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GROWTH IN CULTURE OF ADENOCARCINOMA CELLS FROM THE SMALL INTESTINE OF SHEEP

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Summary.—Explant cultures were initiated from adenocarcinoma of the small intestine in sheep and from various metastases. Several cell types grew, most being fibroblastic in nature. However, 2 cultures yielded mixed cells which arranged themselves into areas of epithelial-like cells surrounded by fibroblast-like cells and this pattern was consistent over 30 subcultures and several months of culturing. The epithelial-like cells were separated from the others by the use of a modified medium containing citrulline or by sedimentation through a bovine serum albumin solution. Various properties, including their growth rate in 5% and 0·5% serum, the absence of surface fibronectin and their ability to grow in semi-solid agar, indicated that they may represent carcinoma cells. Screening for virus production from these cells and all other explant cultures proved negative.

ADENOCARCINOMA of the small intestine in sheep has been reported particularly in older ewes in New Zealand (Simpson and Jolly, 1974), Australia (Ross, 1980), Iceland (Georgsson and Vigfusson, 1973), Scotland (Norval, personal communication) and Northern England (McCrea and Head, 1978). In those parts of the world at least this tumour is found relatively frequently, but it should be noted that prevalence in abattoir surveys is controlled not only by the number of tumours in the sheep population of the district but also by the age and type of sheep sent for slaughter. Thus, in animals of about 1 year old and older (lambs and ewes) it is given as 0·97% in Iceland (Georgsson and Vigfusson, 1973) and 0·2% in Scotland (Norval, personal communication). Simpson (1972*a*) reported a range of from 0·2% to 1·58% of ewes slaughtered in 10 slaughterhouses in 7 geographic areas of New Zealand. Abattoir prevalence figures in New South Wales, Australia, ranged from 0·093% to 0·487% for adult sheep (Ross, 1980). Because there are difficulties in ensuring that the abattoir samples are comparable it is not certain if there is a geographical distribution in incidence (Head, 1967). The aetiology of the disease is not known. Simpson (1972*b*) demonstrated 16 factors were statistically associated with the tumour in New Zealand. It has been suggested that exposure to carcinogens in fodder (Georgsson and Vigfusson, 1973) or in bracken, *Pteridium aquilinum* (Evans and Mason, 1965) may be important but other factors, in particular viruses, may be involved. In this context, the recent work by Jarret *et al.* (1978) is of interest where a correlation has been drawn between bovine papilloma virus, an environmental carcinogen thought to come from bracken and alimentary carcinoma in cattle.

Ovine adenocarcinoma is found at any site along the length of the jejunum and ileum, but is not found in the duodenum. In sheep from this country, it is usually present as a solitary primary lesion. The cell type involved is thought to be either adsorptive cells or goblet cells (Simpson and Jolly, 1974). Tumours may spead from the intestinal site to the mesenteric

lymph node by lymphatic permeation, and there may be transcoelomic metastasis leading to lesions on the surface of many organs such as the peritoneum of the anteroventral flanks, diaphragm and spleen. In the final stages, a large volume of ascitic fluid is present.

This study was undertaken to examine the cells which grew *in vitro* from the primary adenocarcinoma tissue and from various metastases, especially to look at their morphology, ultrastructure and whether they were infected with viruses. One cell line of particular interest was obtained, ST–6, which produced a mixture of epithelial-like and other cell types on primary culture. The epithelial-like cells were separated from the others using sedimentation in bovine serum albumin solution and growth in citrulline-containing medium, and their characteristics examined in detail.

MATERIALS AND METHODS

Tumour material

Tissues from 11 sheep suspected by the meat inspectors of being examples of adenocarcinoma of the small intestine were obtained from the abattoir in Edinburgh. As soon as possible after slaughter, the primary lesion and/or any metastases were used for culturing purposes. The specimens were also the subject of morbid anatomical examination. Tissues were processed to paraffin wax, cut at 5 μm and stained with haematoxylin and eosin; in addition, where appropriate, Gordon and Sweet's stain for reticulin fibres, Alcian Blue and P.A.S. for mucin were also used.

Culture methods

(a) *Primary culture of tumour cells.*—The tumour specimen was cut into small pieces approximately 1 mm in size and several placed beneath a glass cover-slip in a plastic Petri dish (Sterilin). The medium used was Earles-based Eagles' complete medium containing 200 iu/ml penicillin, 200 μg/ml streptomycin, 50 iu/ml mycostatin and 100 μg/ml gentamicin, and supplemented with 10% newborn calf serum. All culturing was performed at 37° in 5% CO_2. Cells were subcultured using trypsin–versene. After subculture, the gentamicin and mycostatin were omitted from the medium and the serum content reduced to 5%. The cells were tested at intervals for the presence of mycoplasmas as described by Mackay *et al.* (1974).

(b) *Citrullin medium.*—ST-6 cells were cultured in Sun's modified Waymouth MB 752/1 medium containing either arginine or citrulline and supplemented with dialysed newborn calf serum (Sun *et al.*, 1979).

(c) *Separation of cell types using sedimentation in bovine serum albumin (BSA).*—The method described by Moore and McBride (1980) was used whereby cell suspensions were fractionated by velocity sedimentation at unit gravity. Briefly, 10^7 cells were sedimented for 90 min through a 1–2% BSA gradient in Dulbecco's solution contained in an 18cm diameter "Staput" chamber (Johns Scientific, Toronto).

(d) *Rate of growth.*—The rate of growth of ST-6 cells and its subpopulations was measured by putting 2×10^5 cells into 50mm Petri dishes in 5 ml Eagles' medium containing either 5% or 0·5% newborn calf serum, and counting the number of viable cells present, after removal with trypsin–versene, on each day for a period of incubation of 8 days.

(e) *Growth in semi-solid agar.*—The epithelial cells derived from the ST-6 culture were cloned in Eagles' medium with 10% newborn calf serum and containing 0·275% agarose (LGT pure agarose powder, 49-056, Miles Laboratories), with 0·55% underlay containing 10^5 human embryo lung cells/ml as feeder cells. The sheep cells were seeded at densities of 5×10^4 and 10^4 in 50mm Petri dishes.

Fibronectin immunofluorescence

An indirect immunofluorescence test to detect fibronectin was carried out as described by Chen, Gallimore and McDougall (1976), using cells grown in monolayers and fixed with 2% paraformaldehyde. Rabbit antiserum, specifically cross-reacting with mouse, rat and human fibronectin (data not shown), was produced by injecting purified human cold insoluble globulin into rabbits, and was kindly donated by Dr J. Kinross. It was used at a dilution of 1/40, while the FITC anti-rabbit conjugate was used at dilutions of 1/8–1/16.

Ultrastructure

Cells grown on plastic and glass flasks were harvested by gentle mechanical scraping using a rubber policeman or by enzyme digestion using neutral protease (Dispase II, Boehringer).

Some samples were fixed for 1 h in 2·5% glutaraldehyde before removal from flasks and others were fixed as cell suspensions after removal. After removal and fixation, cell pellets were formed in polyethylene conical-tipped tubes (Azlon) by centrifuging at 3000 *g* for 10 min. Pellets were washed twice in 0·1M sodium cacodylate buffer, post-fixed in 2% osmium tetroxide and then embedded in Spurr resin (EMscope Laboratories) before sectioning on an LKB Ultratome III microtome. Sections stained

with lead citrate and uranyl acetate were examined in a Phillips 400 TEM at an accelerating voltage of 100 Kv.

For scanning electron microscopy cells were cultured on 10mm diameter glass cover-slips in Petri dishes. When confluent, monolayers were fixed in 2·5% glutaraldehyde and prepared for scanning electron microscopy by critical-point drying using a Polaron critical-point drying apparatus. Samples were examined in a Cambridge Stereoscan 180 SEM.

Chromosome analysis

Sixteen hours after subculture into 25cm^2 flasks from a confluent monolayer, ST-6 cells were pulsed with 0·2 ml 2% colchicine (BDH) in saline, and incubated for a further 2 h at 37°. The cells were removed from the flask by trypsinization, and processed through hypotonic KCl and fixative (Hungerford, 1965). Chromosome spreads were made on clean chilled slides and stained for 10 min with a 1/20 dilution of Giemsa.

Screening for virus production

All cell cultures at Pass 4 or 5 were labelled with ^{3}H-thymidine and ^{3}H-uridine for 24–48 h followed by concentration of the culture supernatant and sucrose density gradient centrifugation as outlined by Norval and Marmion (1976). In one case, ST-4, the primary tumour disaggregated by the use of trypsin and the resulting single cells cultured in the presence of ^{3}H-thymidine and ^{3}H-uridine directly.

In addition, for the ST-6 cells only, the cultures at Pass 10 immediately before labelling were induced with IUDR (20 μg/ml) for 3 days followed by 2% DMSO for 3 days (Stewart *et al.*, 1972), mitomycin C (1 μg/ml) for 18 h in the dark (Weiss *et al.*, 1971), or cycloheximide (10 μg/ml) for 16 h (Aaronson and Dunn, 1974).

ST-1, ST-2 and ST-3 cells and cell lysates from Pass 2 were also examined by co-cultivation and infection of human embryo fibroblasts, RK_{13}, HEp-II and Vero cells followed by microscopic examination over a period of 4 weeks for evidence of any viral cytopathic effect.

RESULTS

Morbid anatomy and light microscopy of tumour cases

All 11 cases of suspected adenocarcinoma occurred in female sheep and they were from a variety of breeds including Blackface, Border Leicester and Halfbred. Their ages by dentition and skeletal

TABLE I.—*Source and investigation of cells cultured from explants of suspect adenocarcinoma of the small intestine of female sheep*

Case No.	Age of host	Tissue used for explant	Time between slaughter (S) or death (D) and culture		Morphology of explant culture
ST-1	Adult	Primary	I	(S)	A
ST-2	Adult	Mesenteric lymph node	I	(S)	A
ST-3	Adult	Primary	I	(S)	A
ST-4	Adult	Primary	II	(S)	Fungal contamination
ST-5	Adult	Primary	I	(S)	No growth
ST-6	ca. 9 months	Posterior mediastinal lymph node	I	(S)	B
ST-7	ca. 9 months	Flank lesion. Rumen serosa	II	(S)	C C
ST-8	Aged	Diaphragm lesion. Posterior mediastinal lymph node	II	(S)	B No growth
ST-9	Aged	Intestinal serosa	II	(S)	C
ST-10	Aged	Primary Flank lesion Ascitic fluid	II	(D)	No growth No growth No growth
ST-11	Adult	Flank lesion Intestinal serosa Rumen serosa	I	(S)	A A C

Key: A = Large fibroblast-like cells with stretched out processes; B = mixture of two sizes of epithelial-like cells, large fibroblast-like cells with long processes and long thin fibroblasts; C = long thin fibroblasts; I = on same day as animal's death; II = on the day following the animal's death.

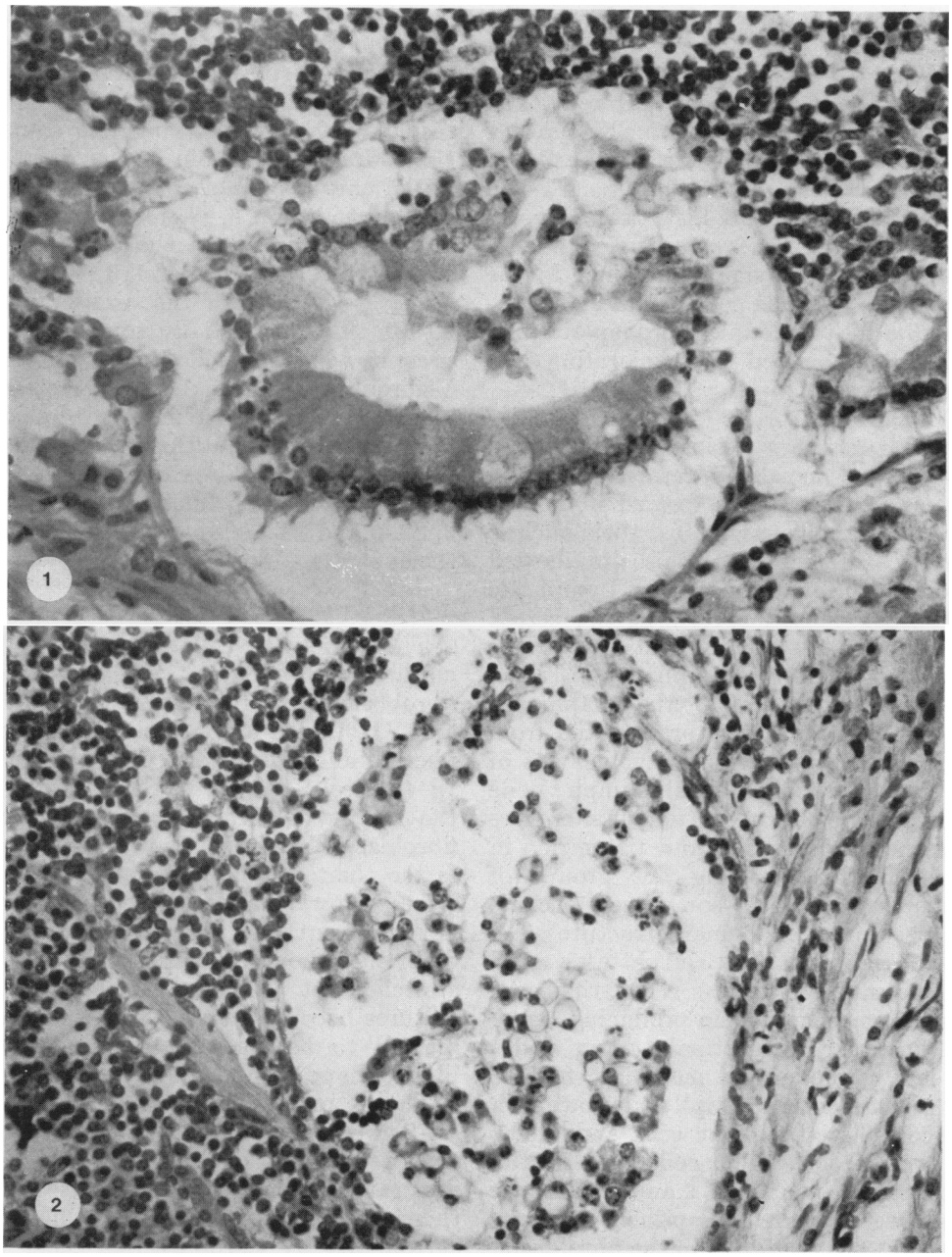


FIG. 1.—Photomicrograph of a metastasis in the subcapsular sinus of the posterior mediastinal lymph node of ST-6. Note the acinar pattern with basal nuclei and goblet-cell formation. Paraffin wax. H. & E. ×456.

FIG. 2.—Photomicrograph of metastatic cells in the subcapsular sinus of the posterior mediastinal lymph node of ST-6. Note many of the cells are signet-ring cells and there is some perinodal fibrosis. Paraffin wax. H. & E. ×380.

development ranged from 9 months through adult (over 4 years old) to aged (probably 7 years old or more) (Table I).

Ten of the cases were slaughtered at the abattoir, the eleventh died from respiratory distress caused by metastasis to the pleura and subsequent hydrothorax (ST-10). Morbid anatomy and histopathology revealed 10 of the cases were typical adenocarcinoma of the small intestine; in the eleventh (ST-7) the peritoneal lesions proved to be due to a nodular fibrous peritonitis probably associated with migrating helminth larvae.

The tumour cases had an annular stenosing primary lesion. The mucosal surface was usually ulcerated and the tumour in the mucosa formed ill-defined tubules, the cells having lost their nuclear polarity. The serosa at this site showed fibrosis with few tumour cells. Lymphatic metastases caused spread in the serosa proximal to the tumour and also to the drainage mesenteric lymph node. Transcoelomic metastases to the diaphragm and flanks were always present and from these sites lymphogenous involvement of the posterior mediastinal lymph node occurred. The peritoneal tumour sites were mainly composed of fibrous tissue rich in fibroblasts but showing few groups of tumour cells which sometimes became organized into an acinar structure with basal nuclei. Both these cell patterns could produce mucin either as signet-ring cells or sometimes as mucin-producing acini. The posterior mediastinal lymph nodes had less perinodal and medullary fibrosis than the mesenteric lymph nodes but both showed isolated tumour cells and a few well organized tumour-cell acini in the subcapsular sinus (Figs 1 and 2). Mitotic figures were rare in the epithelial tumour cells and were not found in the fibroblasts. The rumen was not always available for inspection but, in those cases where it was examined, half had rumen fibropapilloma present.

Primary culture of tumour material

At first, in addition to explant culture, the tissues were disaggregated by the use either of trypsin or of a mixture of collagenase and dispose, but it was found that explant cultures gave more satisfactory results with cells growing out from the tissue fragment within a few days. The initial cultures could be split into 2 after 2 weeks to 1 month. Thereafter, the rate of growth increased and the cells could be subcultured at a ratio of 1:3 every week. No cells grew from the ascitic fluid of ST-10 although initially some cells were present which looked like macrophages. Vigorous washing of the flank metastases was also tried in an attempt to dislodge surface tumour cells, but this was not successful. In one case, ST-4, the cultures became contaminated with fungi, and in another 2, ST-5 and ST-10, no cells grew out from the explants.

Morphology of cultured cells

The variety of cell types obtained on culture was of great interest. Typical fibroblasts grew from 2 specimens, ST-7 and ST-9, with long, thin, tapering morphology and which were contact-inhibited. In 4 cases, ST-1, ST-2, ST-3 and ST-11, the cell type which grew also looked fibroblastic although it was shorter and fatter than the fibroblasts already described. However, when the cultures were not confluent, the morphology was quite different. The cells were large and had stretched-out processes (Fig. 3a). As the cultures become more confluent, the cells seemed to be compressed into a fibroblastic shape (Fig. 3b).

The 2 remaining cultures, ST-6 and ST-8, were very different. In both there was a mixture of cell types which is illustrated in Fig. 4. It may be seen that there are patches of epithelial-like cells surrounded by fibroblast-like cells. This appearance was noticed in the primary culture from the explant (Fig. 5) and continued as a regular pattern throughout more than 30 subcultures which were performed weekly at a ratio of 1:2 or 1:3. In this respect, these cells may mimic the behaviour of cells within the tumour mass

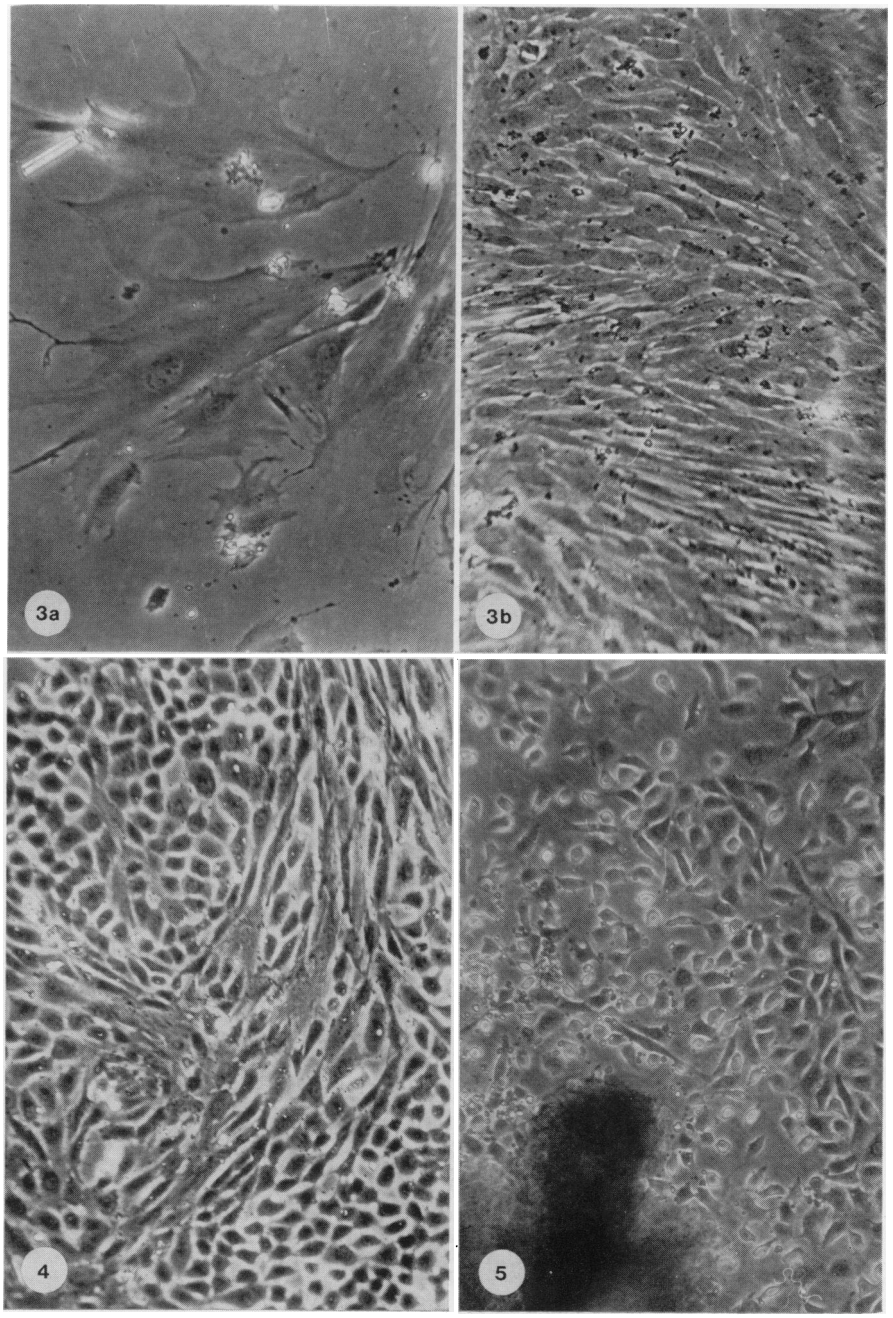
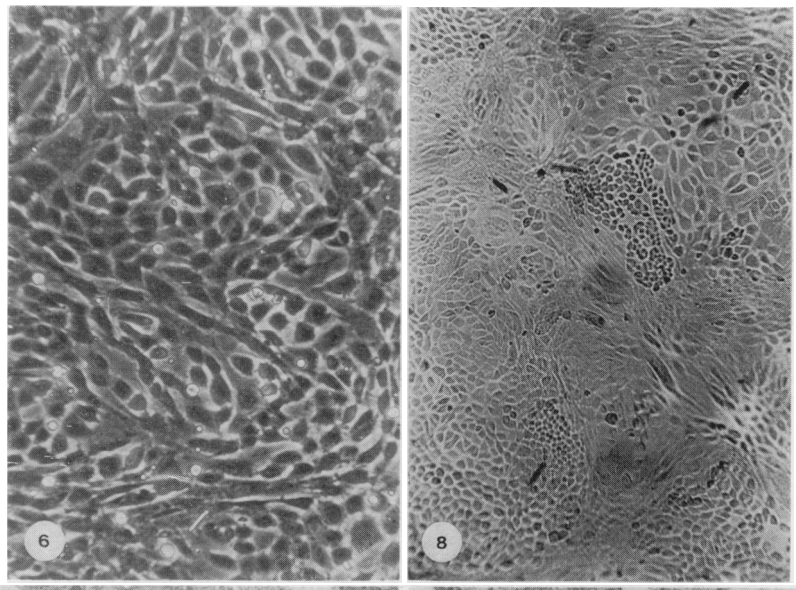


FIG. 3.—Cells from culture ST-1 (a) at low density and (b) when fully confluent. Phase contrast. × 75.
FIG. 4.—Cells from culture ST-6, Pass 3, showing mixed cell types. Phase contrast. × 75.
FIG. 5.—Explant culture of ST-6. Phase contrast. × 75.



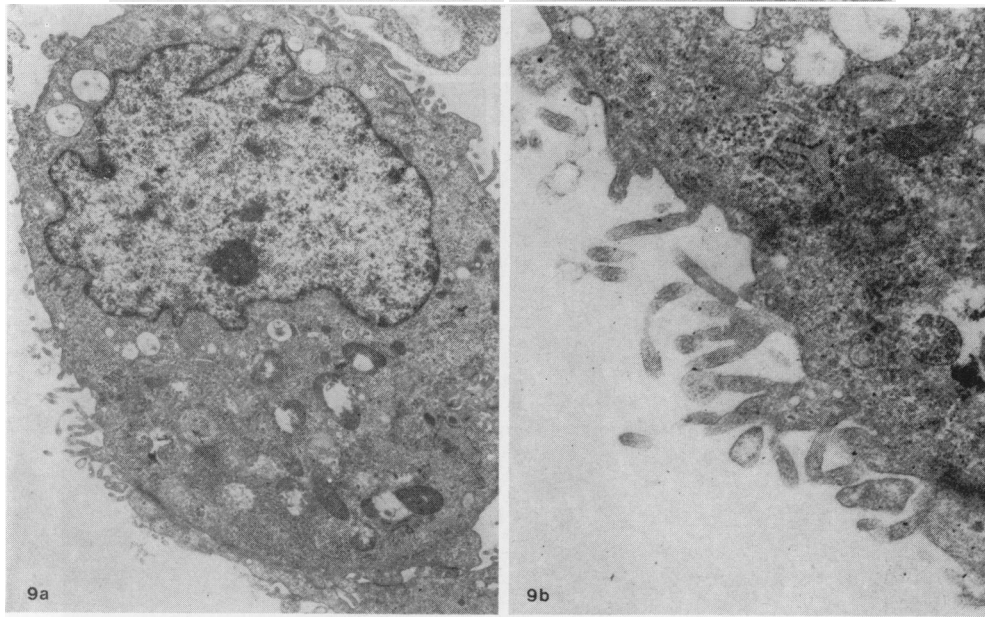


FIG. 6.—Fraction 4 cells from ST-6 after separation on BSA. Phase contrast. × 66.
FIG. 8.—ST-6 cells after 5 passages in Sun's modified medium with citrulline. Phase contrast. × 46.
FIG. 9.—Transmission electron micrograph of cell pellet from Fraction 4. (a) Typical cell; note numerous vacuoles with osmophilic material and prominent surface villi. × 4000. (b) Close-up of same cell showing surface villous structures. × 14,333.

and it was decided to attempt to separate the cell populations present within the ST-6 culture and to study their properties further.

Separation of ST-6 subpopulations

(a) *Culture in citrulline medium.*—The first approach was to culture the cells in a medium containing citrulline which was designed to inhibit the growth of fibroblasts as described by Sun *et al.* (1979). ST-6 cells were passaged 5 times in this medium and the appearance of the cultures compared with growth of these cells in Eagles' medium supplemented with the same serum.

In the modified medium with citrulline, the fibroblast-like cells gradually disappeared with each passage until none were seen after 5 passes. Two distinct populations of epithelial-like cells which differed in size remained, as shown in Fig. 8. The fibroblast-like cells persisted in the modified medium containing arginine and in the original Eagles' medium. It was very striking that, during the course of passage in the modified medium, the ST-6 cells seemed to rearrange themselves into cell types and to grow in discrete areas.

(b) *Sedimentation through B.S.A.*—In the second method ST-6 cells at Pass 10 were sedimented through B.S.A. and aliquots collected, with the result shown in Fig. 7. Each fraction was cultured after washing the cells. Fractions 1, 2 and 3 showed fibroblast-like cells of the type already described, *i.e.* they were large and spreading at low confluence with long processes. These cell fractions were grown up separately and then pooled for subsequent investigations and identified as Fraction 1–3. Fraction 4, on the other hand, contained a very large percentage of epithelial-like cells and these are illustrated in Fig. 6. Fractions 5–8 contained a mixture of epithelial cells and fibrobasts like the original culture and the remaining fractions had cells of typical fibroblast appearance, no epithelial cells being present.

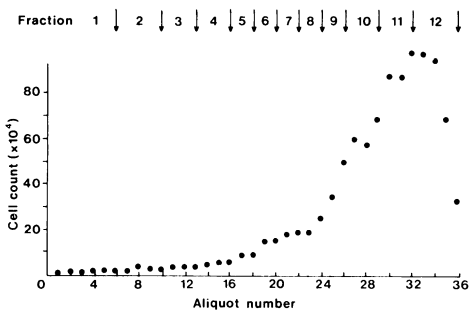
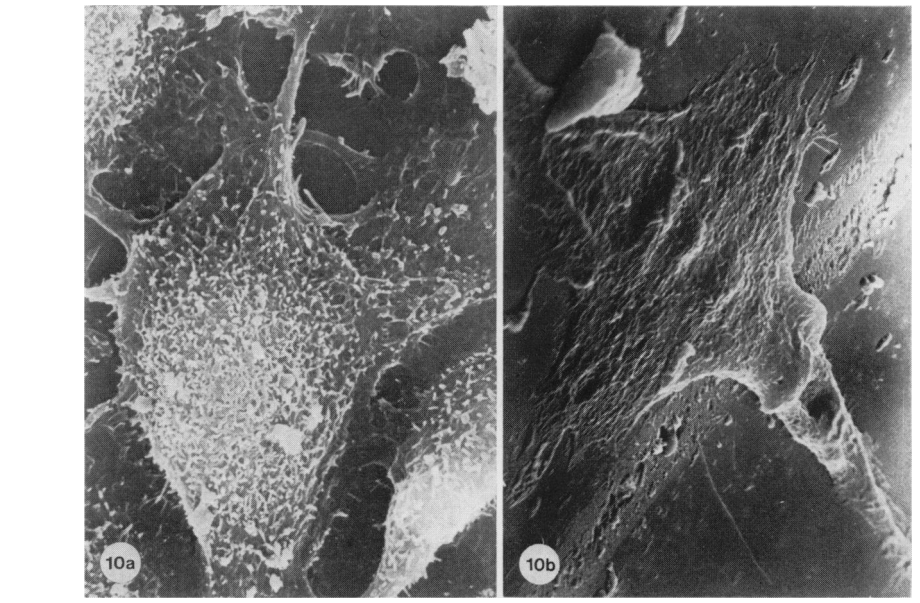


FIG. 7.—Profile of cell numbers in each aliquot after sedimentation of 10^7 ST-6 cells, Pass 10, through BSA solution. Fractions for culture were made from pooled aliquots as shown.

Ultrastructure of Fraction 4 and Fraction 1–3 subpopulations of ST–6

Thin sections of Fraction 4 cell pellets showed both individual cells and groups of cells. The nuclei were irregular in shape with strongly indented nuclear margins. where the cells had remained in groups, desmosomes were seen in tight junctional sites. At free cell surfaces, and where cells were loosely associated, there were prominent microvilli (Fig. 9). There were many cytoplasmic vacuoles which were apparently devoid of contents. At high power many of these structures contained sparse amounts of osmophilic material only, whilst others contained granular, more strongly osmophilic material. Relatively few mitochondria were present and these were usually degenerate. Other cells showed larger, more irregular vacuoles containing finely particulate, weakly osmophilic material characteristic of mucus. Some of the larger structures had villus-like cytoplasmic processes extending into their lumina. SEM observations showed numerous microvilli on the surface of Fraction 4 cells (Fig. 10a).

Examination of Fraction 1–3 cells by TEM showed cells with irregular, indented nuclei. Cell membranes were irregular but had markedly fewer microvilli than the Fraction 4 cells (Fig. 11). Round, and more irregular, vacuoles were observed in the endoplasmic reticulum. In addition, many cells showed accumulations of fibrillar material. The SEM appearance of a typical



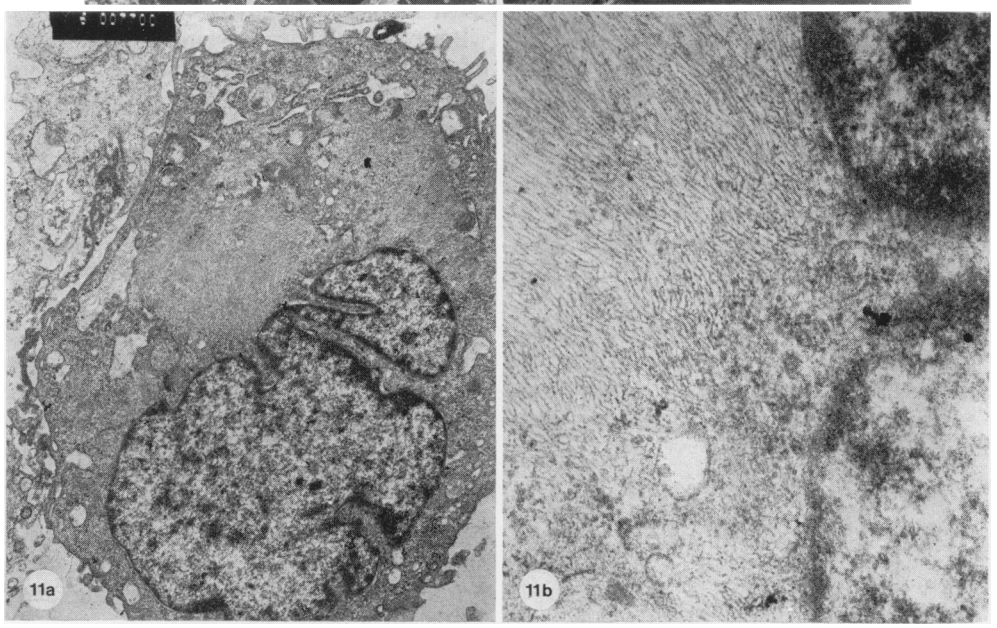


FIG. 10.—Scanning electron micrographs of cultured ST-6 subpopulations. (a) Typical cell with multiple villous processes on the cell surface from Fraction 4. Note apparent cell processes due to shrinkage artefact. × 2000. (b) Typical flattened cell with long cell processes and relatively smooth cell surface from Fraction 1–3. × 2000.

FIG. 11.—Transmission electron micrograph of cell pellet from Fraction 1–3. (a) Typical cell with filamentous inclusions. × 5132. (b) Close-up of same cell showing filamentous structures. × 30,666.

Fraction 1–3 cell is shown in Fig. 10b. No microvilli were seen on these cells and the cytoplasmic processes were extensive and, clearly, not due to shrinkage artefact.

No evidence of viral particles was observed in any of the sections examined.

Cultural characteristics of ST-6 and its subpopulations

(a) *Growth rate.*—The growth rate of Fraction 1–3, Fraction 4 and ST-6 cells was compared in the presence of 5% and 0·5% newborn calf serum, and the time taken for a cell division to occur was calculated. This is shown in Table II, in

TABLE II.—*Generation time of ST-6 cells and its subpopulations, Fraction 4 and Fraction 1–3 cells, in Eagles' medium containing 5% and 0·5% newborn calf serum*

	Generation time in 5% serum (days)	Generation time in 0·5% serum (days)
ST-6 (Pass 20)	2·8	3·6
Fraction 1–3 (Pass 5)	3·0	Did not divide
Fraction 4 (Pass 5)	1·2	1·6

which it may be seen that Fraction 4 cells grew about 3 times as quickly as Fraction 1–3 cells in medium containing 5% serum. In addition, they grew well in medium containing only 0·5% serum, while the Fraction 1–3 cells did not divide under these conditions.

(b) *Fibronectin immunofluorescence.*—In addition to the growth rate, the presence of fibronectin was tested by indirect immunofluorescence on ST-6 cells and its subpopulations. The unseparated ST-6 cells revealed a mixture of fibronectin-positive and fibronectin-negative cells. Fraction 4 cells had little or no fibronectin staining, while the Fraction 1–3 cells remained strongly positive.

(c) *Culture in semi-solid agar.*—As a further test of the possible tumour origin of Fraction 4 cells, they were cloned in semi-solid agar, being seeded at a density of 5×10^4 and 10^4 per plate. These numbers gave rise to 12 and 3 clones respectively, giving a cloning efficiency of 2–3 cells in 10^4 Fraction 4 cells. The clones which grew out contained epithelial-like cells.

(d) *Chromosome analysis.*—Chromosome analysis of ST-6 cells from Pass 12 was performed on 30 well spread cells and 25 of these possessed 54 chromosomes, the normal diploid number for sheep. Three cells had 51 chromosomes, one 52 and one 53 but the same chromosome was not missing consistently in these. Complete karyotypes were set out on 4 of the cells containing 54 chromosomes and all were shown to be normal (Fig. 12). No marker chromosomes or structural abnormalities were seen in any of the cells examined. 400 cells in mitosis were looked at and 32% of these possessed twice the normal diploid complement.

Screening for virus production

All cell strains which grew from the explant cultures (see Table I) were examined at Pass 4 or 5 for the presence of virus by light microscopy and electron microscopy, and none showed any evidence of this. In addition, ^{3}H-thymidine and ^{3}H-uridine were added to these cultures at Pass 4 or 5 in an attempt to label any virus which might be released into the fluid phase of the culture without an apparent cytopathic effect. No labelled material was found on subsequent sucrose gradient centrifugation of the concentrated culture supernatant with a density typical of virus particles. Also, in the case of the ST-6 cells, treatment with IUDR, cycloheximide and mitomycin C before labelling did not induce any virus. Finally, using 3 cultures, ST-1, ST-2 and ST-3, no cytopathic effect was detected after cocultivation with a variety of cell lines or infection of these with cell lysates.

No culture contained Mycoplasmas.

DISCUSSION

Explant cultures were used in an attempt to grow tumour cells from adenocarcinoma of the small intestine of sheep and a variety of cell types emerged.

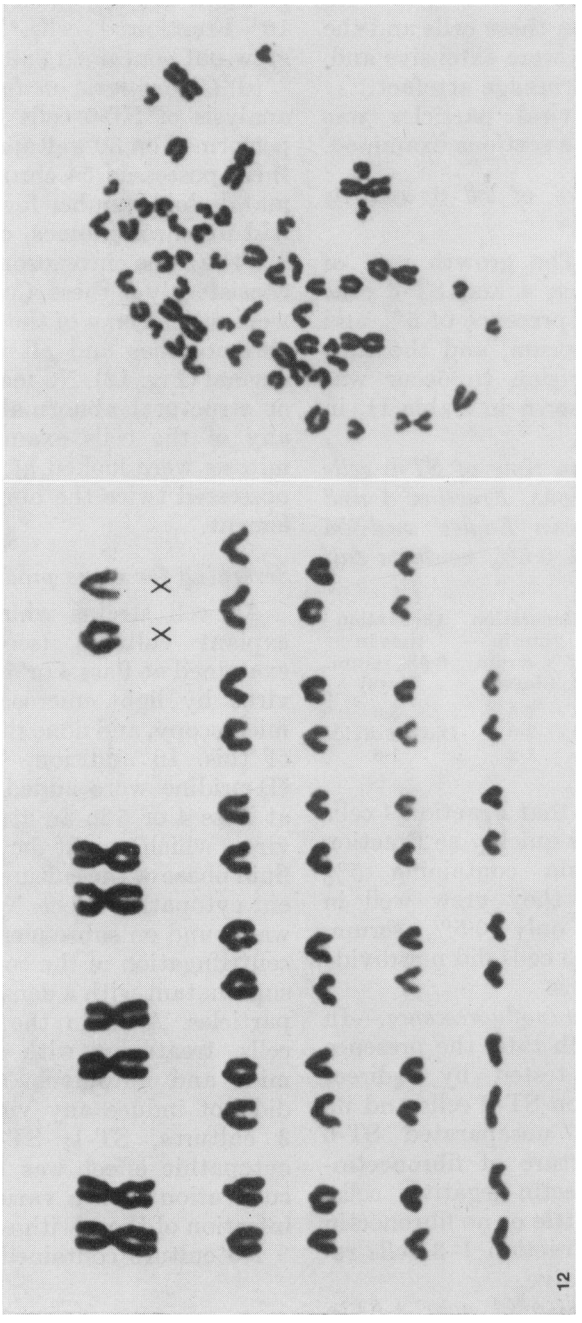


FIG. 12.—Chromosome analysis of ST-6.

Table I shows that the success of a culture could not be correlated with the speed of establishing the explant provided the sheep had been slaughtered and not left unopened for hours before postmortem examination. Likewise, the site from which the explant was taken did not seem to influence the successful outgrowth of cells. Three main cell types were found in the cultures: typical small fibroblasts, large fibroblast-like cells and epithelial-like cells. The small fibroblasts were recovered from both the non-neoplastic inflammatory reaction and from the tumour tissue. However, the fibrosis associated with the tumour when cultured soon after the death of the sheep often gave rise to large fibroblast-like cells. The fact that the most successful culture (ST-6) was obtained from a lamb might be taken to indicate that the case was unusual because of the age of the tumour-bearer. We do not think that this is tenable, first, because the other sheep to give a mixed epithelial-like and fibroblast-like culture (ST-8) was aged, *i.e.* at least 6 years old, and secondly, occurrence in lambs, although rare, has been previously reported; Georgsson and Vigfusson (1973), for example, stated that the youngest of their affected sheep was a yearling.

It has been suggested by Simpson and Jolly (1974) that this tumour arises from either adsorptive or goblet cells. Recently an ultrastructural study has been undertaken by Ross and Day (1979) and they reported that the tumour cells at the primary site, and in the metastases of the draining lymph node, form small acini which invade deeper tissue and provoke a fibrotic reaction. They suggested that, as all cell types found in the normal mucosa are present in the tumour, the lesion originates from undifferentiated intestinal epithelial cells.

This was of special interest in the present study as explants from 2 adenocarcinoma specimens gave rise to cell types of mixed morphology which persisted as such through several months of culture and over 30 passages. The cells seemed to form themselves into distinct growth patterns on subculture with the epithelial-like cells in discrete areas being surrounded by fibroblast-like cells. This mimics the appearance of the tumour *in vivo*. Separation of these cell types was achieved in 2 ways using methods which may also be applicable to other systems. The large fibroblast-like cells (Fraction 1–3) were observed ultrastructurally to have accumulations of fibrillar material in their cytoplasm together with mucin-containing vacuoles. It is not known what cell type they represent. On ultrastructural study, the epithelial-like cells (Fraction 4) showed extensive microvilli and frequent desmosomes between adjacent cell groups. In their cytoplasm there were vacuoles some of which contained mucus. On SEM cells of varying size could be seen as in the phase contrast examination of culture samples, but the morphology of these cells did not allow separation into 2 clearly defined subpopulations.

There were several factors which may indicate the oncogenic nature of these epithelial-like cells. In the first place, their growth rate was high and was little affected by reduction of the serum concentration to 0·5%. Secondly, a correlation has been drawn between the expression of fibronectin (LETS protein) and the oncogenicity of virus-transformed cell lines where transformed lines which produce tumours have little or no fibronectin, while in normal cells it is distributed over the surface (Chen *et al.*, 1976). An immunofluorescence test for the presence of fibronectin on the Fraction 4 cells showed little or no staining, while the Fraction 1–3 cells were strongly positive. In addition, the epithelial-like cells had the ability to form colonies in semi-solid agar. Therefore, it seems likely that these epithelial-like cells may represent the undifferentiated tumour cells described by Ross and Day (1979) and that they can be successfully cultured *in vitro*.

The original cells of this culture and all the other cell types which grew from explants of other specimens were screened for

virus production and all proved negative. It is possible that a virus is not involved in the aetiology of this tumour or, alternatively, that it may be present in a non-productive state, perhaps integrated into the host cell genome. The epithelial cells obtained here will be further studied to examine this possibility, especially with regard to ovine papilloma virus, in case the same correlation may apply in sheep as has been suggested in cattle (Jarrett *et al.*, 1978).

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