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Studies of Three Canine Mammary Carcinoma Cell Lines—I. *In Vitro* Properties*

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Abstract—*Three cells lines, REM* 134, 111 *and* 367, *have been derived from canine mammary carcinomas and their morphological characteristics* in vitro *are described. They are tumorigenic in athymic nude mice, have no demonstrable fibronectin on their cell surfaces and exhibit a varied pattern of lectin binding. They can be cloned in semi-solid agar. One line, REM* 134, *responds to oestrogen and luteotropic hormone* in vitro, *although none of the three had demonstrable oestrogen receptors.*

INTRODUCTION

THERE is a high incidence of naturally occurring mammary carcinomas in bitches [1], and a recent survey of such tumours showed them to be remarkably similar to human tumours in their pathology and receptor status [2]. The bitch has been used as an experimental animal for the testing of human drugs, especially steroid hormones which may increase the risk of mammary carcinomas [3]. Depite this, there have been few reports of *in vitro* long-term culturing of cells from these carcinomas. Owen *et al.* [4] established two cell lines, one fibroblastic in nature, derived from a primary adenocarcinoma, and the other epithelial, from a lung metastatis, and described some of their properties in tissue culture. The induction of retrovirus particles from another canine mammary carcinoma line, after treatment with IUDR and dexamethasone, has also been reported [5]. Recently serial transplantation of four canine mammary tumours in athymic nude mice has been described [6], in addition to a study of the oestrogen receptor status of such experimentally induced tumours [7].

During this work, cells have been cultured from over 130 canine mammary carcinomas using various techniques of explantation and disaggregation. Some specimens yielded cells which grew *in vitro* with epithelioid morphology but only in three instances have cell lines been established which have properties of tansformed cells and which induce tumours in athymic nude mice. These were called REM 134, 367 and 111. The initial characterization of REM 134 cells has already been reported [8]. This communication compares the three lines with special regard to their morphology at the light and electron microscopic levels, their growth in semi-solid agar, their surface properties such as fibronectin content and lectin binding, assay for retroviruses and their stimulation by various hormones. Their tumorigenicity in athymic nude mice is reported in the accompanying paper [9].

MATERIALS AND METHODS

Clinical specimens

Mammary carcinomas and various metastases were routinely obtained from bitches at surgery or within 1 hr of euthanasia. All were classified subsequently according to the WHO International Histological Classification [10]. Within 1–2 hr explant cultures of small fragments, about 1 mm^3, were made in Petri dishes (Sterilin), or small pieces of tissue were disaggregated in collagenase/dispase (Boehringer) according to the manufacturer's instructions for 1–2 hr at 37°C. Single cells were washed and cultured in Falcon flasks (Flow). Eagle's minimal essential medium (EMEM) supplemented with 100 iu/ml penicillin, 200 μg/ml streptomycin, 50 iu/ml fungizone, 100 μg/ml gentamycin and 100% foetal calf serum was used routinely. Incubations were carried out

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at 37°C in an atmosphere of 5% CO_2 in air. Once the cells were established, gentamycin and fungizone were omitted from the medium and the serum changed to 5% foetal calf serum plus 5% newborn calf serum.

Electron microscopy

Cells were harvested by gentle mechanical scraping or by using dispase. After washing in 0.1 M sodium cacodylate buffer, they were fixed in 2.5% glutaraldehyde for 2 hr and pelleted by centrifugation at 3000 *g* for 5 min. Pellets were post-fixed in 2% osmium tetroxide and embedded in epoxy resin prior to sectioning. Thin sections, stained with lead citrate and uranyl acetate, were examined in a Philips 400 TEM. For scanning electron microscopy cells were cultured on 10-mm 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. Specimens were viewed in an ISI 60 or Philips SEM 505.

Growth rate

Cells (4×10^5) were seeded into 50-mm Petri dishes in 5 ml EMEM containing either 5% foetal calf serum plus 5% newborn calf serum, or 0.5% of each. On each day for the next 4 days the number of viable cells present were counted after removal with trypsin–versene.

Growth in semi-solid agar

Cells (10^3) were cloned in 5 ml EMEM containing 10% foetal calf serum and 0.25% agarose (Seaplaque, Marine Colloids Division) in 50-mm Petri dishes with 1 ml medium containing 0.55% agarose as underlay. One mililitre of fresh medium with 0.25% agarose was added after 10 days incubation.

Fibronectin immunofluorescence

An indirect immunofluorescent test to detect fibronectin was carried out using cells grown in monolayers or cryostat sections of tumours which were fixed in 2% paraformaldehyde [11]. Rabbit antiserum was produced by injecting purified human cold insoluble globulin into rabbits, and was kindly donated by Dr J. Kinross. It was used at dilutions of 1/20, 1/100 and 1/250, while the FITC anti-rabbit conjugate (Wellcome) was used at 1/16.

Lectin immunofluorescence

A direct immunofluorescence assessment using FITC-labelled lectins (E-Y Laboratories, California) was carried out on cells grown in monolayers or cryostat sections of tumours fixed in cold acetone for 10 min.

Induction method for retroviruses

Cell cultures were induced with luteotropic hormone (5 μg/ml, Sigma) for 24 hr, progesterone (1 μg/ml, Sigma) for 24 hr and IUDR (20 μg/ml) for 24 hr, then dexamethasone (5 μg/ml) for 72 hr [5]. They were labelled with [^{3}H]-thymidine for 24–48 hr followed by concentration of the culture supernatant and sucrose density gradient centrifugation [12].

Effect of hormones on RNA synthesis

Cells (5×10^4) in 1.5 ml EMEM with 1% newborn calf serum and 1% foetal calf serum were put into tubes containing cover-slips. In some the medium was supplemented with luteotropic hormone in concentrations ranging from 10^{-6} to 10^{-12} M, or oestradiol-17β or testosterone from 10^{-5} to 10^{-11} M. Five microcuries of [^{3}H]-uridine was added to a duplicate set of tubes on the same day and to equivalent sets after the tubes had been incubated for 1, 2 and 3 days. The cover-slips were removed 24 hr after the addition of the label, washed in phosphate-buffered saline, fixed in cold 10% trichloroacetic acid for 10 min, washed in water, air-dried and counted in a PPO-toluene scintillator. An average of the two counts (which were always within 10% of each other) was taken.

Hormone receptors

Cultured cells of REM 134, 367 and 111 were examined for oestrogen and progesterone receptor activity as measured by uptake of [^{3}H]-oestradiol. Culture medium after growth of REM 134 cells was measured for oestrone and oestradiol-17β by a radioimmunoassay [Hawkins, personal communication]. In addition, the primary tumour of REM 134 has been assayed previously for oestrogen receptors [13].

Chromosome analysis

REM 134 and 111 were karyotyped using the method outlined in [8].

RESULTS

Origin of growth of REM 134, 367 and 111 cell lines

After explantation or disaggregation followed by culturing, epithelioid cells from canine mammary tumours were obtained in about 60/130 cases. In some of these the epithelial cells persisted only a short time, a month or two in culture, before detaching from the surface. Sometimes they became overgrown with fibroblastic cells or failed to grow on subculture. However, in some cases epithelial cells were obtained which could be subcultured successfully and maintained through at least 20 passages. Three, REM 134, 367 and 111, persisted as long-term cultures, considered as cell lines. Tumour

cells for REM 134 were obtained from a primary mammary solid carcinoma as described previously [8]. Cells for 367 were cultured from a primary papillary mammary adenocarcinoma obtained at the time of mastectomy. Tumour cells for 111 came from a pleural effusion collected immediately after euthanasia of a bitch with metastatic disease, following a primary mammary anaplastic solid carcinoma. All have been cultured for periods in excess of 1 yr; more than 130 passages of REM 134 cells have been effected and over 40 passages of 111. Cells of 367 exhibit a slower growth rate but have been passaged over 30 times. From the remaining epithelial cell cultures, two strains called A and B were used for comparison throughout, and one with fibroblastic appearance was also used.

The doubling time of REM 134 cells in 10% serum was about 24 hr, about 48 hr for 111 and 60 hr for 367 cells. If the serum content of the medium was reduced to 1%, the doubling time of REM 134 and 111 cells were unchanged for two divisions, and no growth occurred thereafter. The 367 cells grew very poorly in low serum.

The ability to form colonies in semi-solid agar has been reported as a good general index of transformation and subsequent tumorigenesis in suitable experimental animals [14]. Thus the three cell lines, as well as epithelial strains A and B and the fibroblastic strain, were cultured in semi-solid agar. Cells REM 134, 111 and 367 were found to form colonies visible by eye after 14 days of incubation which were picked using micropipettes and cultured separately. The cloning efficiency was 4% in all cases. No colonies were formed from the other cells strains.

Morphology of cell lines

The histopathological appearances of the original primary tumours REM 134, 111 and 367 are shown in Fig. 1, together with the cells from the pleural effusion, 111. Throughout the culture period the morphology of the three lines was typically epithelial (Fig. 2). REM 134 cells, however, showed considerable variation, ranging from polygonal to rounded. Epithelial cells from strain A and the fibroblastic cell strain used throughout are also shown in Fig. 2.

Marked variation in cell size was seen on thin sections of cultured cells, especially for REM 134. Nuclei were usually irregular, with indented nuclear membranes and coarse granular chromatin (Fig. 3a). Mitotic figures were common in REM 134 and 111, but less common in 367. Vacuole-like structures with villi were occasionally seen in REM 134 cells but were absent in 111 and 367. Many vacuoles in REM 134 cells contained electron-dense particles (Fig. 3b). Tonofibrils were common in the cytoplasm but mitochondria were relatively few and sometimes bizarre, particularly in REM 134. There were prominent microvilli in all three lines, but none showed myosin bundles. No evidence was seen of virus particles.

On examination of confluent monolayers in the scanning electron microscope, there were flattened cells with indistinct cell borders and slightly raised nuclear areas. At the periphery of confluent areas the cells were rounded and heaped (Fig. 4). The cells from the three lines showed prominent microvilli on their surfaces with fewer in nuclear zones. Rounded cells had prominent villous structures and some had bleb-like formations (Fig. 5). REM 134 cells had elongated villi (Fig. 6a), whilst both 367 and 111 had a greater density of shorter formations which were sometimes more bulbous (Fig. 6b).

The karyotype of REM 134 has already been reported [8]. Cells 111 at passage 10 showed marked polyploidy, with chromosome numbers ranging fron 126 to 167.

Surface properties

In addition to the ability to grow in suspension, most transformed cell lines exhibit a greatly reduced content or absence of fibronectin [11]. The cell lines and strains were tested by indirect immunofluorescence after growth on cover-slips. The results are shown in Table 1. It may be seen that REM 134, 367 and 111 cells had no detectable fibronectin, while the epithelial strains A and B and the fibroblastic strain had.

The surface of the cultured cells was also analysed by binding of nine different lectins using a direct immunofluorescent technique (Table 2). Wherever possible, the specificity of the binding was demonstrated by the reduction of immunofluorescence in the presence of the sugar to which the lectin attaches. There was no apparent pattern of lectin binding to separate the three lines from the other strains used.

Table 1. *Presence of fibronectin as measured by indirect immunofluorescence*

	Dilutions of antiserum		
	1/20	1/100	1/250
REM 134	-	-	-
367	-	-	-
111	-	-	-
Epithelial strain A	++	++	-
Epithelial strain B	++	+	-
Fibroblastic strain	++	++	-
Human embryo fibroblasts	+++	++	+

+++, ++ and + = degrees of immunofluorescence; - = no immunofluorescence.

Table 2. Binding of FITC-lectins to cell cultures as measured by direct immunofluorescence

	REM 134	111	367	Epithelial strain A	Epthelial strain B	Fibroblastic strain
PNA	-	-	-	-	-	-
GS-1	++	+	+	-	-	++
GS-I + D-gal	-	-	-	-	-	+
BSA-II	+++	+++	+++	++	++	+++
DBA	-	+	+	-	+	+
DBA + NAc-D-gal	-	-	-	-	-	-
SBA	+++	+	++	+	-	-
SBA + D-gal	-	-	-	-	-	-
MPA	-	+	-	-	++	+
MPA + D-gal	-	-	-	-	-	-
RCA	++	+	+	+	+	++
RCA + D-gal	-	-	-	-	+	-
WGA	+++	+++	+++	+++	+++	+++
UEA	-	-	-	+	-	-
UAE + L-fuc	-	-	-	-	-	-

PNA, *Arachis hypogaea* agglutinin; BSA-II, *Bandeiraea simpliciforlia* agglutinin; SBA, *Glycine max* agglutinin; RCA, *Ricinus communis* agglutinin; UEA, *Ulex europaeus* agglutinin; GS-I, *Bandeiraea simplicifolia* agglutinin; DBA, *Dolichos biflorus* agglutinin; MPA, *Maclura pomifera* agglutinin; WGA, *Triticum vulgaris* agglutinin; D-gal, D-galactose; L-fuc, L-fucose; NAc-D-gal, *N*-acetyl-D-galactosamine.
+++, ++ and + = degrees of immunofluorescence; - = no immunofluorescence.

Induction of retroviruses

There have been several reports of retroviruses being associated with mammary carcinoma cell lines from various species, such as human [15], murine [16] and canine, the latter being induced with IUDR followed by dexamethasone [5]. When such a regime was carried out on REM 134, 111 and 367 cells no virus particles were detected using [^{3}H]-uridine labelling. In addition, induction by treatment with progesterone or luteotropic hormone was not successful.

Effect of hormones on rate of growth and hormone receptors

Oestradiol-17β, testosterone and luteotropic hormone at varying molarities were added to REM 134, 367 and 111 to find out if they stimulated the growth rate. This was measured by the uptake of [^{3}H]-uridine over a 4-day period after the addition of the hormone. Cell lines 111 and 367 did not respond to any of the added hormones at any concentration. In the case of REM 134 there was no effect due to the addition of testosterone, but oestradiol and luteotropic hormone stimulated RNA synthesis 72 hr after addition by as much as 2–3 times, as illustrated in Fig. 7.

REM 134 and 111 cell lines were analysed for oestrogen and progesterone receptor activity and both were negative. In addition, the uptake of [^{3}H]-oestradiol (5 nM) into 5×10^8 REM 134 cells was measured in a 1- to 2-hr period at 37°C. The cells were washed once, incubated in oestrogen-free buffer for 0.5 hr and then washed three times prior to counting. Control cells were incubated with [^{3}H]-oestradiol plus a 500-fold excess of diethylstilbestrol, which competes for oetrogen binding sites and would therefore block receptor-binding. Three per cent of the initial radioactivity was found in the cells after incubation but the control cells contained the same amount, thus implying that there were no oestrogen receptors.

DISCUSSION

Three epithelial cells lines derived from canine mammary carcinomas have been described which show tumorigenic properties in athymic nude mice [9]. They have varied morphology but all contain detectable fibronectin on their cell surfaces and have the ability to grow in semi-solid agar, producing colonies at the same efficiency. The clones obtained seem to have identical morphology at the light microscopic level as the parent cells and are presently being tested for their

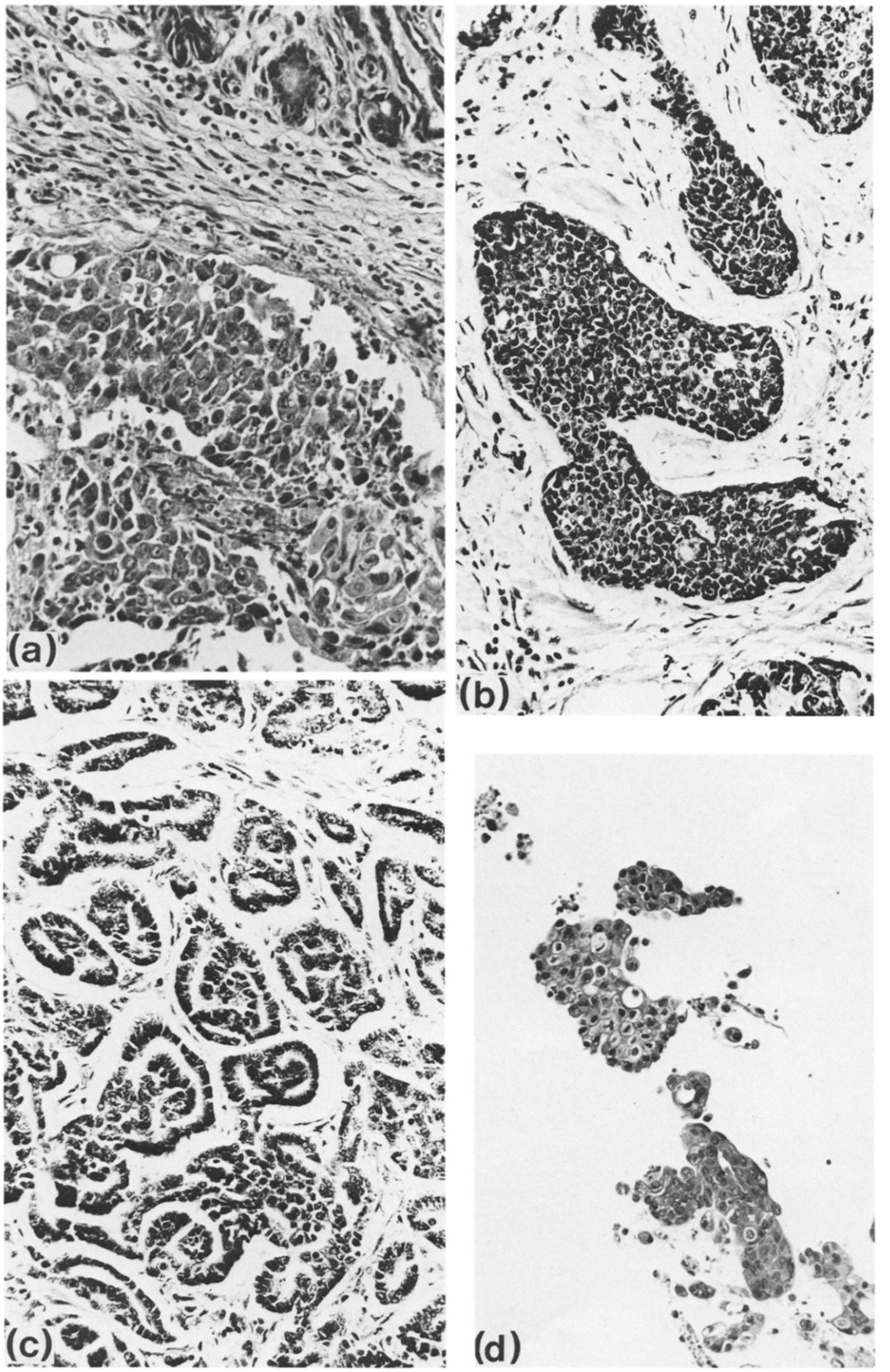

Fig. 1. *Histopathology of original primary tumours,* ×200, *H and E stain.* (*a*) *REM* 134; (*b*) 111; (*c*) 367; *and* (*d*) *pleural effusion* 111.

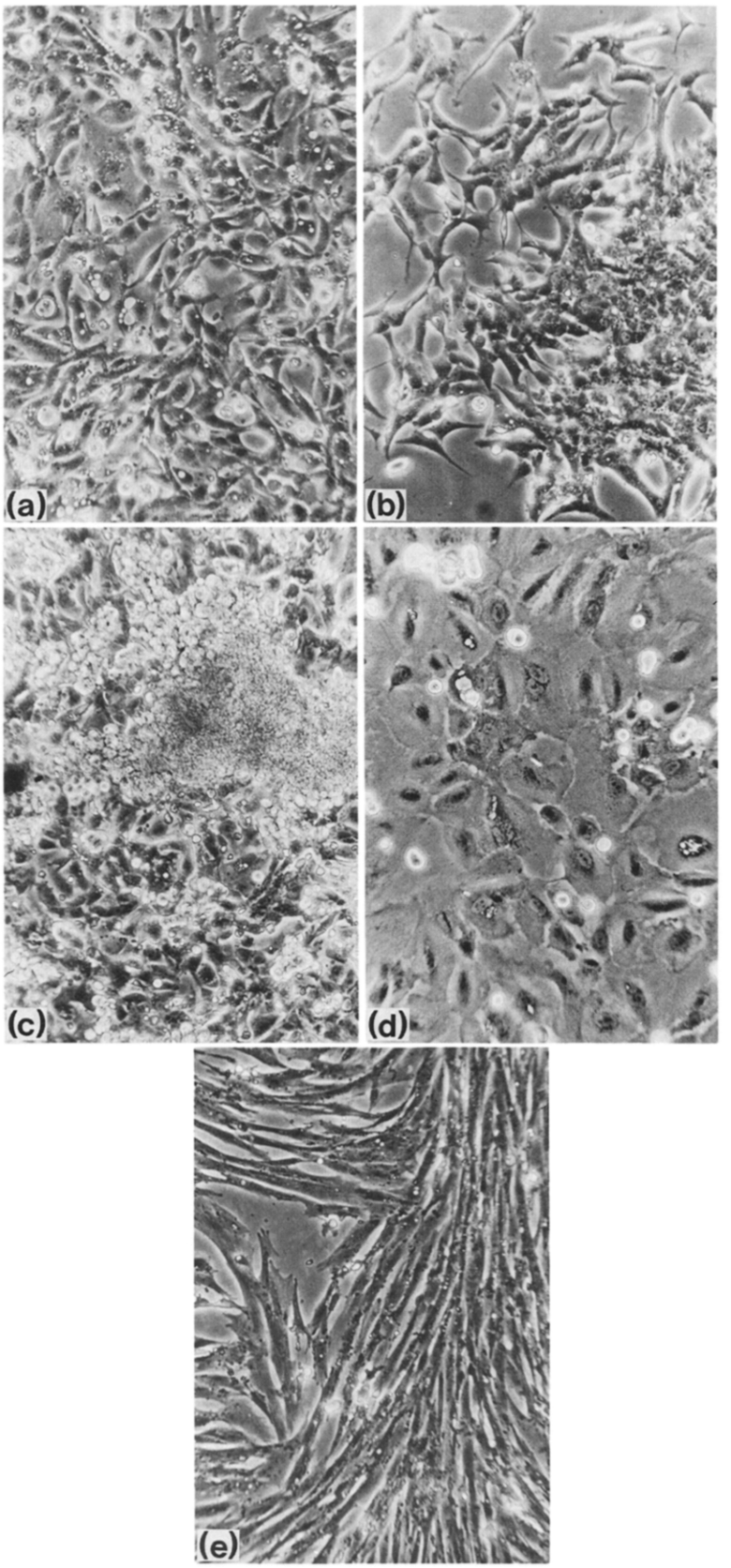

Fig. 2. Phase-contrast microscopy, ×100. (a) REM 134; (b) 111; (c) 367; (d) epithelial strain A; (e) fibroblastic strain.

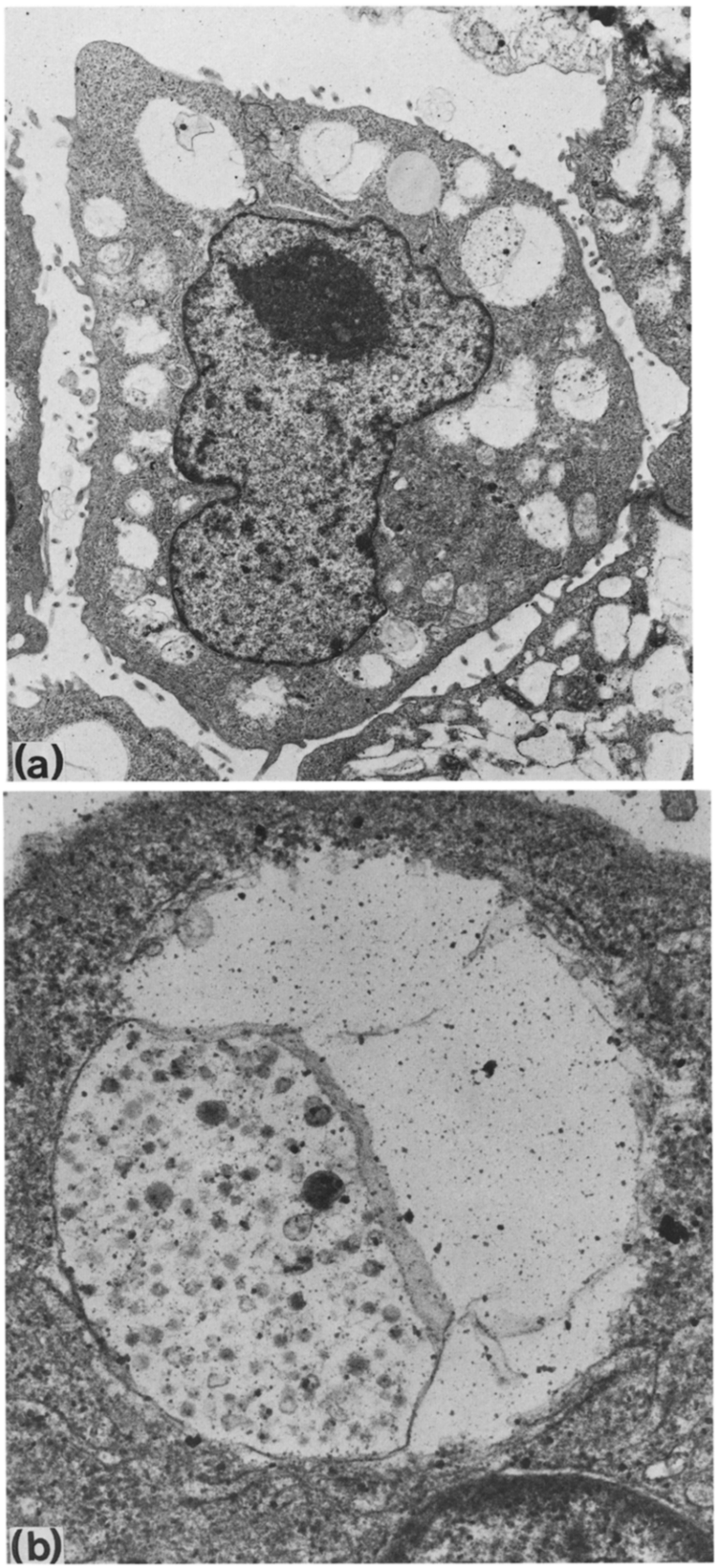

Fig. 3. Transmission electron micrographs of REM 134 cells. (a) Typical cell, ×10,000; *(b) vacuole-like structure containing particles,* ×71,000.

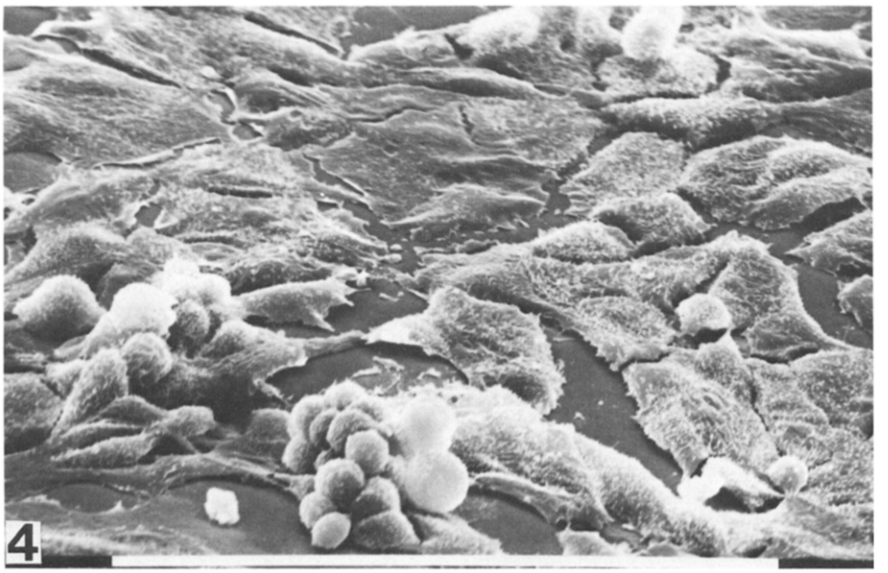

Fig. 4. Scanning electron micrograph (SEM) of 367 cell monolayer, showing rounding and heaping of cells at periphery, ×1850.

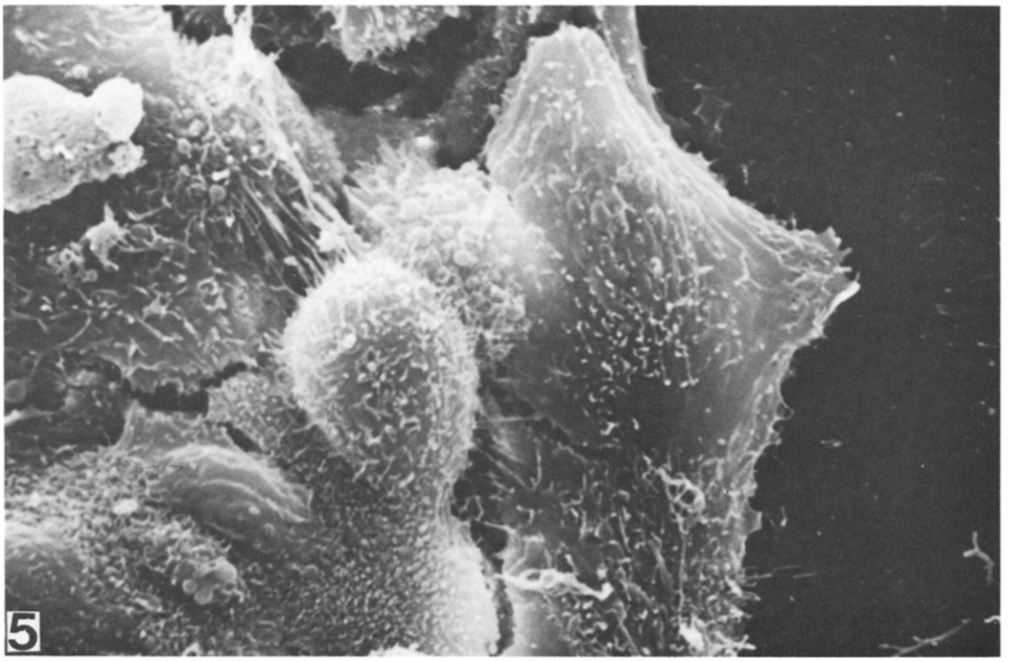

Fig. 5. SEM of 111 *cells showing villi and blebs on rounded cells,* ×4000.

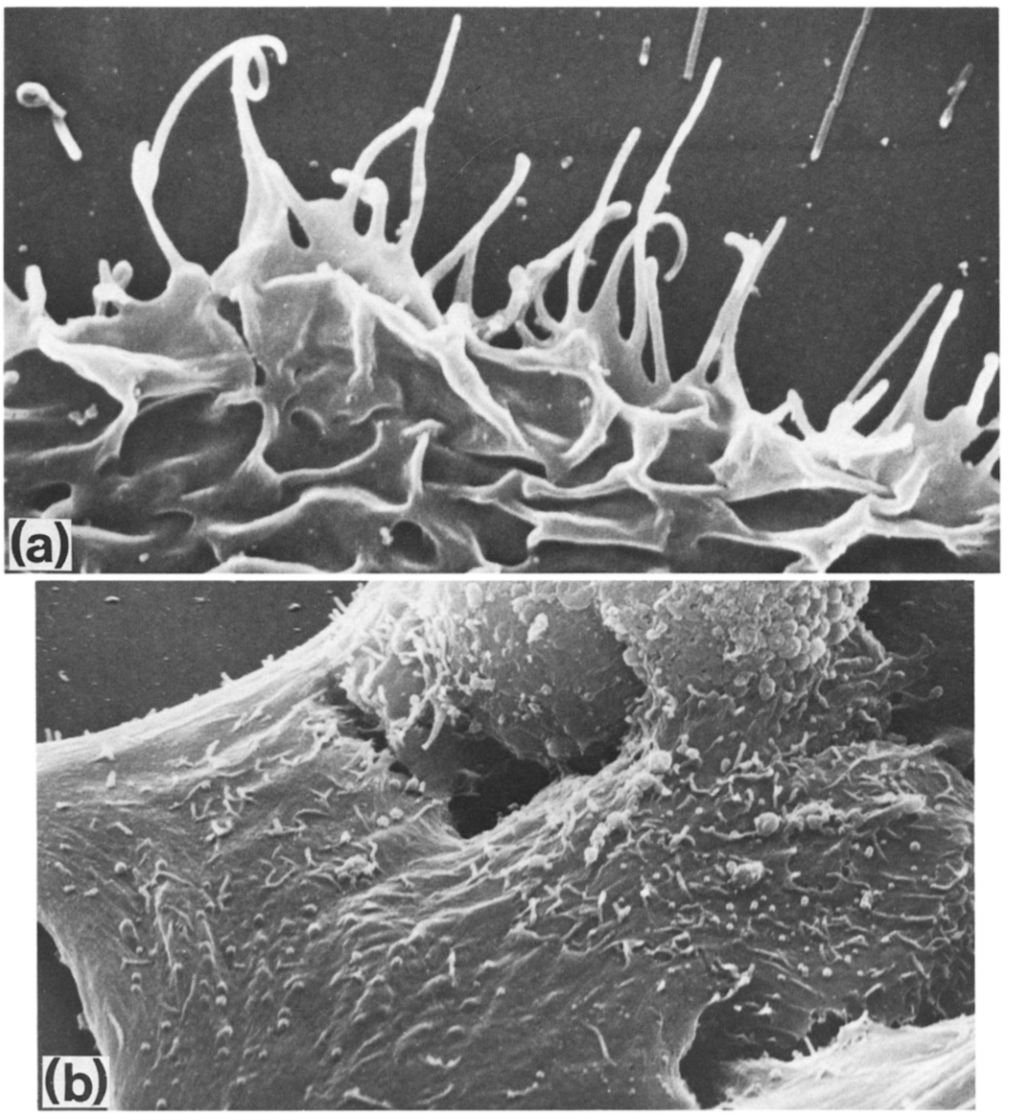

Fig. 6. *SEM of villous structures on cells with elongated villi in* (*a*) *REM* 134, *and bulbous structures in* (*b*) 367; ×11,200 *and* ×7000 *respectively.*

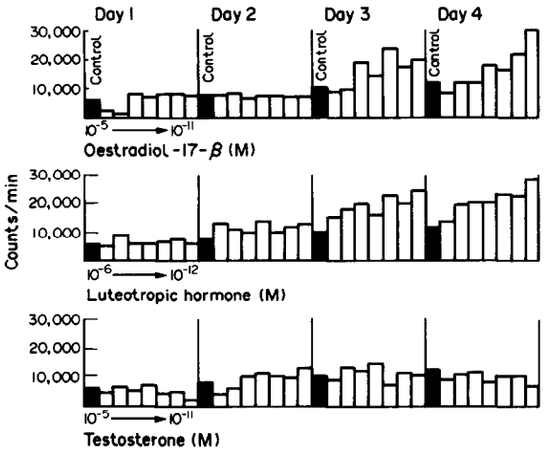

Fig. 7. Incorporation of [^{3}H]*-uridine in REM 134 cells over a 24-hr period on each of four days after the addition of oestradiol-17β, luteotropic hormone and testosterone. Concentrations of hormones are in ten-fold dilutions starting at* 10^{-5} *or* 10^{-6} M, *as shown. Controls for each day with no added hormone are also shown.*

tumorigenic properties and ultrastructure. While the REM 134 cells grew as thick even layers of cells after reaching confluence, 111 and 367 began to heap up at the edges of 'islands' of cells and these had a tendency to detach from the surface, although still viable and capable of starting re-growth in a new flask. The pattern of binding of lectins to the surfaces of the cell lines was not consistent and they could not be distinguished from the epithelial strains on this basis.

Attempts to demonstrate productive infection with retroviruses failed despite the same methods of induction being used as had been reported for a canine mammary carcinoma line [5] and for MCF-7 cells [15]. The lines are presently being tested with anti-gp52 of a murine mammary tumour virus [17]. In addition, an antiserum prepared in rabbits to the membranes of REM 134 cells is being used in Western blotting to detect similarities and differences in polypeptides eluted from the surface of REM 134, 367 and 111, epithelial cell strains and mammary carcinomas themselves.

Oestrogen receptors were detected on the original tumour from which the REM 134 cell line was derived [13]. The tumours which gave rise to 111 and 367 lines were not tested in this way. A recent survey has indicated the presence of such receptors on 61% of canine mammary carcinomas [2]. *In vitro*, no receptors were detected on the cell lines tested. However, while 367 and 111 were not stimulated by oestrogen, luteotropic hormone or testosterone in culture, REM 134 cells showed an increase in [^{3}H]-uridine uptake in the presence of oestrogen and luteotropic hormone at physiological levels. Thus there seemed to be a response of REM 134 cells to female hormones, although no receptor activity could be detected. This may be due to low levels of such receptors and an insufficiently sensitive assay system, or to some other reason. The rate of growth of tumours induced by the three lines in male and female nude mice is reported in the accompanying paper [9]

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