SALIVARY EPITHELIAL CELLS IN PRIMARY CULTURE: CHARACTERIZATION OF THEIR GROWTH AND FUNCTIONAL PROPERTIES

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SUMMARY

Mouse submandibular salivary gland cells were grown in primary explant culture. After an initial period of degeneration within the explant, surviving epithelial cells proliferated rapidly and duct-like structures recolonized the explant. Autoradiographic studies showed that a peak of DNA synthesis occurred after 4 days *in vitro* and that proliferation was enhanced by insulin and hydrocortisone. These cells retained specialized secretory function (protease activity) for at least 2 weeks *in vitro*. This enzyme is a differentiated product of granular tubule cells *in vivo*. Between 6 and 10 days, explants attached to the substrate. An outgrowth developed, consisting largely of ultrastructurally identifiable epithelial cells which formed pseudoglandular structures in the monolayer. Epithelium survived for over 6 months in primary culture but could not be serially transferred. Secondary cultures were rapidly overgrown by mesenchymal cells.

INTRODUCTION

Many attempts have been made to culture normal adult differentiated epithelial cells. Compared with numerous reports of the growth of highly differentiated neoplastic cell lines (Buonassisi, Sato & Cohen, 1962; Yasumura, Tashjian & Sato, 1966; Richardson, Tashjian & Levine, 1969) and some instances of specialized mesenchymal cells being grown for long periods (Holtzer, Abbott, Lash & Holtzer, 1960; Konigsberg, 1960), there are very few cases where prolonged growth *in vitro* of normal epithelial cells has been convincingly demonstrated (Wigley, in press). Many critical studies came to the conclusion that differentiated epithelium either died or lost its specialization rapidly *in vitro* (Sato, Zaroff & Mills, 1960; Sandstrom, 1965; Le Guilly, Launois, Lenoir & Bourel, 1973a; Le Guilly, Lenoir & Bourel, 1973b).

In this study, the behaviour of adult male mouse submandibular gland was investigated *in vitro*. The adult mouse gland is a complex, hormonally-dependent (Lacassagne, 1940; Grad & Leblond, 1949), tubulo-acinar structure (Hollmann & Verley, 1965) which secretes both mucous and serous components of saliva (Junqueira, Fajer, Rabinovitch & Frankenthal, 1949; Shear, 1972). Several authors have investigated the behaviour of young or adult submandibular gland in organ culture (Trowell,

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1959; Tapp, 1967; Lucas, Peakman & Smith, 1970) and found that acinar cells degenerated and ductal epithelium became hyperplastic. This response resembled the *in vivo* response to arterial ligature (Standish & Schafer, 1957). The cultured gland did not respond *in vitro* to testosterone, thyroxin or somatotrophin however, which are thought to act directly on these cells *in vivo*, but hydrocortisone and insulin markedly improved epithelial cell survival.

Various authors have attempted to grow submandibular epithelium in cell culture systems (Kreider, 1970; Gallagher, Marsden & Robards, 1971; Marcante, 1973; Brown, 1973) and most believed their cell lines to have been derived from mucin-secreting acinar cells but in one instance (Brown, 1973), tumours produced after treatment of such cells with chemical carcinogens resembled typical myxoid or epithelioid forms of transformed cell sarcomas (Franks, Chesterman & Rowlatt, 1970), suggesting that the cells were mesenchymal.

In this study, an attempt has been made to identify positively the cells which survive and proliferate in a tissue culture system using adult male mouse submandibular gland. Both morphological and functional criteria have been correlated to demonstrate the *in vivo* cell type from which long-term primary epithelial cell cultures derive.

METHODS

Culture techniques

Young adult (4–6 month) male C57/BL/Icrf a^t mice were used in all experiments. The submandibular glands were chopped, using fine curved scissors, in a few drops of nutrient medium (Waymouth's MB 752/1) (Waymouth, 1959) plus 10 % calf serum (Flow Labs., Irving, Scotland), to obtain a homogeneous preparation of explants of 0·5–1·0 mm^3. Fifty-millimetre Petri dishes (Nunclon Nunc, Denmark) were prepared with or without Melinex coverslips (Boyden Data Papers Ltd, London) and 5 ml Waymouth's medium plus 10 % calf serum. Explants adhered poorly to glass. In some experiments hormonal supplements were included at the following final concentrations: insulin (Boots Pure Drug Co., Nottingham, England, sterile acid solution) at 40 μg/ml, 1·0 i.u./ml; hydrocortisone (Steraloids, Croydon, Surrey, England) at 1·0 μg/ml; testosterone (British Drug Houses, Poole, England) at 0·01 μg/ml; and thyroxin (Sigma Chemical Co., London, England, Sodium salt) at 0·2 mg/ml. Isoproterenol (IPR-HCl – a gift from Professor K. Hellmann, Imperial Cancer Research Fund) was used in one series of experiments at final concentrations of 0·1, 0·01 and 0·001 mg/ml. Fifteen to twenty explants were distributed to each Petri dish. These were kept in a gassed incubator (5 % CO_2:95 % air) at 37 °C in a humidified atmosphere. Medium was changed twice weekly.

Histology and autoradiography

Sections of explants were stained with Ehrlich's haematoxylin and eosin (H & E) for histological investigation. In some experiments, growth characteristics of cells within the explants were studied. Explants were fixed after various 24-h exposures to 1·0 μCi/ml ([*Me*-^{3}H]-thymidine in the culture medium (sp.act. 5 Ci/mM, Radiochemical Centre, Amersham, England). Radioactive label was given to groups of explants on days 0–1, 1–2, 3–4, 5–6, 7–8, and 9–10. Medium was changed at the usual times, so that only on days 3–4 and 7–8 was thymidine added to fresh medium.

Sections were exposed to Ilford L4 emulsion (Ilford, England) at 4 °C for 3 days. Developed, stained autoradiographs were counted as follows. Each whole explant section was scored separately (one observation) and the percentage of labelled epithelial cells calculated for

each (L.I.). All cells except isolated single cells or cells in blood vessels were included. From 5–12 explants counted at each culture time, average labelling indices and standard deviations were calculated and the results plotted graphically.

Histochemistry

Mucins were demonstrated with the Alcian blue/periodic acid/Schiff (AB/PAS) method (Lev & Spicer, 1965). Sections were then counterstained with orange G.

'*Protease*' activity was demonstrated using 7-μm cryostat sections of pelleted explants cultured for various times. Sections were then picked up on substrate films (Daoust, 1965) of developed photographic emulsion (Kodak AR 10, Kodak Ltd, Kirby, England) and incubated in a humidified atmosphere at 36 °C for 1·5 h. Slides were fixed, dehydrated and mounted for comparison with adjacent, H & E stained sections. Clear areas in a dark background indicated the localization of proteolytic activity.

11β-*hydroxysteroid dehydrogenase* (11β-HSD) activity was demonstrated using the method of Baillie, Ferguson & Hart (1966). Experimental hydrocortisone and control cortisone (lacking the 11β hydroxyl group) substrate solutions (Steraloids Ltd, Croydon, England) were used at 0·125 mg/ml, dissolved in a minimal amount of dimethylformamide. The reaction was performed in phosphate buffer at pH 8, containing 2·0 mg/ml nicotinamide adenine dinucleotide (Sigma Chemical Co., Kingston-upon-Thames, England) and 0·5 mg/ml nitroblue tetrazolium (Sigma); 7-μm cryostat sections of pelleted explants were incubated on coverslips in 0·5 ml of substrate solution for 2–2·5 h at 37 °C. Insoluble reduced tetrazolium salt was seen as a deep blue coloration at sites of 11β-HSD activity.

All histochemical experiments included positive controls using sections of uncultured mouse submandibular gland.

Electron microscopy

Explants or cell monolayers on Melinex coverslips were fixed in 2·5 % glutaraldehyde, postfixed in osmium tetroxide, dehydrated through graded acetones and embedded in Araldite. Coverslips were removed and selected areas prepared for sectioning in the plane of the monolayer. Where sections were required in the plane perpendicular to the monolayer, a second layer of Araldite was poured on to the undersurface of the cells. When possible, semithin 3-μm sections were cut and stained with toluidine blue (1 %) at 60 °C. Ultrathin sections were double stained with uranyl acetate and lead citrate, coated with a thin carbon film and viewed in a Hitachi HS-7S or Siemens Elmiskop 1A electron microscope.

RESULTS

Histological changes within the explant

Acinar cells rapidly became degenerate and were lost from the explant during the first 24–48 h *in vitro*. Granular tubule cells gradually lost their secretory granules and assumed a simple cuboidal or slightly flattened shape. Relatively few explants contained striated duct cells in the plane of section but, where present, these remained unchanged during the early culture period. Intercalated ducts were difficult to identify histologically. After 2 days *in vitro*, most viable epithelial cells existed as small groups in part of otherwise degenerate tubular structures which resembled granular tubules in size and distribution. Isolated mesenchymal cells and capillary structures were also seen.

From the third day onwards, epithelial cells began to increase in number and form rudimentary duct-like structures or ribbons of cells. The surrounding acellular areas became fibrous and were gradually infiltrated by regenerating ducts. After 8 days, explants had the appearance shown in Fig. 2. Here, the cultures were grown

in insulin and hydrocortisone-supplemented medium, which improved the organization of cells into glandular structures and also increased the height of some cells to a low columnar shape. After 8 days, numbers of cells within explants were higher in hormonally treated groups. Hormones thought to act specifically on granular tubule cells (testosterone and thyroxin) did not affect growth or survival of proliferating epithelium. Isoproterenol, a β-adrenergic drug which primarily stimulates acinar cells *in vivo* (Barka, 1965), did not improve survival of these cells *in vitro*.

Growth pattern within the explant

Fig. 1 shows the growth pattern of epithelial cells within the explant during the first 10 days in culture, expressed as the percentage of cells taking up tritiated thymidine in a given 24-h period. Growth in basic medium can be compared with
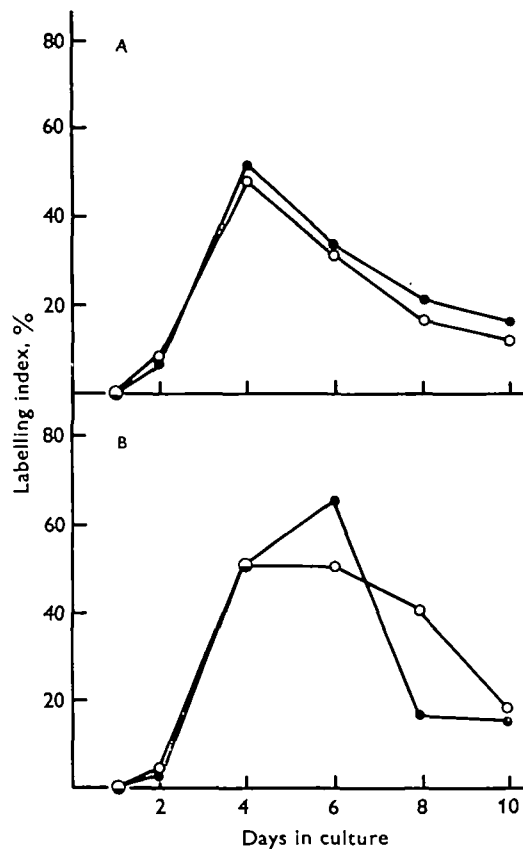

Fig. 1. Growth pattern of ductal epithelial cells within the explant during early culture in A, basic medium, or B, medium supplemented with insulin and hydrocortisone; ●, ○ indicate separate experiments.

growth in insulin- and hydrocortisone-supplemented medium. It can be seen that the hormonal supplements enhance the proliferative activity of ductal epithelium, shown in one experiment as an extended period of high labelling index and in the other as an increased peak value for the labelling index. See Tables 1 and 2.

At the time when regenerated ducts had repopulated the explant and formed a cellular layer around the periphery, the explant attached to the substrate. An outgrowth of cells followed, the majority of which appeared epithelial in morphology. Although thymidine incorporation and cell division continued at a slow rate, outward migration of epithelial cells was the predominant means of establishing the outgrowth. This usually reached a few millimetres distance from the explant before observable migration ceased.

Table 1. *Duct cell growth pattern in basic medium*

Labelling period, days	No. of explants	Total no. of cells counted	Average L.I. ± s.d.
0–1	9	—	0
	12	—	0
1–2	5	829	6·8 ± 2·5
	8	2201	6·1 ± 2·7
3–4	8	1848	47·4 ± 3·3
	6	2254	51·0 ± 2·9
5–6	6	2423	31·7 ± 5·1
	7	2387	33·6 ± 8·4
7–8	7	2211	15·8 ± 5·1
	7	2091	20·7 ± 6·3
9–10	9	2823	11·2 ± 4·1
	6	1899	15·6 ± 3·5

Each pair of results represents separate experiments, plotted as 2 graphs in Fig. 1A.

Table 2. *Duct cell growth pattern in insulin- and hydrocortisone-supplemented medium*

Labelling period, days	No. of explants	Total no. of cells counted	Average L.I. ± s.e.
0–1	10	—	0
	10	—	0
1–2	7	1277	3·5 ± 1·6
	7	1770	2·6 ± 0·9
3–4	5	1034	50·3 ± 4·5
	7	1891	50·3 ± 5·5
5–6	6	1504	65·3 ± 6·4
	7	1733	50·4 ± 3·5
7–8	9	4408	16·4 ± 4·2
	7	2826	41·4 ± 8·8
9–10	5	2850	16·0 ± 4·0
	7	4252	19·2 ± 6·2

Each pair of results represents separate experiments, plotted as 2 graphs in Fig. 1B.

Histochemical evidence for the origin of proliferating cells

Proliferating cells were strongly positive for orange G, a counterstain used in the AB/PAS method to demonstrate mucins synthesized by acinar cells. This supports a ductal rather than acinar origin (Hoshino & Lin, 1971). No blue (acid mucin) or magenta (neutral mucin) material was seen in the proliferating cell cytoplasm or the lumina they surrounded. The addition of insulin, hydrocortisone, testosterone and thyroxin in various combinations did not alter these results.

After 24 h in culture, a few explants contained ducts which reacted positively with the hydrocortisone substrate to give a moderately intense blue coloration in the cytoplasm, typical of striated duct cells. This indicated the presence of 11β-hydroxysteroid dehydrogenase activity (Baillie *et al.* 1966) and was absent from sections incubated with control cortisone substrate. By 3 days, one or two explants contained a 'positive' duct but these ducts were degenerate in appearance. 11β HSD-positive cells were never found in proliferating ducts. Hormone supplements had no effect on the survival of cells synthesizing this enzyme.

Explant sections from cultures grown in basic medium or with the addition of all 4 hormones, showed proteolytic activity at every time investigated up to 14 days. During the early degeneration phase (0–3 days) activity was not localized to any particular area but was diffuse over most of the explant tissue. As duct cells began to regenerate, proteolytic activity (shown as clear digested areas in the dark emulsion) was increasingly localized to the lumina of new duct structures (see Fig. 3). The cells themselves showed as incompletely digested areas of emulsion, with an intermediate tone. *In vivo* protease activity is a specific function of granular tubule cells (Shear, 1972).

In hormone-treated explants, regeneration was more rapid and this was reflected in the time at which proteolytic activity first became localized to duct lumina. The hormone-treated explants at day 6 were comparable to explants cultured in basic medium at day 8. Apart from this, there was no difference between these groups. The level of activity at this stage was comparable to that in granular tubules of male mouse submandibular gland, demonstrated under identical conditions. No protease histochemistry was attempted on cells after outgrowth on to the culture substrate.

Outgrowth of epithelial cells from the explant

Explants attached to the substrate between 6 and 10 days after culture initiation, earliest in the insulin- and hydrocortisone-treated groups. Outgrowth of cells on to the plastic substrate followed rapidly, particularly in hormone-supplemented medium. In many cases, isolated fibroblast-like cells were the first to migrate out but these were usually followed by a contiguous sheet of epithelial cells. These were small cells with large, round nuclei and phase-dark cytoplasm. In the multilayered area near the explant, whole duct-like structures could be seen which then flattened out on to the substrate. This is clearly seen in Fig. 4. Epithelial cells in the monolayered region occasionally reorganized into 2-dimensional, duct-like structures which were large enough to be visible at the light-microscope level (Fig. 6). After

prolonged periods *in vitro* (up to 6 months), epithelium remained healthy in appearance but showed no further net growth.

Ultrastructural characteristics of cells in the explant and outgrowth

After the initial period of degeneration, where mucus-secreting acinar cells and many duct cells atrophied and were lost from the explant, increasing numbers of simple cuboidal cells were found in well organized ducts or ribbons of cells. Apart from a few dark granules resembling those found in granular tubules, cells showed no evidence of active secretory function. Intercellular spaces became filled with mature banded collagen fibrils during the regeneration phase. This was probably derived from the polymerization of existing precursor molecules.

Cells were low columnar or, more usually, cuboidal in shape. The addition of insulin and hydrocortisone increased the height of most duct cells and decreased intercellular oedema. Where cells were polarized around a lumen, typical junctional complexes (Farquhar & Palade, 1963), luminal microvilli and occasional intracellular canaliculi were found. Lateral plasma membranes were folded into interlocking microvilli. Basement membrane remained intact around ducts or ribbons of cells, separating them from mesenchymal cells and collagen.

In the outgrowth, where cells were arranged in a duct-like pattern (Figs. 8, 9), cells were easily recognizable as epithelial from the well defined junctional complexes at luminal intercellular junctions and from pronounced desmosomes and their associated 7-nm diameter tonofilaments. Lateral plasma membranes often interlocked, as in cells within the explant. Arrangement of cells into organotypic structures was unaffected by addition of hormonal supplements to the medium.

Establishment of cell lines

When well established primary cultures with a high proportion of epithelium were trypsinized or mechanically dispersed and replated, many small sheets of epithelium attached to the new substrate. No further proliferation was observed in these cells, however, and most cultures were eventually taken over by progeny of the few mesenchymal cells present. Two mesenchymal cell lines were established which displayed typical morphology and growth characteristics.

Ultrastructurally, cells resembled those of other mesenchymal cell lines derived from various mouse tissues (Franks & Wilson, 1970). Intermediate junctions were the only specialized intercellular contacts and although confused with desmosomes by some authors, at high magnification they were easily distinguishable. Filaments associated with intermediate junctions were finer (approximately 5 nm in diameter) than epithelial tonofilaments.

After about 200 days in culture, both cell lines produced subcutaneous tumours after injection of 2×10^6 cells into the flanks of syngeneic mice. These were typical transformed cell fibrosarcomas with myxoid and leiomatous regions (Franks *et al.* 1970).

DISCUSSION

It has been shown that explantation *in vitro* of adult male mouse submandibular gland stimulated a pronounced proliferative response in surviving epithelial cells. These then recolonized the degenerate explant and eventually formed a large proportion of the primary outgrowth. This response closely resembled that seen in organ culture and *in vivo* after arterial ligation (see Introduction). There was also a similarity to the response to excretory duct ligation (Junqueira, 1951), partial excision (Hanks & Chaudhry, 1971) and intraperitoneal grafting (Hoshino & Lin, 1971). This suggests that the results seen in explant culture were largely those of a response to trauma.

Since there is no known stem cell population in this gland, duct cells which survive the initial insult assume a morphologically 'dedifferentiated' form and enter a phase of rapid proliferation. Anoxic stress may be a common causal factor in all systems where submandibular gland regeneration is seen, although attempts to minimize this stress in organ culture failed to prevent completely the changes described (Tapp, 1967). *In vivo*, acinar cells and mature granular tubule cells occasionally reappear in regenerated regions. This was not seen *in vitro*, even after medium supplementation with specific hormones.

There were no true squamous changes in proliferating cells in the explant as described by Trowell (1959). Cells often appeared flattened but these were ultrastructurally identical to their cuboidal counterparts. Some ultrastructural features of squamous cells were seen occasionally in monolayered cells after extended periods *in vitro*.

Results of experiments involving addition of specific hormones to the medium were in general agreement with the organ culture experiments of Lucas *et al.* (1970). None of the sex steroids or thyroid hormones used in the experiments of these workers affected the survival of epithelium. Insulin and hydrocortisone, however, were found to improve glandular survival in organ culture considerably; most of the effect was due to hydrocortisone. In the present study, proliferation was enhanced by these 2 hormones but the response was largely attributable to insulin.

There was good agreement between the 2 experiments measuring the growth pattern of duct cells cultured in basic medium. Differences in the degree of damage suffered by the tissue during explantation affected the total numbers of surviving cells capable of proliferation, but not the percentage of surviving cells synthesizing DNA at a given time (see Table 1). Standard deviation values showing variability between explants were highest at later times (days 5–6 and 7–8), where some explants within a group were more 'advanced' histologically. They showed a high cell density, more typical of explants fixed at a later culture time and a correspondingly lower labelling index.

The discrepancy between the 2 experiments showing the growth pattern of duct cells in hormone-supplemented medium could be explained as follows. In one case, cells capable of further DNA synthesis labelled earlier and more synchronously than in the other experiment, where the increase is seen as a slower decline in labelling

index with time than is shown by cells grown in basic medium. Since cell turnover rate in the submandibular gland is very low (Barka, 1965), conditions of explant preparation and culture have significantly stimulated DNA synthesis in surviving epithelium.

No convincing evidence exists in the literature which identifies the duct cell type involved in the regeneration process. It has been shown here that regenerating tubules retain histochemically demonstrable proteolytic activity for at least two weeks *in vitro*. The level of enzyme activity was comparable to that seen in sections of intact submandibular gland, where it is localized to the granular tubules. No evidence of 11β-HSD activity was seen in proliferating duct cells and it was concluded that striated ducts either lose this enzyme rapidly during proliferation, or take no part in the regeneration process. The latter explanation seems more likely. An acinar origin for proliferating cells was considered improbable but the participation of intercalated duct cells could not be ruled out. It is concluded then, that the majority of cells in submandibular gland explant outgrowths derive from the epithelium of granular tubules. The organization of epithelial cells in the outgrowth into organotypic structures closely resembled that described for thyroid cell cultures (Fayet, Michel-Bechet & Lissitzky, 1971) after stimulation with thyrotropin. Here, no hormonal stimulus was required.

Identifiable epithelial cells were lost from the cell line at the second or third transfer, in spite of the prolonged survival of healthy epithelium in primary culture. It seems, therefore, that loss of epithelial cells on transfer was due to their low capacity for proliferation under these conditions rather than to any lack of ability to survive *in vitro*.

These results conflict to some extent with those of other authors reporting the growth of epithelial cell lines from rodent salivary glands. They believed these lines to have derived from acinar (Kreider, 1970; Gallagher *et al.* 1971) or granular tubule cells (Marcante, 1973) but most of the evidence for their claims is open to reinterpretation. The epithelial-like tumours obtained from Brown's cell lines (Brown, 1973) appeared similar to epithelioid or myxoid forms of transformed cell sarcomas (Franks *et al.* 1970).

Although differentiated epithelial cell lines could not be established, this explant system seems suitable for the study of the *in vitro* action of both hormones and polycyclic carcinogenic hydrocarbons on a defined epithelial cell type. Squamous carcinomas are readily induced in mouse submandibular gland *in vivo* by benzo(a)-pyrene, dibenz(a,h)anthracene (Steiner, 1942) and dimethylbenz(a)-anthracene (Matsumura, 1966). It has been shown that these derive from granular tubule cells (Wigley, 1974). Preliminary results show that these same carcinogens but not their weak or non-carcinogenic analogues, can induce changes in ductal epithelial cells *in vitro* which may correspond to early changes seen *in vivo* during hydrocarbon carcinogenesis.

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Fig. 2. Explant cultures in medium supplemented with insulin and hydrocortisone for 8 days, showing regenerated duct structures. A metaphase cell is indicated (arrow). × 300.

Fig. 3. Cryostat section of explant cultured in basic medium for 8 days, exposed to developed photographic emulsion. Clear areas indicate the location of protease activity in duct lumina. × 150.
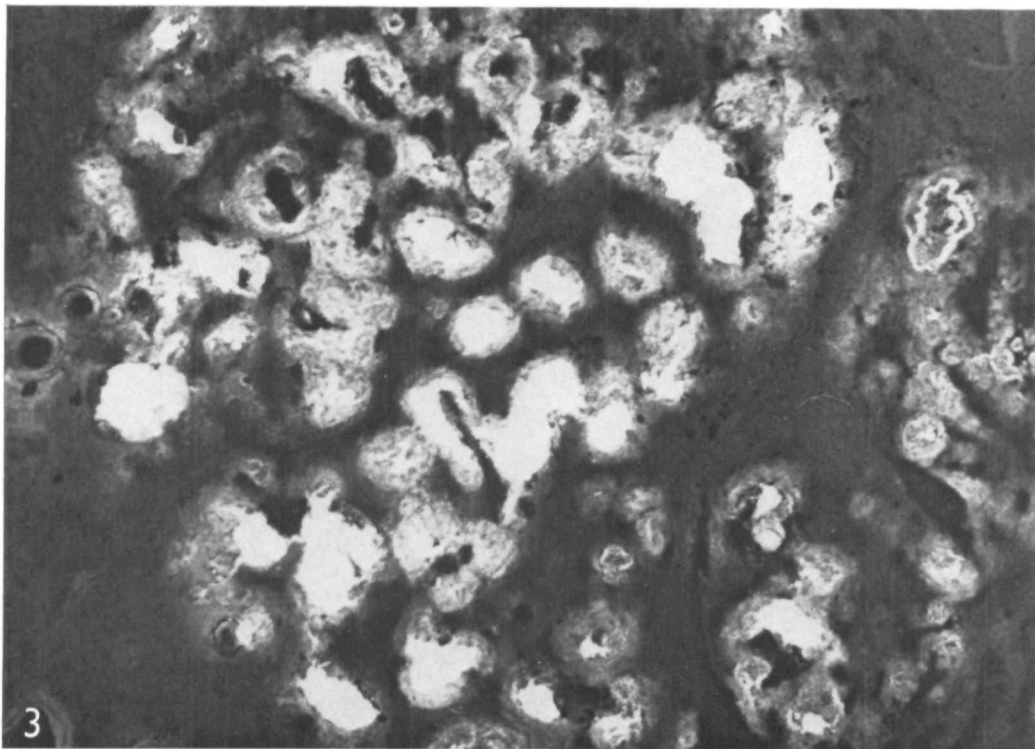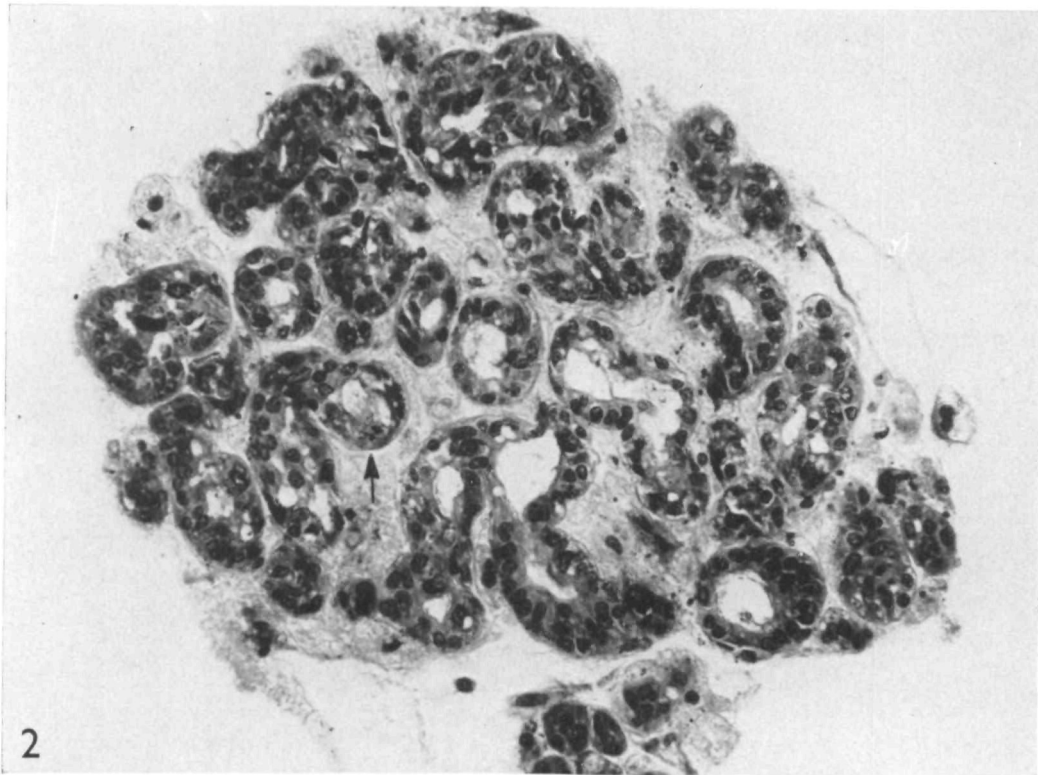
Fig. 4. Vertical section through explant and outgrowth after 4 weeks *in vitro*. Many regenerated ducts have migrated on to the Melinex substrate as intact structures which then flatten out to form a monolayer. The explant consists largely of collagen (c) at this stage. Toluidine-blue stained Araldite section. × 100.

Fig. 5. Phase-contrast photograph of an epithelial cell monolayer after 5–6 weeks *in vitro*. × 160.

Fig. 6. As for Fig. 5. Cells in this area show reorganization into 2-dimensional pseudoglandular structures in the monolayer. × 160.
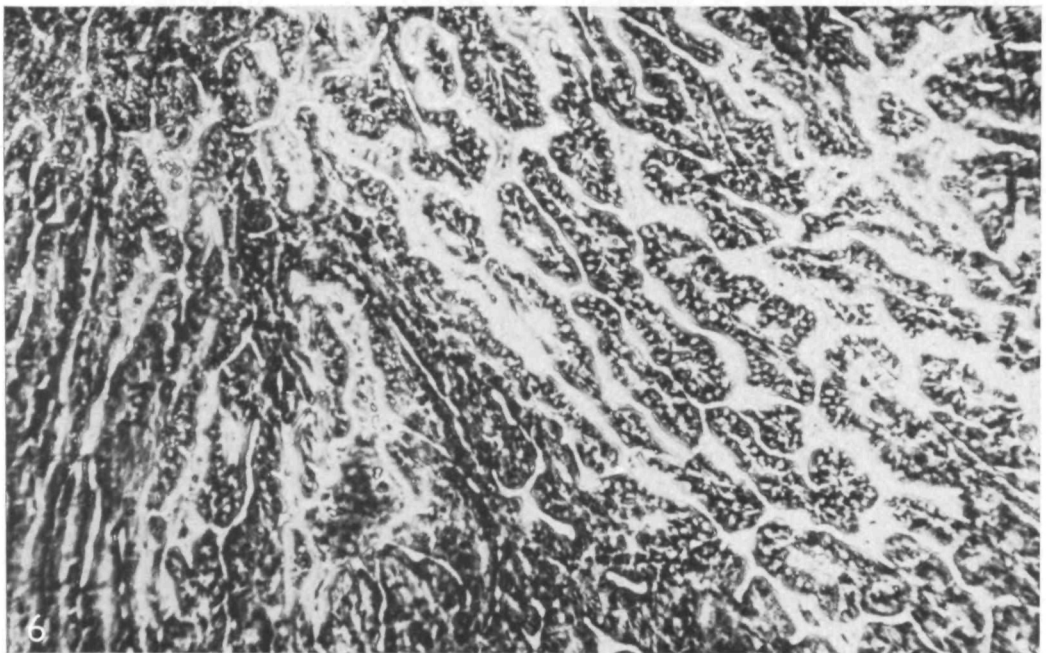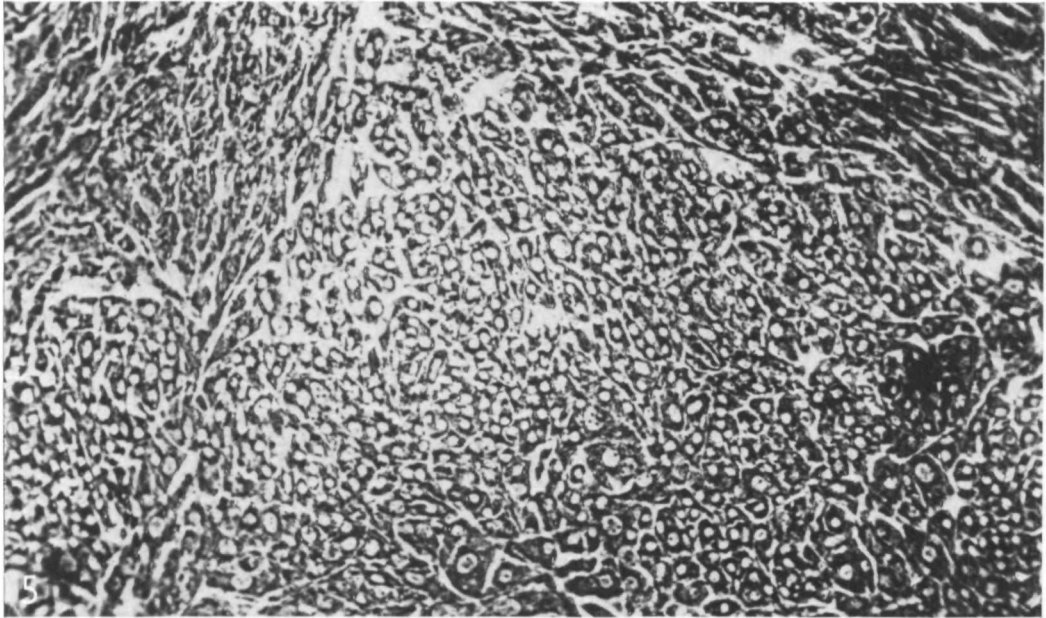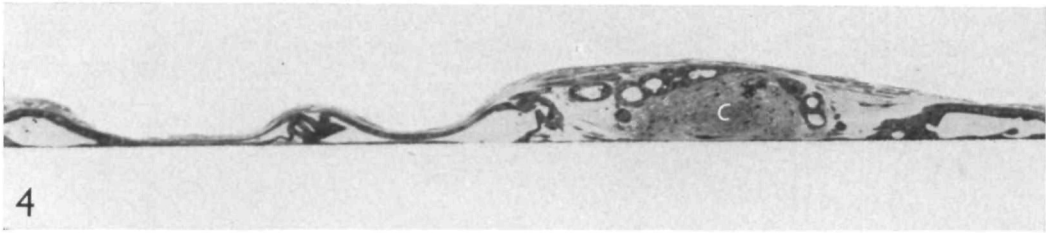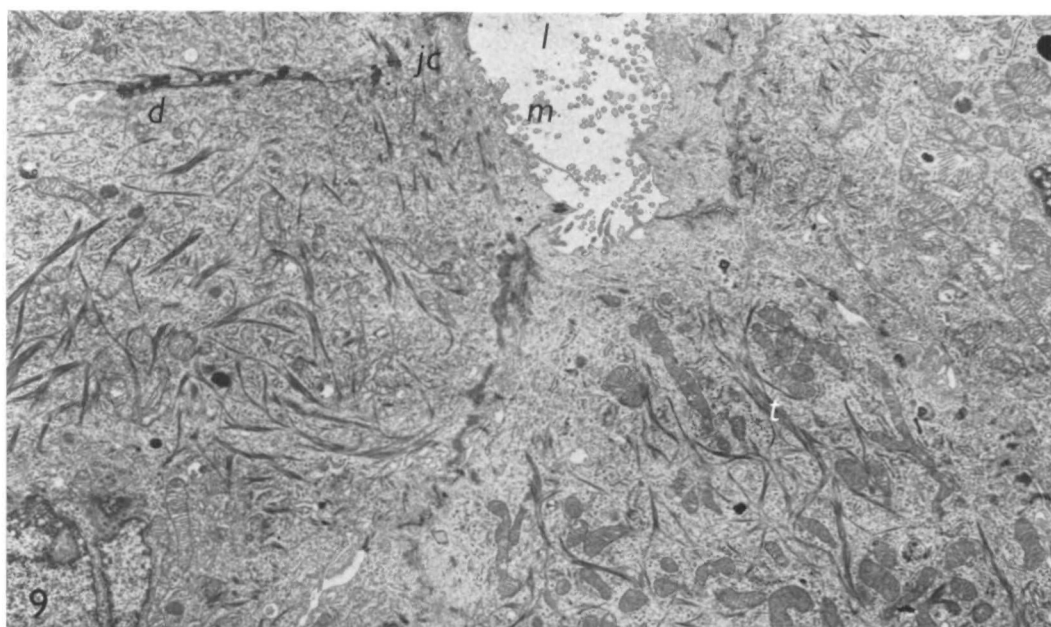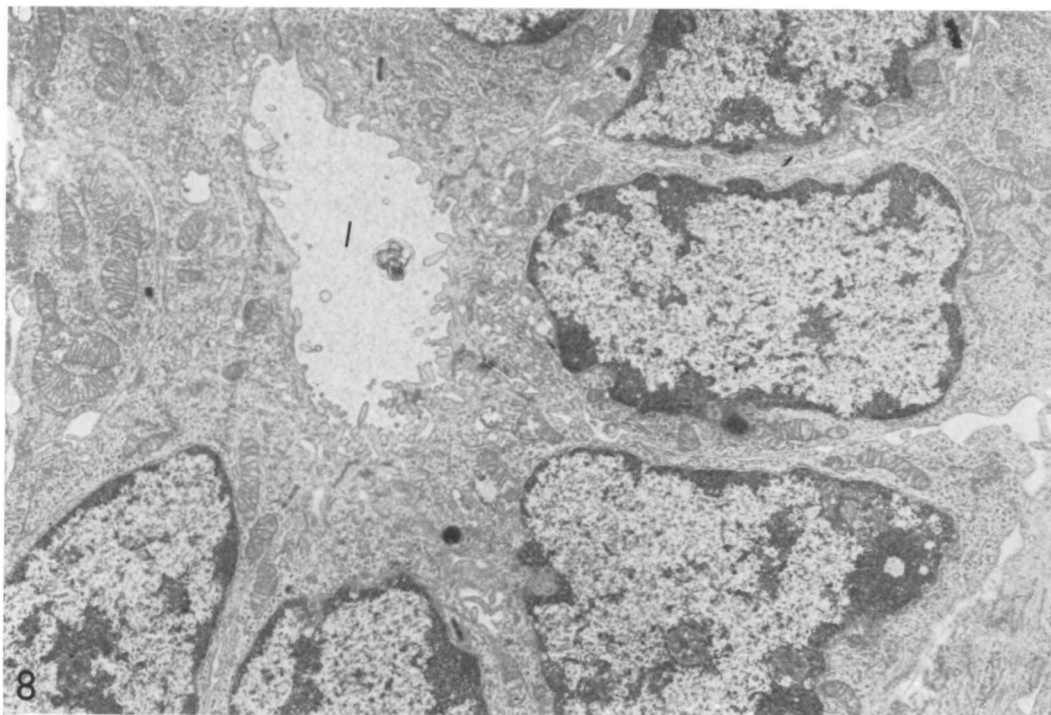
Fig. 7. Electron micrograph of epithelial cells in the outgrowth, sectioned in the plane of the monolayer. A small 'lumen' (*l*) is shown, indicating pseudoglandular organization of the cells. Tonofilaments (*t*) and desmosomes (*d*) are present. × 6000.

Figs. 8, 9. Electron micrographs of epithelial cells, sectioned in the plane of the monolayer, showing pseudoglandular organization around a lumen (*l*). Microvilli (*m*) project into the lumen and junctional complexes (*jc*) are found between cells near the lumen. Desmosomes (*d*) and tonofilaments (*t*) are pronounced in Fig. 9, which is sectioned near the substrate. × 6000.