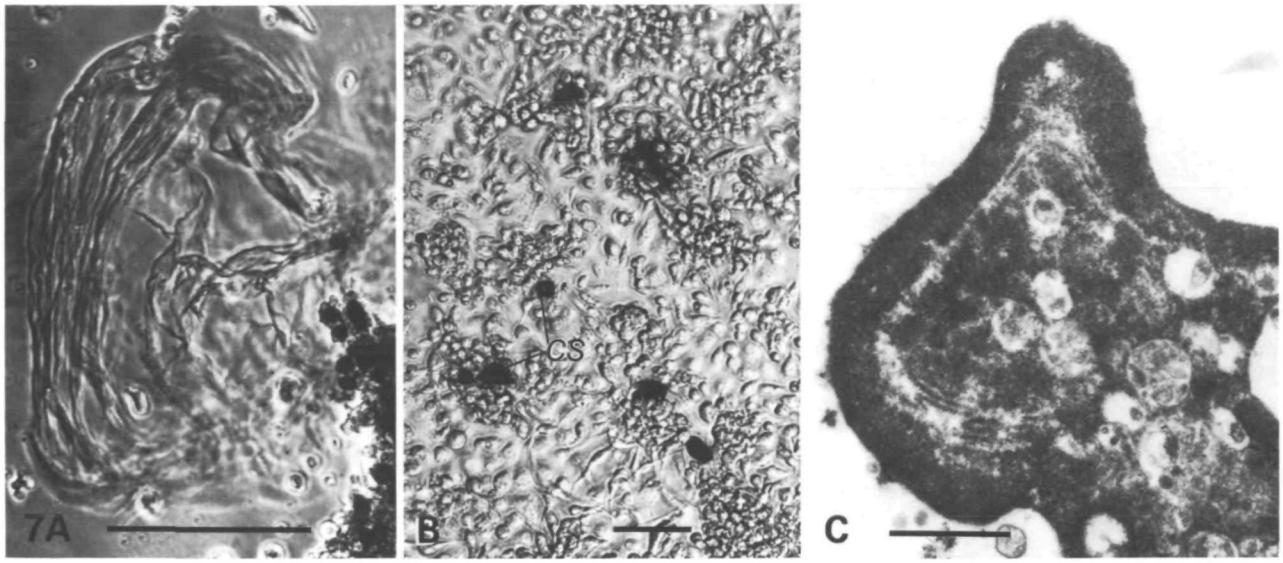

Fig. 7. Cuticle secretion in culture. (A) Thin, untanned cuticular sheet released from wing cell colonies after 5 weeks of culture. (B) Melanized, cuticle-secreting cells (*cs*) in colonies of dividing leg cells after 5 weeks of culture. (C) Thin section of a cuticle-secreting cell showing the dense, cuticle-like material which has encapsulated the cell. Bars, 100 μm in A & B, 0·5 μm in C.

that some adepithelial cells divide and persist. We are at present attempting to clone our existing cell lines in order to establish homogeneous cell populations.

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