HISTO-CYTOLOGICAL CHARACTERIZATION OF HUMAN BREAST CARCINOMA CELLS CULTURED ON PIGSKIN AND PLASTIC SUBSTRATES.

by

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LIST OF IMPORTANT ABBREVIATIONS

AEC	=	amino-ethylcarbazole
AgNORs	=	silver-stained nucleolar organizer regions
DNA	=	deoxyribonucleic acid
EA50	=	eosin-azure
ECM	=	extracellular matrix
EMA	=	epithelial membrane antigen
ER	=	oestrogen receptor
ERICA	=	peroxidase immunochemistry
FNA	=	fine needle aspirate
H&E	=	haematoxylin and eosin
IF	=	intermediate filament
IGSAM	=	immunogold streptavidin enhancement method
IGSS	=	immunogold silver stain
IU	=	international units
LCA	=	leukocyte common antigen
MCF-7	=	breast carcinoma cell line
MDA-MB-231	=	breast carcinoma cell line
PAP	=	peroxidase-anti-peroxidase
PBS	=	phosphate buffered saline
PEG	=	polyethylene glycol
rRNA	=	ribosomal ribonucleic acid
SCF	=	slide culture flasks
T-47D	=	breast carcinoma cell line
WF	=	washing fluid

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1 INTRODUCTION

1.1 MOTIVATION

Breast cancer remains the leading cause of mortality among western women and its incidence is increasing (Tjandra and McKenzie, 1988; Allred *et al.*, 1990). Although several different and effective therapeutic treatments for breast cancer are used, all suffer to some extent from lack of specificity. At present, chemotherapeutical treatment of cancer is done very much on a "touch wood" basis as far as the drug mixture and dose is concerned. Currently breast cancer cells appear to be one of the most difficult cancers to establish in culture (Fogh, 1975; Hainsworth and Garson, 1990).

The fact that cancers are classified as identical according to their histopathological characteristics but are nonetheless highly individual in their drug sensitivities, poses a major clinical problem (Vescio *et al.*, 1987). Ample documentation exists confirming that most cancers consist of subpopulations of tumour cells which differ with regard to numerous properties. Subpopulations of single tumours often have differential drug sensitivities. The growth characteristics and sensitivity to chemotherapeutic agents of individual tumour subpopulations can also be influenced by the presence of other subpopulations of the same tumour (Miller *et al.*, 1981). There is currently no way to predict clinical outcome of chemotherapy for individual patients. There is also no optimum assay to screen for new human anticancer agents, especially for solid tumours, which presents a second problem. Many attempts have been made to develop *in vitro* drug-sensitivity tests for individual cancer patients about to undergo chemotherapy and to screen for new anticancer agents (Vescio *et al.*, 1987). However, most of these attempts have suffered in one form or another from their inability to support growth of human tumours such that they reflect the *in vivo* situation. In many instances plating of dissociated tumour cells in soft agar and monolayer cultures does not allow the growth of tumour cells (Vasiliev and Gelfland, 1981). Furthermore, Schor *et al.* (1982) stressed that, "there is a growing awareness that cell migratory behaviour *in vitro* should be examined on biologically relevant macromolecular matrices, rather than on the artificial surfaces commonly used in such studies. Collagen is a major constituent of the extracellular matrix *in vivo*." Often, when some tumour cells do grow under culture conditions, other cell types present in the original tumour probably have

not grown (Vescio *et al.*, 1987). Overgrowth by fibroblasts is often a problem, especially in long term cultures (Emoto *et al.*, 1992). Since it has been shown that interactions between cell types can alter their drug sensitivities, it is critical that the cell types present in the original tumour also be present in the assay (Vescio, *et al.*,1987; Jung *et al.*, 1991). An assay system representing the original tumour for individual cancer patients who will respond differently, according to his or her own metabolic capabilities, is therefore needed to determine the sensitivity of the cancer cells to different drugs as well as combinations of drug types. The aim should also be focussed on any kind of short-term assay which will improve the ability of scientists to do chemical testing (Klausner, 1987; Vescio *et al.*, 1987).

1.2 BREAST CARCINOMA

Degenerative disorders and cancer are still a major challenge facing medicine today. In Western society, cancers of the breast and colon are the most important malignancies among non-smokers (Willett, 1989).

Although enormous advances have already been made in the classification and management of haematological malignancies, management of common adult carcinomas has progressed slowly over the past 45 years. This is largely due to the inherent complexities of these tumours and our limited understanding of the differences between normal and malignant cells (Hainsworth and Garson, 1990). Intra-tumour heterogeneity exists in most, if not all, human solid tumours. This is reflected by the differences in morphology, antigenicity, chromosomal and biochemical markers, invasion, metastatic spread and sensitivity to therapy (Denk, 1988).

The familial aggregation of breast cancer was first recorded in the Roman medical literature around 100 A.D. In spite of this long historical experience, we remain abysmally ignorant about its aetiology and pathogenesis (Lynch *et al.*, 1984). Another important characteristic of human breast cancer is its aetiologic heterogeneity due to factors that differ between patients regarding their history and physiological status. Factors i.e. ionizing radiation, diet, socio-economic status, menstrual status, reproductive history, family history, long-term treatment with oestrogens, genetic factors and previous atypical benign breast disease, provide a selective environment for the clonal outgrowth of cells which contain

somatic mutations. One biological consequence of these mutations may be the development of neoplasia through the uncoupling of normal mammary gland development. Because of this heterogeneity and the multiple events necessary for most epithelial tumours, it seems likely that multiple genetic mutations act in concert to produce an invasive breast carcinoma with the ability to metastasize to distant organ sites (Russo *et al.*, 1990; Callahan *et al.*, 1991).

Numerous studies, based on examination of surgical and autopsy tissues, have been undertaken in an effort to determine the nature and site of origin of neoplastic growth in human breast. Tumours differ considerably in the pattern of invasive growth adopted *in vivo* (Schor *et al.*, 1982). Two general patterns of invasive behaviour on the basis of histological data have been defined. Tumours displaying pattern I behaviour tend to invade surrounding normal tissue by the penetration of a coherent mass of tumour cells, usually along available tissue spaces. This particular pattern of invasive growth is often observed with tumours of endothelial origin (i.e. adenocarcinoma). Tumours that display pattern II behaviour, on the other hand, tend to invade surrounding normal tissue by the active movement of single tumour cells and often involves the infiltration of tumour cells between connective tissue elements.

Studies using sub-gross sampling of whole human breasts with histologic confirmation, allowing the identification and quantitation of essentially the entire pathologic content of each breast, proved the most useful. It is postulated that there are four major possible sites of origin of mammary carcinomas: ducts, terminal ducts, ductules and "acini". Infiltrating ductal carcinoma originates in the large and medium ducts of the breast, and is the most common carcinoma of the breast constituting approximately 80% of all types of breast carcinoma. Medullary carcinomas probably also arise from the medium and large ducts. Infiltrating lobular carcinoma arises within the lobules from the ductules and acini and constitutes about 10% of all breast carcinomas. Mucinous carcinomas (colloid carcinomas) constitute about 5 % of all breast carcinomas and occur in pure or mixed form, often as part of an infiltrating ductal carcinoma. When these neoplasms are homogeneous, they are associated with a good prognosis (Kline, 1988). Papillary carcinoma occurs homogeneous in about 2% of all breast carcinomas and affected patients have a favourable prognosis, similar to that associated with colloid carcinoma (Kline, 1988).

1.3 CULTURING OF MALIGNANT TUMOURS

Much of the knowledge concerning mechanisms involved in the growth regulation of breast cancer comes from the use of continuous cell cultures, particularly cell lines (Leibovitz, 1986). However, the available cell lines which are supposed to be well characterized are few and in many ways putative due to a lack of standardized criteria certifying it's human, mammary and malignant origin. Often cloning and growth of cell lines and tumours are claimed but the methodology on which to establish the validity of these claims has not been reported on (Engel and Young, 1978). During the last few years, various methods have been described allowing the growth of normal and carcinoma derived breast epithelial cells in monolayer, clone or organotypic form. Substrates for the growth of cells include soft agar, pigskin, extracellular matrices, glass and plastic substrates. A vast number of different media compositions and supplements necessary to culture different tumour cells have also been developed. In many cases i.e. breast tumour cell lines derived from similar tumours, the media composition to culture these cell lines are different (Kruse and Patterson, 1973; Paul, 1975; Freeman *et al.*, 1979; Friedman and Glaubiger, 1982; Biran *et al.*, 1983; Crickard *et al.*, 1983; Finlay and Baguley, 1984; Freeman and Hoffman, 1986). A prerequisite for the proper interpretation of growth control mechanisms identified in short-term cultures is the outgrowth of a representative fraction of tumour cells under conditions which preserve the physiological state of the cells comparable to the state *in vivo*. Careful characterization of the cells present in short-term cultures reflecting the heterogeneity of the tumours at a cellular level is also required (Renegass *et al.*, 1989). Unfortunately breast cancer currently appears to be one of the most difficult cancers to establish in culture (Hainsworth and Garson, 1990). Although reports of attempts to culture breast cells appeared as early as 1937, it was not until 1958 that the first successful long-term culture of a breast tumour was reported (Engel and Young, 1978).

Several investigators have reported on the technical difficulties in achieving continuous cultures of these tumours. Numbers of malignant cells provided by tumour samples may not be great (often less than 1%), and the viability of these cells is often low. Furthermore, tumour cells often comprise only a minority of the population of cells in a lesion. Most breast tissue resected from cancer patients consisted mainly of stroma and supporting cells (Crickard *et al.*, 1983;

Baker *et al.*, 1986). In culture, fibroblasts often tend to outgrow the less prolific epithelial cells as shown in Fig. 2 (Van Huyssteen, 1994) Some investigators have attempted to recreate the *in vivo* milieu by providing connective tissue substrates using attachment factors i.e. collagen, fibronectin, gelatin, laminin and cell adhesion molecules i.e. cadherins and integrins, while others have focussed on nutritional requirements. Hormones (i.e. oestrogen and other contraceptive steroids) especially may play a role, while serum additives further contribute variable and unknown amounts of steroids and proteins necessary for growth (Murray *et al.*, 1980; Calaf *et al.*, 1986; Longman and Buehring, 1987; Hart and Saini, 1992).

Attempts to culture breast cancer cells have been successful more often when cells from malignant effusions were used, than with cells from solid tumours. Effusions provide large numbers of dissociated, viable tumour cells with little or no contamination by fibroblasts (Engel and Young, 1978). This may be due to the fact that the less adhesive tumour cells are preferentially sampled over more cohesive stromal and epithelial cells. Mechanical spill out methods of disaggregation are probably the simplest and best source of obtaining tumour cells by the least traumatic method with minimal stromal contamination (Kruse and Patterson, 1973; Leibovitz, 1986). Tumour cells are often loosely adherent to their stromal matrix and are easily released into the surrounding medium when mechanical spill out methods are used. Mechanical methods entail the slicing of tumours with surgical blades into 1-2mm cubes or forcing the tumours through metal sieves into a medium from which the less adherent tumour cells can then be retrieved for culture (Leibovitz, 1986). Several studies were done using collagen gel which had been extracted from rat-tail tendons (Chambard *et al.*, 1981; Schor *et al.*, 1982; Chaplowski *et al.*, 1983; Miller *et al.*, 1985). These authors found that the migration of tumour cells into the gel matrix is affected by the concentration of collagen in the gel, as well as by the initial density of cells plated onto the gel and the density of cells in the primary culture dishes.

Chemotherapy predictive assays are presently based mainly on cultures in soft agar where only the malignant component is grown as illustrated in Figs. 1 and 3 (Van Huyssteen, 1994). Vescio *et al.*, 1987 developed a three-dimensional tumour culture method by which most human tumour biopsies can be grown. The culture system meets important criteria of *in vivo* growth, including maintenance of tissue structure. They report that with the use of their tumour culture system, tumours respond to drugs in a manner that reflects the *in vivo*

response (Vescio *et al.*, 1987). However, several problems have been identified in culturing human tumour cells in soft agar. Approximately 50% of tumours cannot be cultured adequately to enable testing for drug sensitivity. Preparing cultures for morphologic, cytogenetic and histochemical analysis is difficult due to technical problems associated with embedding the cells in agar (Elsdale and Bard, 1972; Baker *et al.*, 1986).

When explants of human skin were cultured on glass or plastic surfaces, epithelial cells grew or migrated out and formed a small, self-limiting island around some of the explants (Smith *et al.*, 1985). For the most part the predominant cell type was fibroblastic. Upon subculture of these primary cultures only fibroblasts were cultured. In contrast, explants cultured on the dermis of pigskin produced a layer of cells consisting mostly of epithelial cells. Thus the dermal collagen bed seemed to select for epithelial cell growth while a glass or plastic surface favoured fibroblastic growth (Freeman *et al.*, 1976).

Yoshida *et al.*, (1980) made use of organ or organ bit cultures which has severe limitations, of which the most critical are short-term survival and lack of new growth. All *in vitro* studies are hampered by the difficulties of identifying the origin and functional properties of the surviving cells. Their approach to these problems was to grow organotypic mouse foetal lung cultures on pigskin dermis and then to identify the cell elements by morphological and histochemical evidence. A question concerning the lung foetal cell population on pigskin dermis is whether they are the result of new growth or whether they merely represent a migration of cells from the explant. Evidence for growth consists of the ever enlarging field of cell area surrounding the original explant, the detection of mitotic figures, and the progressive incorporation of tritiated thymidine into DNA. According to these authors the pigskin culture method has advantages over monolayer cultures grown on plastic because growing cells form organoid structures that permit cell to cell interaction. Cells grown on pigskin can be sectioned, fixed, and stained by classic histochemical methods. Because of its long-term growth potential, the pigskin culture method has advantages over most organ and organ bit cultures, which can usually be maintained for a few days only and are essentially dying systems (Yoshida *et al.*, 1980).

Methods for culturing rabbit and human adult skin epithelium on the dermal collagen surface of dead, sterile pigskin have been reported (Freeman *et al.*,

1974; Igel *et al.*, 1974; Freeman *et al.*, 1976; Freeman *et al.*, 1979). The pigskin substrate method has been further developed to produce primary, proliferating, organotypic cultures of embryonic mouse liver, lung, kidney and pancreas, pregnant mouse mammary gland and rabbit blastocysts. It was found that this method appears to select against the outgrowth of fibroblasts. Crickard *et al.*, (1983) investigated the ability of culture dishes coated with an extracellular matrix (ECM) to act as a suitable substrate for human ovarian carcinoma cells *in vitro* and considered this method as superior to plastic. However, mammary epithelial cells have been successfully cultured on plastic culture surfaces with a mixture of Dulbecco's and Ham's F12 nutrients in their study to determine the effect of calcium ions on the attachment of cells in primary cultures (Soule and McGrath, 1986).

Most of the techniques described in the literature that involved enzymatic dissociation of tumour biopsies in our laboratory produced few viable cells. This led to long periods (2-4 weeks) to establish primary cultures before subcultures could be attempted. This time factor was deemed unacceptable for an assay. It was also found that selection took place in the first two weeks in culture, favouring fibroblast type cells which tended to overgrow the epithelial type cells. Therefore the cells present in the final cultures did not represent the original tumours. The aim should be focussed on any kind of short-term assay representing the original tumour, which will improve the ability of scientists to do chemical testing (Klausner, 1987; Vescio *et al.*, 1987). Because carcinoma cells have more often been successfully cultured from malignant effusions than from solid tumours, with the advantage of providing larger numbers of dissociated, viable tumour cells with little or no stromal contamination, it was decided to use a mechanical spill out method to culture cells on a plastic substrate in this study (Kruse and Patterson, 1973; Leibovitz, 1986). This method provided sufficient material for experimentation with immunochemical techniques.

1.4 HISTOCYTOLOGICAL CHARACTERIZATION

Although many biological and biochemical properties have been associated with malignancy of cancer cells, none of them are ideal for routine use, most of them apply to only a limited proportion of cancer cells or are exhibited by a range of

normal cell types also, and often they require specialized techniques or involve a significant time lag before results are available (Gilvarry *et al.*, 1990).

Cytological studies are of value in the development of systems for predictive drug testing. The heterogeneity of tumours both in terms of the spectrum of cell types which they contain and the ability of the tumour cells to survive *in vitro* make such studies difficult to perform routinely. Contamination of carcinoma cultures by fibroblasts or macrophages can generally be recognized microscopically and confirmed by immunofluorescence, but normal and malignant epithelial cells are difficult to distinguish (Gilvarry *et al.*, 1990). By far the most frequently encountered malignant lesions of the breast are carcinomas arising from the duct epithelium. Connective tissue tumours or lymphomas are rarely encountered. According to Suen (1990) an increased cellularity in breast aspirates is the first prerequisite for the diagnosis of breast carcinoma. The cellular pattern in case of malignancy should show numerous tumour cells present, isolated as well as in clusters. As far as cellular morphology is concerned, malignant cells generally show nuclear enlargement and anisonucleosis. Nuclei are hyperchromatic with irregularly distributed chromatin. Although prominent nucleoli may be present in poorly differentiated carcinomas, this is not required for diagnosis. However, nucleoli, when large and multiple, are a useful feature of malignancy. Cytopiasm is visible, as opposed to the naked bipolar nuclei of myoepithelial cells seen in benign lesions. Large tumour cells are obviously a useful marker of malignancy but size alone is not a reliable criterion as many malignant breast carcinomas, particularly in old patients, are composed of cells which differ very little in size from normal ductal cells. The chromatin of the normal breast ductal cells is rather coarse but regular, whereas many of the tumour cells show a fine chromatin pattern. Chromatin patterns are not reliable as a single marker for malignancy.

The malignant features looked for cytologically are:

a) Very cellular smears.
b) Loss of cohesiveness of epithelial cells.
c) Pleomorphism of cells and cell nuclei.
d) Irregular chromatin.
e) Multiple nucleoli.

In breast carcinoma, with the emphasis on the pattern approach, the special criteria of malignancy include:

a) Cellularity.
b) Dyshesion.
c) Polymorphism.
d) Nuclear membrane irregularity.
e) Anisonucleosis.
f) Macronucleoli.

Although some benign lesions display cellularity or anisonucleosis (i.e. the fibroadenoma), the presence of all six of these criteria, demonstrated in sufficient numbers in well-preserved cells, indicates a malignant neoplasm (Kline, 1991). For aspiration biopsy cytology, a preliminary diagnosis is made based on pattern using cellularity, dyshesion (loosely bound or isolated cells), and monomorphism (a single family of epithelial cells) as criteria. The pattern impression generally is confirmed by the individual cell examination for nuclear membrane thickening and irregularity, anisonucleosis and macronucleoli (Kline, 1991).

Gilvarry *et al.*, (1990) in their study concentrated on criteria which can be quantified and analyzed in most cell culture laboratories without special histopathological expertise. They rejected altered cytoplasmic acidophilia/basophilia and nuclear hyperchromasia as criteria because of the subjective nature of these criteria and used criteria which they considered more objective i.e.. number of nucleoli, number of normal, tripolar and multipolar mitosis. Some preliminary work on nuclear/cytoplasmic ratio was also done.

Histopathologists have developed a range of relatively simple criteria to detect malignancy in tissue sections. While some of these (such as metastasis and invasiveness) are not readily applicable to cells in culture, several of the characteristics used involve intrinsic properties of the cells and therefore might be relevant *in vitro* (Gilvarry *et al.*, 1990). Among the histopathologic criteria of malignancy the first one is the loss of the tubulo-alveolar pattern of the normal mammary gland, a pattern maintained in adenomas and fibroadenomas. Second, as in other carcinomas, the epithelial cells are larger than normal and have an increased nuclear-cytoplasmic ratio. The nuclear chromatin tends to be coarse, and nucleoli are prominent. The heterogeneity of the nuclear elements found in benign tumours, in which myoepithelial, dark and intermediate or clear

cell types are found, is not observed in malignant lesions. The number of mitosis generally is higher in malignant tumours than in benign ones, but benign tumours can have significant numbers of mitosis. Finally invasion of the stroma and neighbouring fat, muscle and dermis is the hallmark of malignant tumours. Stromal response, as demonstrated by fibrosis and inflammatory cell infiltration is generally more prominent in invasive malignant tumours than in non-invasive or benign lesions (Russo *et al.*, 1990).

Although most breast lesions can be diagnosed by routine light microscopy, immunochemistry is useful or necessary for the proper classification of these lesions (Johnston *et al.*, 1987). Immunochemical or cytochemical techniques provide information regarding changes in the cells which are not available using standard histopathological or cytopathological techniques (Kline, 1991).

1.5 AIMS WITH PRESENT STUDY

A large number of methods reported in the literature failed to yield successful cultures in all patients. Therefore it was attempted, in the present study, to culture human breast and cervix carcinomas on pieces of sterile pigskin and breast carcinoma cells dissociated by a mechanical spill out method on a plastic substrate. The cultured cells in the two methods used were characterized as human and of mammary origin using histological, cytological and immunocytochemical methods only. The cultured cells were also compared to the original tumour (histologically, cytologically and with immunocytochemical methods) in order to ascertain if the cultures corresponded to the original tumour. The two culture methods were evaluated using criteria such as culture time, histocytological characterization, biopsy size, culture success and microscopic analyses of the cells in culture. Because cervical specimens are plentiful and readily obtainable, biopsies from the uterine cervix were used to develop the method for pigskin cultures before the culturing of mammary tumours was attempted.

Cells from known breast cell lines obtained from the National Repository for Biological Materials of the National Cancer Association of South Africa were also cultured and used as controls. The cell lines obtained were MCF-7, T-47D and MDA-MB-231. MCF-7 and T-47D were both derived from infiltrating breast

ductal carcinomas while MDA-MB-231 was derived from adenocarcinoma (Engel and Young, 1978; Pancino *et al.*, 1990).

The use of immunohistochemistry will also be assessed as a diagnostic tool to compare the *in vivo* receptors to the *in vitro* receptor status of tumour cells.

As the degree of silver staining of nucleolar organizer regions seems to reflect the activity of the rRNA genes and active cells have a larger number of AgNORs compared to inactive cells (Egan and Crocker, 1992), AgNOR staining was deemed to be useful to determine culture growth and as an indicator of malignancy.

2 MATERIALS AND METHODS

2.1 CULTURES ON PIGSKIN

2.1.1 PIGSKIN SUBSTRATE

2.1.1.1 Acquisition

Pieces of soft, hairless pigskin cut from the groin or axillary of pig carcasses with dermis intact was obtained from a local butcher.

2.1.1.2 Sterilization

Method 1: Antibiotics

Pigskin was removed from the refrigerator and placed in washing fluid (WF). After boiling for 10 minutes the pigskin was placed in a sterile Petri dish containing fresh WF. The yellow hypodermis was trimmed away leaving a thin layer of white fluffy dermis. The pigskin was then placed into fresh WF and allowed to stand for at least 18 hours. The washing fluid consisted of 1L of phosphate buffered saline (PBS) to which was added 10ml of Penicillin (100IU/ml), 2.4ml of Streptomycin (0.5mg/ml) and 0.6ml of Gentamycin (0.050mg/ml), (Van Huyssteen, 1994).

Method II: Autoclaving

Pigskin was cut into 6mm x 6mm pieces, placed into McCartney bottles containing saline and autoclaved for 5 min at 120°C and 100kPa.

Method III: Alcohol

Pigskin was placed in 70% alcohol for 24 hours, rinsed repeatedly with PBS and placed into culture medium (51ml Ham's F12 + 9ml foetal calf serum + 100IU/ml Penicillin).

2.1.2 ORGANOTYPIC CULTURES

2.1.2.1 Petri dishes and grids

Plastic Petri dishes (90mm) were used. The grid consisted of a circular stainless steel wire mesh 50mm in diameter, bent down round the edge to provide a stand 4mm in height (see Fig. 4). Before use each grid was washed and scrubbed using a bottle brush and Hibiscrub (Zeneca). A final rinse was done using distilled water. The grids were then placed in alcohol overnight, dried and flamed before being placed into the sterile Petri dish.

2.1.2.2 Biopsy procedure

In the case of the six uterine cervical tumours, a crocodile forceps was used to remove a small piece of the tumour (punch-biopsy) which was immediately placed in WF. In the case of the ten mammary tumours, part of the excised tumour was placed in WF and the rest placed in methacarn (Carson, 1990) for histology. The tumour was then transported in a coolbag to the laboratory where it was cut into three parts with a sterile scalpel blade. The largest piece was placed into fresh WF for tissue culture. The other two pieces were placed in methacarn at 4°C for Kulzer (Kulzer & Co GmbH, Wehrheim, Germany; Carlemalm *et al.*, 1982) and paraffin wax embedding.

2.1.2.3 Growth medium and incubation

The growth medium consisted of 51ml Ham's F12 (Sigma Chemical Company, St. Louis, U.S.A.), to which was added 9ml foetal calf serum and Penicillin (50 IU/ml).

Nine sterile pieces of pigskin 0.5 cm square were placed with dermis side up on wire grids (3 per grid) in Petri dishes. Incisions were made in the pigskin to facilitate the infiltration of tumour cells. The mammary and uterine cervical tumours which had been collected in washing fluid, were cut into 1-2mm square pieces and a piece placed on each of the pieces of pigskin in the Petri dishes. Growth medium sterilized through a 0.22µm filter membrane (Sartorius) was added till the level was just above the top of the grid and touching the pigskin

(Fig. 4). The cultures were then incubated at 37°C in 5% CO_2 in air and humidified (78%). Growth medium was changed once a week and the tumours harvested at one, two and three weeks respectively. On each occasion one piece was taken from each grid and a squash biopsy made. The other two pieces were put into methacarn at 4°C for embedding in Kulzer resin and paraffin wax for haematoxylin and eosin staining.

2.2 CELL CULTURES ON PLASTIC

2.2.1 CULTURED CELLS FROM TUMOURS

The tumour was received in theatre and placed in filter sterilized medium which consisted of 200ml Dulbecco's modified Eagle's medium (Sigma), 400ml Medium 199 (Sigma), 40ml foetal bovine serum (Sigma), 4ml ITS (insulin, transferrin and sodium selenite supplement, Sigma I1884), 0.48ml Premarin (1.1 mg/L) and 4ml Gentamycin (10mg/ml, Sigma G1272).

In the laboratory, growth medium and tumour was separated by pouring the former into a conical tube and then centrifuging for 5 minutes at 800 x g to collect any loose cells. The tumour was placed in a sterile Petri dish, cut into small pieces (1-2mm blocks) and put into two conical tubes. Sterile Medium 199 (10ml) was added and the tubes were vortexed well for 5 minutes. After vortexing the medium containing single cells and clumps of cells was aspirated off using a syringe fitted with a 40 gauge needle. This cell rich medium was then centrifuged for 5 minutes at 800 x g in a centrifuge using a swing out rotor. Fresh Medium 199 (10ml) was added to the tubes containing the 1-2mm blocks and vortexed again for 5 minutes after which the supernatant was again collected to pellet loose cells. The process was repeated until the medium was clear after vortexing. The pellets were then pooled into 1 or more conical tubes and washed and centrifuged successively until no more fat was visible. The cells were then dispersed in 1ml growth medium and equally divided between the number of tissue culture flasks (Nunc, Roskilde, Denmark) used (see Table 2 for further information). To each slide-, T25- and T75- culture-flask was added a further 3ml, 5ml and 15ml medium respectively. Each flask was gassed with CO_2 for 3, 5 and 11 counts respectively and capped. Caps were tightened and placed into a CO_2 incubator. After 15 minutes the caps were turned back half a

turn. The cultures were then incubated at 37°C in a CO_2 incubator (5% CO_2) and 78% humidity.

2.2.2 CULTURED CELLS FROM KNOWN CELL LINES

MCF-7, T-47D and MDA-MB-231 cell cultures (in T25 flasks) almost confluent (70-80%) obtained from the National Cancer Association of South Africa were placed in a incubator at 37°C for 2 days in order to check for any microbial infections before subcultures (in T25, T75 and slide culture flasks) were made. The split ratio for subcultures were 1:3. Subcultures were made by decanting spent medium and adding 5ml fresh medium to each flask. The flasks were then shaken vigorously in order to detach cells from the plastic substrate. Detachment of cells was confirmed with an inverted microscope using a 10 x magnification lens. The cell rich suspension was then equally divided between the appropriate number of flasks. The medium required by each cell line was used to detach cells and was also used to fill each flask to the final volume required for each type of flask (2.5ml, 5ml, 15ml for slide-, T25- and T75- culture flasks). After gassing with 5% CO_2 for 3, 5 and 11 counts respectively, the flasks were capped and incubated at 37°C. Subcultures were again made when cultures reached ~90% confluency. Medium used for MCF-7 , T-47D and MDA-MB-231 cell lines was Roswell Park Memorial Institute 1640(RPMI 1640, Sigma) and 5% foetal calf serum, Ham's F10 (Sigma) and 5% foetal calf serum with 0.2IU/ml insulin and Ham's F10 and 10% foetal calf serum enriched with glucose respectively.

2.3 HISTOCYTOLOGICAL CHARACTERIZATION

2.3.1 FIXATION

All primary tumours and pigskin cultures were fixed in methacarn (methyl alcohol, chloroform and glacial acetic acid 6:3:1) as this fixative is preferred by some investigators (Derenzini *et al.*, 1990) both for immunohistochemical staining as well as for AgNOR staining.

ready for cutting after 5-10 minutes.

2.3.3 SECTIONING

Paraffin wax sections 3µm and 5µm thick were cut on a rotary microtome and heat fixed overnight at 37°C. The Kulzer resin sections were cut 1-3µm thin on a rotary microtome using a tungsten carbon knife. Sections were stretched on a waterbath, picked up on glass slides and dried at 37°C for 23 hours.

2.3.4 STAINING TECHNIQUES

2.3.4.1 Haematoxylin and Eosin for use on paraffin sections

Sections 3µm thick from the biopsy as well as the pigskin cultures were stained with haematoxylin and eosin (H&E) as described by Coleman and Chapman, (1989).

Sections were dewaxed in xylene for 2 x 5 min, rinsed in absolute alcohol and hydrated using decreasing concentrations of ethyl alcohol (96%, 80% and 70%). After rinsing in water, sections were stained in Harris's haematoxylin (Merck Chemicals) for 5 minutes and rinsed in water. After differentiating in 1% HCl diluted in 70% alcohol, sections were rinsed in water and placed in Scott's tapwater according to Bancroft (1982) (10g magnesium sulphate, 2g sodium bicarbonate per litre of tapwater) for blueing. Sections were then counterstained in 1% Eosin Y (Merck Chemicals) for 2 minutes. After dehydrating through increasing concentrations of ethyl alcohol (70%, 80% 96% and 100%), sections were cleared in xylene and mounted in Entellan (Merck Chemicals).

2.3.4.2 Haematoxylin and Eosin stain for use on Kulzer resin sections)

Resin sections were stained in Gill's Haematoxylin (Merck Chemicals) for 15 minutes, washed in running tapwater for 10 minutes and rinsed in distilled water. After counterstaining in Eosin Y (0,5gm Eosin Y-(alcoholic) C.I. 45380, 100ml 96% ethanol and 2 drops of glacial acetic acid) sections were dehydrated

through increasing concentrations of ethanol (70%, 80%, 96% and 100%), cleared in xylene and mounted in Entellan (Merck Chemicals).

2.3.4.3 Papanicolaou stain

Smears and tumour imprints of all primary tumours and their cultures made on glass slides were stained for cytology using the method described by Coleman and Chapman, (1989) using Gill's haematoxylin. The polyethelene glycol fixative (PEG) that was used in spray-fixing smears was removed by placing smears in 50% ethanol for 2 minutes. Smears were rinsed in water and stained in Gill's haematoxylin (Merck Chemicals) for 5 minutes. After rinsing in water, smears were dehydrated through increasing concentrations of ethanol (70%, 80% and 96%), and stained in Orange G6 (Merck Chemicals) for 1 minute. Smears were then rinsed in 96% alcohol and stained in eosin-azure (EA50, Merck Chemicals) for 5 minutes. After clearing in xylene, sections were mounted in Entellan (Merck Chemicals).

2.3.4.4 Silver-Stained Nucleolar Organizer Regions (AgNORs)

The AgNOR staining was performed using the method described by Dervan *et al.*, (1989). Air-dried imprints were used as the whole nucleus is seen and not only a portion as is the case in 5µm sections (Raymond and Leong, 1989). For tissue sections methacarn (methyl alcohol, chloroform, glacial acetic acid 6:3:1) was used as fixative since silver staining of NOR is less intense when formalin is used as a fixative (Griffiths, 1989; Derenzini *et al.*, 1990). Two volumes of 50% silver nitrate solution were mixed with 1 volume of 2% gelatin in 1% formic acid. Smears and sections were stained in this solution for 1 hour at 20°C in darkness. After rinsing in water the stain was fixed in 5% hyposulphite for 5 minutes, rinsed in water and counter-stained with 0.3% methyl green for 30 seconds. Smears and sections were then dehydrated using increasing concentrations of ethyl alcohol (70%, 80%, 96% and 100%), cleared in xylene and mounted in Entellan.

2.3.5 IMMUNOCHEMISTRY

2.3.5.1 Fixation and embedding

Tissues from primary tumours and pigskin cultures for immunochemistry were fixed in methacarn and embedded in Kulzer resin as well as paraffin wax. In the case of paraffin wax care was taken that the temperature of the wax did not at any point exceed 58°C.

2.3.5.2 Immunostaining

Immunogold-silver stain on primary breast and pigskin cultures for oestrogen receptors was performed using Amersham's Auroprobe LM Streptavidin (Amersham International, England). The silver enhancement was done using Amersham's IntenSE M kit. Both paraffin wax sections and Kulzer resin sections were used.

The following reagents were used and were obtained from Amersham.

Auroprobe LM Streptavidin	RPN457
Intense M	RPN491
Biotin GARa	RPN485
ER D5 Mouse monoclonal	RPN710
Normal goat serum	RPN480

The ER-D5 monoclonal antibody was raised against affinity-purified cytosolic oestradiol receptor from human myometrium and recognizes a 29kD non-hormone binding protein associated with the oestrogen receptor which has a cytoplasmic location rather than the nuclear locus of the oestradiol binding unit (Colston *et al.*, 1989).

IntenSE M

The IntenSE M kit is specially tailored for use in microscopy and is composed of 15ml ready to use Reagent A (Enhancer) and 15ml ready to use Reagent B (Initiator). Reagent A and Reagent B have to be stored at 2-8°C in the dark. Glassware was decontaminated with Deacon soap in order to remove all heavy

metals in elemental form and heavy metal salts. Sections and smears were washed for 2 x 5 minutes in phosphate buffered saline (PBS). A tissue paper was used to wipe around the section after which it was covered with 50 to 200µl of 5% heat inactivated normal goat serum for 20 minutes. Excess normal goat serum was removed by wiping around the section and 50 to 200µl of primary antibody was placed on the section for 1 hour at room temperature in humid conditions. Preparations were rinsed in PBS by placing sections in a coplin jar and washing for 3 x 10 minutes in this solution under constant agitation.
Sections were dried around the sample area, covered with 100µl of biotinylated secondary antibody and incubated for 60 minutes at room temperature in a humid environment. Slides were rinsed in PBS, placed in a coplin jar and washed thoroughly for 3 x 10 minutes with PBS. After drying by wiping around the section, the section was covered with 100µl of Auroprobe LM (diluted 1:40 with PBS containing 0.1% Bovine Serum Albumin (BSA) and 0.5% sodium azide at pH 7.2) and incubated for 1 hour at room temperature. Sections were then rinsed for 3 x 5 minutes with PBS in a Coplin jar and 3 x 3 minutes with distilled water with agitation. The silver enhancement reagent was prepared immediately before use by mixing an equal amount (in drops) of enhancer solution and initiator solution. The sample area in each of the slides was covered immediately with 4 drops of the silver enhancement mixture and incubated at 22°C for 15 minutes. Slides were washed for 2 x 5 minutes in excess distilled water and the preparations counter-stained with Harris's haematoxylin (Merck Chemicals), dehydrated in ethanol, cleared in xylene and mounted in Entellan (Merck Chemicals).

Procedure using DAKO PAP KITs

Staining for the following was done using Dako PAP KITs (Dako A/S, Glostrup, Denmark): oestrogen receptor, vimentin, cytokeratin, epithelial membrane antigen, desmin and leukocyte common antigen. The procedure was basically the same in each case and differed only in the monoclonal antibody used.

Where available, positive control sections were included in the procedure. After removal of paraffin wax and rehydration of tissue, a circle was drawn around the section using a DAKO Pen. Excess liquid was wiped away outside the circle and the slides placed on a flat surface in a humidity tank. The specimens were covered with 3% hydrogen peroxide and incubated for 5 minutes at room temperature. After rinsing with distilled water from a wash bottle, the slides

were placed in a PBS buffer bath for 5 minutes. Slides were then wiped around the marked area, covered with blocking serum and incubated for 20 minutes at room temperature. After wiping the slides again, the appropriate antibody was applied (for the negative controls, negative control reagent was used) and slides incubated for 20 minutes at room temperature. Slides were wiped, the linking antibody was applied and the slides were again incubated for 20 minutes. Slides were rinsed with PBS buffer from a wash bottle and placed in a PBS buffer bath for 5 minutes. After wiping again, slides were covered with peroxidase-anti-peroxidase reagent (PAP) and incubated for 20 minutes at room temperature. Slides were rinsed with PBS buffer from a wash bottle and placed in PBS buffer bath for 5 minutes. After rinsing the slides, the substrate consisting of amino-ethylcarbazole (AEC) and hydrogen peroxide was applied and sections incubated for 30 minutes. Imprints were incubated for 10 minutes only. Slides were rinsed with distilled water and counter-stained with Mayer's haematoxylin (Bancroft, 1982), blued with ammonia water, rinsed with tap water and mounted in glycerin gelatin.

2.3.6 MICROPHOTOGRAPHY

A Leitz Laborlux microscope with Wild camera system was used. Kodak ektachrome 160 tungsten for color transparencies was used for all prints (EPT 135 -36< ASA 160/23DIN).

3 RESULTS

3.1 ORGANOTYPIC CULTURES ON PIGSKIN

3.1.1 PIGSKIN STERILIZATION

3.1.1.1 Boiling

Sepsis occurred in only 3 out of 15 cultures when this method of sterilization was used. Fungal growth occurred in one case (Figs. 5, 6). Pigskin that was boiled for ten minutes and then placed into washing fluid, often contained smudged nuclei from hair follicles or capillaries (Fig. 7). The smudged nuclei were easy to distinguish from the nuclei of the tumour cells which had clear, crisp nuclear membranes with well discernible chromatin pattern and macronucieoli. Boiling softened the pigskin which seemed to improve attachment and infiltration of the tumour cells.

3.1.1.2 Autoclaving

No contamination was experienced but the four cultures on the autoclaved pigskin were not successful. After autoclaving, the pigskin was almost translucent and had a gel-like consistency. The pieces of tumour did not attach and tended to slide off when placed into fixative after culturing. It proved to be a poor substrate as it often broke up on the culture grids. Autoclaved pigskin generally show no nuclei or identifiable cells, consisting mainly of collagen "gel" (Fig. 8).

3.1.1.3 Alcohol

With this method of sterilization, sepsis occurred in only two out of thirty cultures. Skin placed in alcohol for 24 hours contained many more nuclei as a result of the alcohol acting as a fixative (Fig. 9). Alcohol sterilized pigskin which was not boiled for 5 minutes after the alcohol sterilization, proved to be too hard for tumour attachment (Table 1). Alcohol sterilization with subsequent boiling provided the best substrate with regard to tumour attachment and infiltration. In histological sections of these cultures, nests of tumour cells could be seen in the

pigskin. Of the three different methods of sterilizing pigskin, the alcohol sterilization followed by boiling gave the best results. The use of alcohol alone resulted in pigskin that was too hard for the tumour cells to infiltrate into (6 cultures) whereas the gel that was formed after autoclaving did not provide sufficient support or structure for the tumour cells to adhere (6 cultures).

Using the cytological criteria for malignancy which were readily identifiable i.e. variation in nuclear size, uneven nuclear membrane, irregular nucleoli, irregular chromatin pattern and parachromatin clear areas, cultured tumour cells were easily distinguishable from the smudged nuclei of the pigskin epithelium (Figs. 9, 10).

3.1.2 GROWTH SUCCESS

In all cases viable malignant cells could be observed in at least two out of four of the cultures per patient. In some cases the malignant cells were contained in the tumour with no invasion of the pigskin (Fig. 11) while in other cases (5 cultures from 2 patients) nests of migrating tumour cells could be seen in the pigskin (Figs. 12,13). After fixation macronucleoli were observed indicating active growth.

Table 1
Tumours cultured on pigskin

CULTURES ATTEMPTED	GROWTH SUCCESS	HISTO/CYTOLOGICAL TECHNIQUES APPLIED
CERVIX - 54 Boiled pigskin - 54	30 (56%) 30 (56%)	H&E, PAP, AgNOR
BREAST - 90 Boiled pigskin - 18 Alcohol - 6 Autoclaved - 6 Alcohol + boiling - 60	60 (67%) 9 (50%) 1 (17%) 0 (0%) 50 (83%)	H&E, AgNOR, ER, PAP

(1 culture = 1 x 0,5mm square tumour block).

In the 60 breast tumour cultures that were grown on pigskin that was sterilized in alcohol and boiled in washing fluid, the tumour growth seemed to peak at 3 - 5

weeks. Thereafter it often seemed as if the tumour was maintained *in vitro* with little further cell division or infiltration (Fig. 14). In 5 cultures the growing tumour cells actually infiltrated the pigskin and nests of viable tumour cells could be seen in the pigskin collagen.

As might be expected with biopsies from various primary breast tumours the growth rates on pigskin were variable. Immunochemistry requires large amounts of cells/tissue and growth on pigskin in this study was insufficient to make tumour characterization using this method possible.

3.1.3 EMBEDDING

The Kulzer resin technique was found to be extremely time consuming and although very thin sections were obtained and the staining results were good (Figs. 15, 16), the advantages did not outweigh those of paraffin embedding. Kulzer resin sections were difficult to cut and a tungsten knife had to be used. The reagents used are also toxic and special conditions and apparatus had to be used. Although the results obtained were good as far as sectioning and staining are concerned, this did not justify the cost, time and apparatus needed.

Paraffin wax processing was much easier and faster than processing using cold curing resin. The immunochemistry was successful on paraffin wax sections provided the temperature of the wax used for impregnation and embedding was kept below 58°C. Methacarn was used as a fixative in both methods as this fixative seemed to have very little effect on immunoreactivity.

3.1.4 STAINING

Haematoxylin and eosin stain.
All pigskin cultures and original tumours were stained using this routine stain. The expected results were obtained; nuclei stained purple and cytoplasm and collagen stained pink (Fig. 17).

Papanicolaou stain
Tumour imprints tended to take up more of the haematoxylin stain and less eosin with the result that all cell components stained basophilic and

macronucleoli were not very prominent.

Silver stain for Nucleolar Organizer regions

This stain was experimented with using sections of blocks from cervical carcinomas. The AgNORs in cases of infiltrating carcinoma (Figs. 19, 20, 21) were more numerous and had a larger total area than was the case with AgNORs in sections of normal cervix (Fig. 18). AgNORs present in the nuclei of the tumour cells in the pigskin, also occupied a relatively large area of the nucleus and their presence was indicative of active growth, as well as malignancy. AgNORs in the known cell lines i.e. MCF-7 were also large and multiple (Fig. 22).

3.2 CELL CULTURES ON PLASTIC

3.2.1 GROWTH SUCCESS

Results of breast tumour cells dissociated by the mechanical spill out method grown on a plastic substrate are summarized in Table 2.

Table 2

Results of cell cultures on plastic substrate

Tumour designation	Culture flasks used	Growth on plastic substrate	Suspension growth noted	Time in culture	% Con-fluency	Sub-culture ratio
*MDA-MB-231♣	T25,T75, SCF	+	-	Cont.	80-90%	1:3
*MCF-7♣	T25,T75, SCF	+	-	Cont.	80-90%	1:3
*T-47D♣	T25,T75, SCF	+	-	Cont.	80-90%	1:3
T1	T25,T75	+	+	5 wks	70%	1:3
T2	T25,T75	+	+	7 wks	60%	1:3
T3	T25,T75	+	+	6 wks	50%	1:3
T4	T25,T75	+	+	9 wks	70%	1:3

T5	T25,T75	+	+	8 wks	60%	1:3
T6	T25,T75	+	+	10 wks	50%	1:3
T7	T25,T75	+	+	12 wks	70%	1:3
T8	T25,T75	+	+	9 wks	60%	1:3
T9	T25,T75	+	+	2 wks	80%	1:3
T10	T25,T75	+	+	2 wks	50%	1:3
T11	T25,T75	+	+	2 wks	70%	1:3
*T12	SCF(14)	+	+	4 days	40%	♦
T13	T25,T75	+	+	2 wks	80%	1:3
*T14	SCF(14)	+	+	5 days	60%	♦
T15	T25,T75	+	+	2 wks	80%	1:3
T16	T25,T75	+	+	2 wks	90%	1:3
*T17	SCF(14)	+	+	7 days	70%	♦
T18	T25,T75	+	+	2 wks	90%	1:3
T19	T25,T75	+	+	2 wks	80%	1:3
*T20	SCF(14)	+	+	5 days	50%	♦
*T21	SCF(14)	+	+	7 days	60%	♦

♣ cell lines

* tumours randomly selected to be cultured in tissue slide culture flasks(SCF) to provide specimens for cytology and immunocytochemistry.

♦ All slide culture flasks were harvested for analysis.

() number of cultures set up as indicated otherwise 3 cultures in T25 flasks

SCF slide culture flasks

T25 plastic culture flasks with $25cm^2$ growth surface

T75 plastic culture flasks with $75cm^2$ growth surface

Growth was determined using cell adhesion, mitotic activity and confluency rate

as indicators.

In all cases a 1 x 1cm^2 breast tumour biopsy was used to set up cultures.

3.2.2 STAINING

Papanicolaou
Smears of imprints, cell lines and cultures tended to overstain with the nuclear stain haematoxylin, with the result that no eosinophilic cytoplasmic staining was observed. Nucleoli also did not stain eosinophilic (Figs. 23 - 26).

IGSS
The immunogold-silver stain proved successful and a positive reaction was seen as a dark brown to deep black reaction product in the cytoplasm of cells (Fig. 27). In the negative control this stain was absent (Fig. 28). ER usually has a nuclear locus but due to the fact that the ER-D5 antibody used recognizes 29kD non-hormone binding protein associated with the oestrogen receptor which has a cytoplasmic location, the positive stain was seen in the cytoplasm.

Immunocytochemistry
A reddish brown cytoplasmic stain was observed in cases of positive immunoreactivity (Fig. 39) while this stain was absent on the negative controls (Fig. 40).

With tumour cells cultured on a plastic substrate sufficient growth was present after 5 days to allow staining for cytological characterization. In all cases fibroblastic overgrowth was not observed or else was not a factor due to the short duration of the culture time (4 days to 2 weeks). Applying the cytological criteria for malignancy, the tumour cells could be readily distinguished from any normal cells or fibroblasts, if present.

3.3 HISTOCYTOLOGICAL CHARACTERIZATION

Using cytological criteria for malignancy, it was evident that the cells growing on and into the pigskin were malignant cells; no normal, viable epithelial cells could be distinguished. Because the pigskin had been boiled, the possibility of

confusing the pigskin nuclei with those of normal epithelial cells was minimal. The pigskin nuclei were smudged and bland and showed no discernible chromatin.

Haematoxylin and eosin stain

This stain was used on paraffin wax sections as well as resin sections of breast carcinoma tissue as well as on pigskin cultures. In both cases collagen and cytoplasm stained pink while nuclei stained purple/black. Malignant cells were readily distinguishable from the pigskin nuclei and showed anisonucleosis, irregular nuclear membranes, irregular and multiple macronucleoli, chromatin clumping and parachromatin clearing (Figs. 29, 30, 31). Three dimensional groups of malignant cells growing in the pigskin were also visible (Fig. 32). In some of these groups the adenocarcinomatous origin of the tumour could be seen by the acinar arrangement of the malignant cells (Figs. 12, 14). On some sections nuclear beading and mitosis were seen (Fig. 33).

Papanicolaou stain

Malignant cells in cultures showed the cytological criteria for malignancy i.e. variation in size, uneven nuclear membrane, irregular nucleoli, multiple nucleoli, irregular chromatin pattern and parachromatin clear areas (Figs. 34, 35). The malignant cells in cultures were comparable to the original tumour cells (tumour imprints) although in the cultured cells the nuclear/cytoplasmic ratio seemed less disturbed (Figs. 36, 37).

Immunochemistry

Immunochemical techniques were applied only to the cell lines and primary breast tumour cell cultures on plastic and in all cases the results proved the cultures to be similar to the original tumour as far as immunochemical reactivity is concerned (Table 3). In all cases of positive staining reaction for specific monoclonal antibodies, the negative controls showed no staining (Figs. 38 - 56). Positive controls were available only for keratin, epithelial membrane antigen and oestrogen receptors. In all positive controls the correct staining reaction was observed (Figs. 57 - 62).

Table 3

Immunochemistry on cell lines and cell cultures.

	EMA | -C	KER | -C	VIM | -C	DES | -C	LCA | -C	ER | -C
*MDA-MB-231	- -	+ -	+ -	- -	- -	+ -
*MCF-7	+ -	+ -	- -	- -	- -	+ -
*T-47D	+ -	+ -	- -	- -	- -	+ -
♦T12	+ -	+ -	+ -	- -	- -	+ -
♦T14	+ -	+ -	- -	- -	- -	+ -
♦T17	- -	+ -	+ -	- -	- -	+ -
*T17c	- -	+ -	+ -	- -	- -	+ -
♦T20	- -	+ -	- -	- -	- -	+ -
*T20c	- -	+ -	± -	- -	- -	+ -
♦T21	± -	+ -	+ -	- -	- -	± -
*T21c	± -	+ -	+ -	- -	- -	± -
ϕ +ve C	+ -	+ -				+ -

\+ = positive staining reaction

\- = negative staining reaction

± = weak positive

-C = negative control to rule out non-specific staining

C = control

c = cultures

EMA = epithelial membrane antigen

KER = keratin

VIM = vimentin

DES = desmin

LCA = leukocyte common antigen

ER = oestrogen receptor

♦ = tumour imprint on glass slide

* = cells on tissue culture slides
ϕ = paraffin wax sections

In the staining of the cell lines, MCF-7 and T-47D showed similar immunoreactivity and stained positive for EMA and negative for vimentin while MDA-MB-231 stained negative for EMA and positive for vimentin (Figs. 63 - 66). All three cell lines stained positive for keratin and oestrogen receptors (Table 3).

All the breast carcinoma tumour imprints as well as the cultures stained positive for keratin and T12, T17 and T21 (Fig. 55) also stained positive for vimentin. With T20 (Fig. 43) the tumour imprint was interpreted as negative for vimentin as the reddish-brown staining reaction was considered to be background staining. In the cell culture of this tumour, T20c (Fig. 38), the majority of tumour cells stained negative with only the occasional cell staining weakly positive compared to the bright reddish-brown staining of the fibroblast-type cells. Both tumours T17 and T20 stained negative for EMA and positive for keratin. All tumours and cultures showed positive immunoreactivity for keratin. Desmin and LCA was negative in all cases.

Nucleolar Organizer Regions

The AgNORs stained black against a light green background (Figs. 67, 68). Most nuclei contained multiple AgNORs and the total area of AgNORs in carcinoma cells was greater than the total AgNORs in normal cells. In addition to the increased number of AgNORs in malignant nuclei, the AgNORs were also irregularly distributed throughout the nucleus and showed a heterogeneity in their size. In benign cells the AgNORs were few, small and rather uniform in size. AgNORs in cultured tumour cells corresponded well with those in the tumour imprints of the tumour biopsies regarding their number, size, distribution and uniformity.

4 DISCUSSION

Schor (1980) stated that although a wide variety of human tumour types can be grown in colony forming assays not all specimens from all patients with any given type of tumour will grow *in vitro*. For most solid tumours cloning efficiencies of single cell cultures are low at about 1% (Salmon, 1986). This low figure could in part be due to an inappropriate substrate and/or medium for primary tumour culture. This is an additional reason for the present author attempting growth on a pigskin (collagen) substrate.

Knowledge of how tumours spread may yet prove to be essential for the devising of novel therapies, the task in cancer research may shift from treatment of metastasis to prevention thereof (Hart and Saini, 1992). As a result, cell attachment and motility studies have important roles to play in modulating cancer spread. Growth of human tumours on pigskin substrates (collagen) could be of great value in this regard as it probably closely simulates the *in vivo* situation.

Freeman et al. (1976) in culturing human skin on a pigskin substrate found that the first factor that affected growth of epithelial cells was the pigskin itself. Commercially available pigskin is prepared in a variety of ways and packaged differently. Some of the products they used proved to be toxic and prohibited culture growth. Some toxicity could be removed by presoaking the pigskins in Hanks balanced salt solution, but eventually the researchers had to pretest each product by using the skin focus outgrowth test. In the present study the author decided to rather prepare her own pigskin and test various cleansing and sterilization methods combined with their respective growth success rates. As indicated in Chapter 3 (results) the alcohol sterilization followed by boiling gave the best results with the lowest sepsis rate 2/60 (3%) and best growth results 50/60 (83%).

Biran *et al.* (1986) in culturing human breast tumours found that plating of cells on an extra-cellular matrix (ECM) greatly improved and in some cases was essential for cell attachment and subsequent outgrowth. Baker *et al.* (1986) also using ECM reported a very high success rate in culturing breast tumours 35/39 (90%). Collagen substrates appear to provide a more favourable surface on which to attempt primary tumour cultures.

The authors Freeman and Hoffman (1986) stated that monolayer cultures or clones in semisolid (agar) medium are only partly representative of the original tumour. Furthermore cloning eliminates cell-cell interactions among heterogeneous cell types in tumours, which may be critical for expression of many of their properties. They stressed that for a cultured tumour to be representative of the tumour *in vivo*, it is essential that the tumour, as it proliferates *in vitro*, maintains its tissue organization and structure, its oncogenic properties, its differentiated functions, and any cellular heterogeneity that may have been present *in vivo*. If tumours, growing *in vitro* can satisfy the above criteria and, in addition, can be grown at high frequency for long periods of time in culture, they should prove valuable for basic studies in cancer biology as well as for clinically relevant drug testing. Freeman and Hoffman also showed that diverse human tumours exploited and floated on pigskin collagen could be cultured with a high degree of success, lung 8/8 (100%), breast 14/17 (82%), colon 8/25 (32% with a total success rate of 64/89 (72%). The results in the current study are similar with success rates with cervix tumours of 30/54 (56%) and breast 60/90 (67%). It also appeared as if the tumours grown mimicked their *in vivo* organization and structure.

Elsdale and Bard (1972) used substrata of a hydrated collagen lattice simulating the concentration and form of collagen in extracellular connective tissue matrices. They concluded after culturing human diploid and malignant cells that their collagen substrate technique exhibited novel cellular behaviour. These novelties they concluded could provide for a better understanding of how cells function in the body.

Freeman *et al.* (1976) grew and characterized 129 out of 140 human skin cells on the dermal collagen bed of sterile, dead pigskin. This 92% growth success is higher than the 67% reported for tumour cells in this study. Sterilization of the pigskin took place after scrubbing and a rinse with betadine skin cleanser in 70% ethyl alcohol. As in the present study no fibroblastic overgrowth was observed.

Schor *et al.* (1982) found that the migration of tumour cells into the gel matrix is affected by the concentration of collagen in the gel.

The pace at which new technologies develop creates a variety of critical issues

i.e. standardization of methods of which the reproducibility is essential, quality control on reagents and methods used, lack of measurement of environmental factors, evaluation of clinical relevance, comparison with other methodologies and dissemination of methods and knowledge. Assumption that a new method will add clinically useful information or is superior to currently employed methods should be based on proper multivariate statistical analyses (Preisler and Raza, 1992).

The key principle in culture systems will be to employ methods that stimulate growth of malignant cells relatively more than accompanying normal cells. Characterization of cultured cells and the original tumour requires the participation of experts in cytology, histology, immunochemistry, cytogenetics, molecular genetics, electron microscopy, clinical chemistry and imaging techniques. Since none of the criteria used separately allow differentiation of a normal cell from a tumour cell growing *in vitro*, the criteria must be used in combination to characterize the benign or malignant nature of cultured cells (De Clerck and Neustein, 1987; Preisler and Raza, 1992).

Whatever culture method is used, detailed characterization of cultured cells and original tumour cells is a priority to ensure that the tumour culture consists of cells representing the tumour before clinical implementation of any assay can be considered (Miller *et al.*, 1981; Klausner, 1987; Vescio *et al.*, 1987; Denk, 1988; Jung *et al.*, 1991; McPherson, 1992). Various modifications to IGSS showed that an immunogold streptavidin enhancement method (IGSAM) produced sensitivity and specificity equal to that of ERICA (peroxidase immunocytochemistry). Most recent studies have shown that ER positive results can be obtained from 10 year old paraffin blocks (Teasdale and Cowen, 1989). In this study both paraffin blocks and fresh, alcohol fixed impression smears were used with no observable difference in the results obtained.

Contamination of carcinoma cultures by fibroblasts or macrophages can generally be recognized microscopically and be confirmed by immunofluorescence, but normal and malignant epithelial cells are difficult to distinguish (Gilvarry *et al.*, 1990). Histopathologists have developed a range of relatively simple criteria to detect malignancy in tissue sections. While some of these (such as invasiveness and metastasis) are not readily applicable to cells in culture, several of the characteristics used involve intrinsic properties of the cells and therefore might be relevant *in vitro*. In their study, Gilvarry *et al.*,

(1990), investigated whether or not simple histopathological criteria can be used in an objective and quantitative manner to distinguish normal from cancer cell populations in culture. They concentrated on criteria which can be quantified and analyzed in cell culture laboratories without special histopathological expertise and also concentrated on human cells. In choosing criteria for detailed analysis they rejected several, either because estimation would be somewhat subjective (i.e. altered cytoplasmic basophilia/acidophilia, nuclear hyperchromasia) or because such criteria were expected to be relevant only in paired normal-malignant cell sets and not relevant to comparison of different cell types (i.e. variation in cell or nuclear size and shape). They concentrated on criteria which they considered suitable for objective quantitative analysis: (a) number of nucleoli and (b) number of normal, tripolar, and multipolar mitosis. Some preliminary work on nuclear:cytoplasmic ratio was also done (Gilvarry *et al.*, 1990). Cultures were analyzed for the number of nucleoli/cell or for the presence of normal or tripolar or multipolar mitosis. The results indicated that nuclei of normal epithelial cells never contained more than 4 nucleoli, whereas nuclei of some of the cells in carcinoma populations contained 5 or 6 nucleoli. Some normal fibroblast populations also contained cells with more than 4 nucleoli. With regard to mitosis the results indicated that normal cell populations contained only normal mitotic figures, whereas within all of the malignant cell populations examined, some tripolar or multipolar mitosis were observed. In the case of normal human fibroblasts, once again tri-and multipolar mitosis were not observed, but in contrast to normal epithelial cells they may contain more than four nucleoli /nucleus.

Using cytological criteria for malignancy in the current study, it was evident that the cells growing on and into the pigskin were malignant cells; no normal, viable epithelial cells could be distinguished. Because the pigskin had been boiled, the possibility of confusing the pigskin nuclei with those of normal epithelial cells was minimal. The pigskin nuclei were smudged and bland and showed no discernible chromatin.

Malignant cells were readily distinguishable from the pigskin nuclei and showed anisonucleosis, irregular nuclear membranes, irregular and multiple macronucleoli, chromatin clumping and parachromatin clearing (Figs. 30, 31). On some sections nuclear beading and mitosis were also seen (Fig. 33). The malignant cells in cultures were comparable to the original tumour cells (tumour imprints) although in the cultured cells the nuclear/cytoplasmic ratio seemed

less disturbed (Figs. 36, 37).

Three dimensional groups of malignant cells growing in the pigskin were also visible (Fig. 32). In some cases the cells were arranged in a circular or acinar pattern indicative of their adenocarcinomatous origin (Figs. 12, 14). This phenomenon has not previously been reported in the literature for human tumours grown on collagen substrates. Clearly the pigskin cultures closely resemble the *in vivo* architecture.

Immunochemical or cytochemical techniques provide information regarding changes in the cells which are not available using standard histopathological or cytopathological techniques (Kline, 1991). Immunochemistry is therefore playing an increasingly important role in the evaluation of breast lesions where it is used (1) to classify poorly differentiated tumours (i.e. carcinoma vs. lymphoid); (2) to identify the primary site of a tumour; (3) to provide information on the prognosis or optimal therapy; and (4) to determine whether a tumour is benign or malignant (Osamura, 1988; Kline *et al.*, 1991). Monoclonal antibodies like monoclonal antibody B72.3 for instance has shown specificity for carcinomatous cells in effusions and can be used to separate benign and malignant lesions albeit only in conjunction with clinical and mammographic findings (Kline, 1988).

It is well known that the progression of some breast cancers is partially dependent on the interaction of various hormones and growth factors with specific receptors on the tumour cells themselves. Studies have shown that about half of all breast carcinomas possess oestrogen receptors and that the oestrogen receptor status can be useful in predicting the response to hormone therapy as well as disease-free survival (Forrest, 1989; Allred *et al.*, 1990). Breast growth and development is influenced by oestrogens and the growth of many breast cancers is driven by oestrogens, an effect which is utilized in the endocrine treatment of breast cancer. Oestrogens act by binding to the oestrogen receptor, a specific protein which in turn binds to specific regulatory regions of DNA, thereby altering gene expression. Several different forms of oestrogen receptor (ER) have been described. Type I ER is the classical ER about which most of the literature on the subject has been written and is a high-affinity, low-capacity, specific oestrogen binding protein (Pertschuk, 1990). The major proportion of receptor protein resides in the nuclei of target cells although lower-affinity type II ERs appear to be located in the cytoplasm of cells (Nadji

and Morales, 1986; Pertschuk, 1990).

Tumour ER content is an established but not absolute predictor of both response to endocrine therapy and prognosis in breast cancer (Henry *et al.*, 1990). A breast tumour may be heterogeneous in receptor content; some portions of the tumour may be rich in ER whereas in other portions cells may lack appreciable ER content (Carpenter *et al.*, 1989). Oestrogen receptor expression can now be determined by immunocytochemistry using monoclonal antibodies to detect the receptor in histological sections (Denk, 1988; Kung *et al.*, 1989). A monoclonal antibody to human ER was produced in 1984 which makes it possible to detect ER in frozen sections of breast tumours using the peroxidase anti-peroxidase (PAP) technique (King *et al.*, 1985; Teasdale, 1990). This antibody became commercially available a few years later in Kit form, mainly for use on fresh tissue. In this study all specimens, both cell lines and tumours, stained positive for oestrogen receptor using the PAP technique. In one case, however, both the tumour and it's culture were interpreted as only weakly positive.

An immunogold-silver staining method was developed by Teasdale *et al.*, (1989) for use on paraffin-embedded tissue. The sensitivity of this method was later improved by enhancing the amount of gold labelling by substituting a biotinylated secondary antibody followed by streptavidin gold (Jackson *et al.*, 1989; Pietschmann *et al.*, 1989).

In the current study, the immunogold-silver stain proved successful and a positive reaction was seen as a dark brown to deep black reaction product in the cytoplasm of cells (Fig. 27). In the negative control this stain was absent (Fig. 28). ER usually has a nuclear locus but due to the fact that the ER-D5 antibody used recognizes 29kD non-hormone binding protein associated with the oestrogen receptor which has a cytoplasmic location, the positive stain was seen in the cytoplasm. Both IGSS and the DAKO PAP kits were used successfully for the demonstration of oestrogen receptors. However because the IGSS technique proved to be more time consuming and costly, it was discontinued and all further immunostaining for oestrogen receptor was done using the PAP Kit.

"Cytoskeleton" is a collective morphologic term applied to a variety of different intracytoplasmic filaments found in most animal cells. "Intermediate-size"

filaments (IF) are an ubiquitous group of cytoskeletal proteins which are responsible for the integrity of cell structure. These filaments can be divided into five major classes: cytokeratins, desmin, glial fibrillary acidic protein, neurofilaments and vimentin (DAKO manual, 1992). The diagnostic value of IF immunohistochemistry in tumour pathology is based on the fact that neoplastic cells maintain the IF characteristic of their parent tissues.

Epithelial cells share several common features that dictate their characteristic morphology. They contain the keratin type of IF proteins often referred to as tonofilaments. Keratin IF in adjacent cells are linked by desmosomes giving the epithelium tensile strength. The practical value of keratin immunohistochemistry in tumour pathology is to classify undifferentiated malignant neoplasm as carcinoma and to subclassify epithelial neoplasm into a more specific subtype (Nadji and Morales, 1986; Regenass *et al.*, 1989). Cytokeratins have so far only been demonstrated in epithelial tissue and are important for the detection and classification of neoplastic cells of epithelial origin and in distinguishing cells and tumours from epithelial origin from those of non-epithelial origin. Free floating cells from primary cultures tested with anti-keratin antiserum distinguishes epithelium from other cell types (Soule and McGrath, 1986). Immunocytochemical analysis have shown that the expression of cytokeratins is maintained in epithelial cells after neoplastic transformation. The presence or absence of keratins in cells of primary or metastatic tumours serves as an important indicator of cell origin in lesions constituting a diagnostic problem (Russo *et al.*, 1990). In this study all tumours and cultures showed positive immunoreactivity for keratin showing that the expression of cytokeratins is maintained in epithelial cells after neoplastic transformation.

Epithelial membrane antigen (EMA) is a high molecular weight glycoprotein isolated from human milk fat globule membrane. EMA belongs to the polymorphic epithelial mucin family and has been shown to be present in a number of epithelia and neoplasms derived from them. Positive immunoreactivity for EMA has been reported in the majority of carcinomas, mesotheliomas and synovial and epithelial sarcomas. Usually EMA positivity is visualized as membrane staining but in occasional tumours additional cytoplasmic staining has also been reported. In breast the luminary cells show EMA positivity concentrated at the apical cell membranes but myoepithelial cells are negative. Neoplasms that display glandular differentiation are EMA positive, (i.e. adenocarcinoma of the breast, Nadji and Morales, 1986). Although

epithelial cells should stain positively for epithelial keratin and EMA, two of the tumours in this study stained negative for EMA and positive for keratin.

Desmin is a polypeptide expressed by the cells of normal smooth, skeletal and cardiac muscles. The major diagnostic application of desmin immunohistochemistry is in the differential diagnosis of soft tissue tumours since the demonstration of this marker establishes the myogenic nature of any neoplasm (Nadji and Morales, 1986). All sections and culture smears in this study stained negative for desmin.

Vimentin is the most widely distributed of the IF proteins because it can be expressed alone or with any of the other IF types. Vimentin is present in all mesenchymal cells. It may be considered a non-specific IF because it is expressed by a number of different cell types in their embryonal stages to be replaced by their specific IF in the process of differentiation. Cells such as fibroblasts, osteoblasts, osteocytes, chondroblasts and chondrocytes are characterized by abundant vimentin IF's and vimentin is the only IF type expressed in these cells. Vimentin positive tumours include all non-muscle soft tissue tumours, bone tumours, lymphomas, leukemias and melanomas. Most carcinomas are negative for vimentin although some carcinomas are keratin and vimentin positive. Although most carcinomas are negative for vimentin, true co-expression of keratin and vimentin has been shown in 20% of fine needle aspirations of the breast and according to Freshney (1992) epithelial tumour cells in culture can also express vimentin. In this study, all the breast carcinoma tumour imprints as well as the cultures stained positive for keratin and 60% stained positive for vimentin.

Leukocyte common antigen (LCA) is a major membrane glycoprotein restricted to leukocytes and is used for the diagnosis of lymphoma and leukemia. LCA reacts with haematopoietic cells and is useful in distinguishing lymphoreticular malignancies from undifferentiated carcinomas and sarcomas. Haematopoetic cells (except myeloma) would therefore stain positively for LCA but negatively for epithelial keratin, desmin and EMA while epithelial cells would be those cells staining positively for epithelial keratin and EMA but negatively for desmin and LCA. Mesothelial cells would stain positively for epithelial keratin and desmin , but negatively (or weakly) for EMA and LCA (Li *et al.*, 1989). All breast culture smears and sections in this study stained negative for LCA.

At times, antibodies used in immunohistochemistry yield unanticipated results. For example EMA is detected immunohistochemically in non-epithelial cells, staining for keratin is observed in mesenchymal tumours, vimentin is expressed in some carcinomas and LCA occasionally reacts with non-haematopoietic cells. In such cases, the immunohistochemical staining obtained with a single antibody may not accurately reflect the histogenesis of the lesion. Therefore, interpretation must be based on the results obtained with a panel of antibodies, spanning the diagnostic possibilities (Li *et al.*, 1989; Knuechel *et al.*, 1990). The purpose of this study as far as immunochemical reactivity is concerned was not to correctly classify tumours but to compare the immunochemistry of the original tumour to that of the tumour cells in culture. As indicated by the results in Table 3, immunoreactivity was identical in all cases where cell cultures were compared to the original tumour biopsy imprints.

The two cell lines derived from infiltrating ductal carcinomas showed identical immunoreactivity and differed from that of the cell line derived from adenocarcinoma.

Silver-Stained Nucleolar Organizer Regions (AgNORs) are loops of DNA that transcribe to rRNA. AgNORs present active rRNA and the proliferation activity of a given cell can be determined by the number of AgNORs present in the nucleus (Lipponen *et al.*, 1991). Although the AgNORs are located in the centromeric regions of the acrocentric chromosomes and each chromosome therefore has two AgNORs, all AgNORs are not visible in normal histological sections. Usually one or two may be present within the nucleus. Large numbers in malignant cells might reflect increased metabolic activity compared to normal tissues. It has been reported by Smith and Crocker (1988) that the number of silver-stained interphasic NORs in malignant lesions significantly exceeds that in normal and benign lesions. Their results were confirmed by Raymond *et al.*, (1989) who demonstrated a significant correlation between the tumour growth and AgNOR count (Rowlands, 1988). They also found significantly higher AgNOR numbers in malignant breast lesions when compared to benign ones. Derenzini *et al.*, (1989) found that the quantity of AgNORs was higher in cancer cells (both mesothelioma and adenocarcinoma) than in reactive mesothelial cells. AgNORs were also more irregularly distributed within the nuclei and were more variably sized in cancer cells than in reactive mesothelial cells. Although Giri *et al.*, (1989) did not find AgNORs a reliable criterion to be used for routine diagnosis, they found that counts in excess of 4.0 per nucleus are highly

suggestive of malignancy.

The silver stain was experimented with in this study using sections of blocks from cervical carcinomas. The AgNORs in cases of infiltrating carcinoma (Figs. 19, 20) were more numerous and had a larger total area than was the case with AgNORs in sections of normal cervix (Fig. 18). AgNORs present in the nuclei of the breast tumour cells in the pigskin also occupied a relatively large area of the nucleus and their presence was indicative of active growth, as well as malignancy (Fig. 21). AgNORs in the known cell lines i.e. MCF-7 were also large and multiple (Fig 22). Most nuclei contained multiple AgNORs and the total area of AgNORs in carcinoma cells was greater than the total AgNORs in normal cells. In addition to the increased number of AgNORs in malignant nuclei, the AgNORs were also irregularly distributed throughout the nucleus and showed a heterogeneity in their size. In benign cells the AgNORs were few, small and rather uniform in size. AgNORs in cultured tumour cells corresponded well with those in the tumour imprints of the tumour biopsies regarding their number, size, distribution and uniformity.

The mechanical spill out method used to dissociate tumour cells from other stromal complements proved to be effective and overgrowth by fibroblast type cells in particular was not apparent in this study. As indicated in Table 2 in all cases subcultures or harvesting of primary cultures were possible within four to seven days. There is however still one aspect of this method which needs further attention namely the fact that not only single cells are obtained but also clumps of cells. Both single and clumps of cells attached well to the substrate within the first 24 hours in culture. After 48 hours new growth became visible by the presence of mitotic cells and the increase in colony size. It was, however, interesting to note that in all the cultures, as soon as colonies reached a certain size or when cultures became more confluent (50 - 70%), some of the colonies started to detach and continued growth in suspension. The viability of this suspension growth was confirmed when clumps were harvested and placed into new culture flasks with new medium after which the whole process repeated itself. It was not within the scope of the present study to address this problem, but this phenomenon would have to be investigated before an assay to test drugs in culture could be implemented.

Primary malignant cultures on plastic substrates are not used for chemotherapy predictive testing due to the likely presence of normal cells and also fibroblastic

overgrowth. However the use of human cancer cell lines as a primary screening system for anti-neoplastic drugs has support (Finlay and Baguley,1984). Established cell lines in their hands manifested responsiveness to anticancer drugs consistent with that expected from their tumours of origin. However characterization using histo-cytology and immunohistochemistry should be carried out on these cells before drug testing as breast carcinoma cell lines have been reported to be Hela cells or of non-human origin (Engel and Young, 1978). The present author used both histo-cytology and immunochemistry on cell lines and primary cultures on plastic and in all cases the results proved that the cultures were similar to the original tumour. Successful application of these methods will allow their application on pigskin cultures during further development of this method by the present author. Management of solid carcinomas has progressed slowly over the last 40 years. This is no doubt due to the inherent complexities of these tumours and also unfortunately, our very limited understanding of the differences between normal and malignant cells (Hainsworth and Garson, 1990).

5 CONCLUSIONS

The main requirements before a chemotherapy predictive assay can be developed and/or implemented is the ability to grow primary human mammary carcinoma cells *in vitro*. Therefore appropriate conditions for cell dissociation, a proper substrate for cell attachment and outgrowth and growth conditions that support epithelial cell proliferation but at the same time suppress or prevent the attachment and growth of adjoining stromal complements of the tumour have to be established (Biran *et al.*, 1986).

In this study cells cultured on a pigskin substrate were in some cases actively growing after 3 to 5 weeks. The presence of nucleoli was used as an indication of cell viability. In 5 out of 60 pigskin cultures on alcohol sterilized, boiled pigskin, growing tumour cells actually infiltrated the pigskin and nests of viable tumour cells could be seen in the pigskin collagen. Using the criteria for malignancy, it was evident that the cells growing on and into the pigskin were malignant cells; no normal, viable epithelial cells could be distinguished. Malignant cells were readily distinguishable from the pigskin nuclei and showed anisonucleosis, irregular nuclear membranes, irregular and multiple macronucleoli, chromatin clumping and parachromatin clearing. Cytologically, the malignant cells in the pigskin cultures corresponded to those of the original tumour. Although growth and viable cells were present in most cases, the growth rate was not predictable.

Cytological studies are of value in the development of systems for predictive drug testing and in this study a definite correlation was found between the malignant cells of the original tumours and their cell cultures. Cytologically, as far as cellular morphology is concerned, malignant cells generally showed nuclear enlargement and anisonucleosis. Nuclei were hyperchromatic with irregularly distributed chromatin and uneven nuclear membranes. Nucleoli were usually large and multiple. Although the malignant cells in cultures were comparable to the original tumour cells (tumour imprints), in the cultured cells the nuclear/cytoplasmic ratio seemed less disturbed.

The number of AgNORs present in the nuclei of the tumour cells in the pigskin occupied a relatively large area of the nucleus and their presence was indicative of active growth, as well as malignancy. AgNORs in the known cell lines i.e.

MCF-7 were characterized by an irregularly scattered distribution of AgNORs and by a heterogeneity in the size of the AgNORs. Tumour biopsies and their cell cultures also showed numerous, irregularly distributed AgNORs of varying size. These results are similar to those reported in the literature for malignant and normal cells.

Immunochemical techniques were applied only to the cell cultures on plastic and in all cases the results proved the cultures to be identical to the original tumour as far as immunochemical reactivity is concerned (Table 3).

Compared to pigskin cultures (3-5 weeks), cell cultures on plastic have the possibility of being developed into an assay system to obtain drug sensitivity results within only four to seven days. This culture technique also lends itself to flow cytometric assessment, image analyses or trypan blue staining to assess cell viability after chemotherapeutic treatment.

6 SUMMARY

The aim of this study was to culture breast and uterine cervix tumours on a pigskin substrate and then to undertake a histo-cytological characterization including immunogold-silver staining techniques. In addition cell lines were cultured on a plastic substrate to develop and test immunochemical methods that could be employed to further characterize pigskin cultures (large amounts of tissue/cells/smears are needed for immunochemistry to standardize methods). Immunochemical and cytological characterization was also carried out on primary breast cultures on plastic substrates.

Cultures on pigskin (collagen substrate) are likely to be more representative of the in vivo situation than single or dissociated cell cultures. As a result pigskin cultures would be very suitable for short term chemotherapy assays, the study of metastatic processes and local infiltration of cancers and also cell-cell interactions.

Pigskin sterilized in alcohol and then boiled gave the best results in breast tumour cultures 50/60 (83%). Viable cell growth was observed in 60/90 (67%) of breast tumour cultures and 30/54 (56%) of uterine cervix carcinomas grown on pigskin.

This culture technique also illustrated malignant cell infiltration of the pigskin and in addition all cells cultured could be characterized as malignant and comparable to the original tumour. No fibroblastic overgrowth was observed in pigskin cultures. This culture method needs further research and development to ensure more consistent and prolonged growth. It lends itself to flow cytometric analyses if it is employed in a chemotherapy predictive assay. Tumour cells, after cytotoxic drug exposures, could be harvested from pigskin cultures using proteolytic enzymes and then analyzed for growth fractions.

Immunochemical assays for the presence of keratin, vimentin, desmin, epithelial membrane antigen, leucocyte common antigen and oestrogen receptor were successfully completed on plastic substrate cultures of established cell lines (MCF-7, MDA-MB-231 and T-47D) and primary cultures of breast carcinoma. The latter cultures showed comparable immunochemical properties with regard to the original tumours.

In this study two methods to culture mammary tumour cells, i.e. organotypic cultures on a pigskin substrate and a mechanical dissociation method on a plastic substrate, were researched. The cells cultured on pigskin were characterized by histo-cytological and immunochemical means. The cell lines cultured on plastic substrate were used mainly to test immunochemical methods. Immunochemistry and cytology were used to characterize the breast tumour cells cultured on plastic. In both cases the cultured cells were compared to the cells in the original tumour using histological, cytological and immunochemical techniques.

REFERENCES

Allred DC, Bustamante MA, Daniel CO, Gaskill HV, Cruz AB: Immunocytochemical analysis of oestrogen receptors in human breast carcinomas. *Arch Surg* 125:107-113, 1990.

Amersham International, Amersham, United Kingdom, Personal Communication, June 1991.

Baker FL, Spitzer G, Ajani JA, Brock WA, Lukeman J, Pathak S, Tomasovic B, Thielvoldt D, Williams M, Vines C, Tofilon P: Drug and radiation sensitivity measurements of successful primary monolayer culturing of human tumour cells using cell-adhesive matrix and supplemented medium. *Cancer Res* 46:1263-1274, 1986.

Bancroft JD: Theory and Practice of Histological Techniques. Second edition. New York, Churchill Livingstone, p 135, 1982.

Biran S, Horowitz AT, Fuks Z, Vlodavsky I: High-density lipoprotein and extracellular matrix promotes growth and plating efficiency of normal human mammary epithelial cells in serum-free medium. *Int J Cancer* 31:557-566, 1983.

Biran S, Vlodavsky I, Fuks Z, Lijovetzky G, Horowitz AT: Growth of human mammary carcinoma cells from biopsy specimens in serum-free medium on extracellular matrix. *Int J Cancer* 38:345-354, 1986.

Calaf G, Garrido F, Moyano C, Rodriguez R: Influence of hormones on DNA synthesis of breast tumours in culture. *Breast Cancer Res Treat* 8: 223-232, 1986.

Callahan R, Cropp SS, Merlo GR, Liscia DS, Cappa APM, Lidereau R: Somatic mutations and human breast cancer. *Cancer Suppl* 69:1583-1588, 1991.

Carlemalm E, Garavito RM, Villiger W: Resin development for electron microscopy and an analysis of embedding at low temperature. *J Microsc* 126:123-143, 1982.

Carpenter S, Georgiade G, Mc Carty Sr KS, McCarty Jr KS: Immuno-histochemical expression of oestrogen receptor in normal breast tissue. *Proc R Soc Edinb* 95:59-66, 1989.

Carson FL: Histotechnology: A Self-Instructional Text. Chicago, ASCP Press, p 22, 1990.

Chambard M, Gabrion J, Mauchamp J: Influence of collagen gel on the orientation of epithelial cell polarity. *J Cell Biol* 91:157-166, 1981.

Chaplowski FJ, Minsky BD, Jacobs JB, Cohen SM: Effect of a collagen substrate on the growth and development of normal and tumorigenic rat urothelial cells. *J Urol* 140:1211-1216, 1983.

Coleman DV, Chapman PA: Clinical Cytotechnology. London, Butterworths, p 80, 1989.

Colston KW, King RJB, Hayward J, Fraser DI, Horton MA, Stevenson JC, Arnett TR: Estrogen receptors and human bone cells: immunocytochemical studies. *J Bone Min Res* 4:625-631, 1989.

Crickard K, Crickard U, Yoonessi, M: Human ovarian carcinoma cells maintained on extracellular matrix versus plastic. *Cancer Res* 43:2762-2767, 1983.

DeClerck YA, Neustein HB: The contribution of tissue culture to the study of solid tumours of childhood. *Perspect Pediatr Pathol* 9: 214-243, 1987.

Denk H: Immunohistologic heterogeneity of malignant tumors. *Path Res Pract* 183:693-697, 1988.

Derenzini M, Betts CM, Trere D, Mambelli V, Millis RR, Eusebi V, Cancellieri A: Diagnostic value of silver-stained interphasic nucleolar organizer regions in breast tumours. *Ultrastruct Path* 14:233-245, 1990.

Derenzini M, Nardi F, Farabegoli F, Ottinetti A, Roncaroli F, Bussolati G: Distribution of silver-stained interphase nucleolar organizer regions as a parameter to distinguish neoplastic from non-neoplastic reactive cells in human effusions. *Acta Cytol* 33:491-498, 1989.

Dervan PA, Gilmartin LG, Loftuss BM, Carney DN: Breast carcinoma kinetics: Argyrophilic nucleolar organizer region counts correlate with K167 scores. *Am J Clin Pathol* 92:401-407, 1989.

Egan MJ, Crocker J: Nucleolar organiser regions in pathology. *Br J Cancer* 65:1-7, 1992.

Elsdale T, Bard J: Collagen substrata for studies on cell behaviour. *J Cell Biol* 54:626-637, 1972.

Emoto M, Iwasaki H, Kikuchi M, Ishiguro M, Kubota T, Izumi H, Shirakawa K, Kaneko Y: Two cell lines established from mixed müllerian tumors of the uterus. *Cancer* 69:1759-1768, 1992.

Engel LW, Young NA: Human breast carcinoma cells in continuous culture: a review. *Cancer Res* 38:4327-4339, 1978.

Finlay GJ, Baguley BC: The use of human cancer cell lines as a primary screening system for antineoplastic compounds. *Eur J Cancer Clin Oncol* 20:947-954 , 1984.

Fogh J: Human Tumour Cells *In Vitro*. New York, Plenn Press, 1975.

Forrest APM: Endocrine management of breast cancer. *Proc R Soc Edinb* 95B:1-10, 1989.

Freeman AE, Igel HJ, Waldman NL, Losikoff AM: A new method for covering large surface area wounds with autografts. *Arch Surg* 108:721-723, 1974.

Freeman AE, Igel HJ, Herrman BJ, Kleinfeld KL: Growth and characterization of human skin epithelial cell cultures. *In Vitro* 12:352-362, 1976.

Freeman AE, Yoshida Y, Hilborn V, Carey SL: Culturing epithelial cell types on a pigskin substrate. *TCA Manual* 5:1181-1183, 1979.

Freeman AE, Hoffman RM: *In vivo*-like growth of human tumors *in vitro*. *Proc Natl Acad Sci* 83:2694-2698, 1986.

Freshney RI, Editor: Culture of Epithelial Cells. New York, Wiley-Liss Inc, p 8, 1992.

Friedman HM, Glaubiger DL: Assessment of in vitro drug sensitivity of human tumour cells using [^{3}H]thymidine incorporation in a modified human tumor stem cell assay. *Cancer Res* 42:4683-4689, 1982.

Gilvarry U, Farrel D, Lynch V, Moriarty M, Dooley M, Law E, Clynes M: Cytological differences between normal and malignant human cell populations in culture. *Cancer Res* 50:3390-3393, 1990.

Giri DD, Dundas AC, Sanderson PR, Howat AJ: Silver-binding nucleoli and nucleolar organizer regions in fine needle aspiration cytology of the breast. *Acta Cytol* 33:173-175, 1989.

Griffiths AP, Butler CW, Roberts P, Dixon MS, Quirke P: Silver-stained structures (AgNORs), their dependence on tissue fixation and absence of prognostic relevance in rectal adenocarcinoma. *J Path* 159:121-127, 1989.

Hainsworth PJ, Garson OM: Breast cancer cytogenetics and beyond. *Aust N Z J Surg* 60:327-336, 1990.

Hart IR, Saini A: Biology of tumour metastasis. *Lancet* 339: 1453-1457, 1992.

Henry JA, McCarthy AL, Angus B, Westley BR, May FEB, Nicholson S, Cairns J, Harris AL, Horne CHW: Prognostic significance of the Estrogen-regulated protein, cathepsin D in breast cancer. *Cancer* 65:265-271, 1990.

Henry JA, Angus B, Horne CHW: Oestrogen receptor and oestrogen regulated proteins in human breast cancer: a review. *Keio J Med* 38:241-261, 1989.

Igel HJ, Freeman AE, Boeckman CR, Kleinfeld KL: A new method for covering large surface area wounds with autographs. *Arch Surg* 108:724-729, 1974.

Jackson P, Teasdale J, Cowen PN: Development and validation of a sensitive immunohistochemical oestrogen receptor assay for use on archival breast cancer tissue. *Histochemistry* 92:149-152, 1989.

Johnston WW, Szpak CA, Thor A, Simpson JF, Schlom J: Applications of immunocytochemistry to clinical cytology. *Cancer Invest* 5:593-611, 1987.

Jung H, Berens ME, Krouwer HGJ, Rosenblum ML: A three-dimensional Micro-organ culture system optimized for *in vitro* growth of human malignant brain tumors. *Neurosurgery* 29:390-398, 1991.

King RJB, Coffer AI, Gilbert J, Lewis k, Nash R, Millis R, Raju S, Taylor RW: Histochemical studies with a monoclonal antibody raised against a partially purified soluble estradiol receptor preparation from human miometrium. *Cancer Res* 45:5728-5733, 1985.

Klausner A: Tissue culture for improved toxicology. *Biotechnology* 5:779-786, 1987.

Kline TS: Handbook of Fine Needle Aspiration Biopsy Cytology. Second edition. Churchill Livingston, New York, pp 215 - 223, 1988.

Kline TS: Survey of aspiration biopsy cytology of the breast. *Diagn Cytopathol* 7: 98-105, 1991.

Knuechel R, Keng P, Hofstaedter F, Langmuir V, Sutherland R, Penney D: Differentiation patterns in two and three-dimensional culture systems of human squamous carcinoma cell lines. *Am J Pathol* 137:725-736, 1990.

Kruse PF, Patterson MK: Tissue Culture Methods and Applications. Academic Press Inc, New York, 1973.

Kung ITM, Chan JKC, Lo F: Application of the Immunoperoxidase Technique to cell block preparations on fine needle aspirates. *Acta Cytol* 34:297-303, 1989.

Leibovitz A: Development of tumor cell lines. *Cancer Genet Cytogenet* 19:11-19, 1986.

Li C, Zeisner SC, Wong Y, Yam LT: Diagnostic accuracy of the immunocytochemical study of body fluids. *Acta Cytol* 33:667-673, 1989.

Lipponen PK, Eskelinen MJ, Nordling S: Nucleolar organiser regions (AgNORs) as predictors in transitional cell bladder cancer. *Br J Cancer* 64: 1139-1144, 1991.

Longman SM, Buehring GC: Oral contraceptives and breast cancer. *In vitro* effect of contraceptive steroids on human mammary cell growth. *Cancer* 59:281-287, 1987.

Lynch HT, Albano WA, Heieck JJ, Mulcahy GM, Lynch JF, Layton MA, Shannon Danes B: Genetics, biomarkers and control of breast cancer: a review. *Cancer Genet Cytogenet* 13:43-92, 1984.

McPherson RA: Data sharing and evaluation. *Cancer Suppl* 69:1572-1577, 1992.

Miller BE, Miller FR, Heppner GH: Interactions between tumour subpopulations affecting their sensitivity to the antineoplastic agents cyclophosphamide and methotrexate. *Cancer Res* 41: 4378-4381, 1981.

Miller BE, Miller FR, Heppner GH: Factors affecting growth and drug sensitivity of mouse mammary tumor lines in collagen gel cultures. *Cancer Res* 45:4200-4205, 1985.

Murray JC, Liotta L, Rennard SI, Martin GR: Adhesion characteristics of murine metastatic and non-metastatic tumor cells in vitro. *Cancer Res* 40:347-351, 1980.

Nadji M, Morales AR: Immunoperoxidase Techniques: A Practical Approach to Tumour Diagnosis. Chicago, ASCP Press, p 42, 1986.

Osamura RY: Applications of immunocytochemistry to diagnostic cytopathology. *Diagn Cytopathol* 5:55-63, 1988.

Pancino G, Charpin C, Osinaga E, Betaille B, Le Roy M, Calvo F, Roseto A: Characterization and distribution in human tissues of a glycoproteic antigen defined by monoclonal antibody IBE_{12} raised against human breast cancer cell line T47D. *Cancer Res* 50:7333-7342, 1990.

Paul J: Cell and Tissue Culture. New York, Churchill-Livingstone, 1975.

Pertschuk L: Immunochemistry for Steroid Receptors. USA, CRC Press, pp 2-6, 1990.

Pietschmann SM, Hausmann EHS, Gelderblom HR: Immunogold labelling of viruses *in situ*. In *Colloidal Gold: Principles, Methods and Applications,* New York, Academic Press, 2:255-283, 1989.

Preisler HD, Raza A: The role of emerging technologies in the diagnosis and staging of neoplastic diseases. *Cancer Suppl* 69:1520-1526, 1992.

Raymond WA, Leong AS: Nucleolar organizer regions relate to growth fractions in human breast carcinoma. *Hum Pathol* 20:741-746, 1989.

Regenass U, Geleick D, Curschellas E, Meyer T, Fabbro D: *In vitro* cultures of epithelial cells from healthy breast tissues and cells from breast carcinomas. *Recent Results Cancer Res* 113:4-15, 1989.

Rowlands DC: Nucleolar organising regions in cervical intraepithelial neoplasia. *J Clin Pathol* 41:1200-1202, 1988.

Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, Van Zwiten MJ: Biology of disease: Comparative study of human and rat mammary tumorigenesis. *Lab Invest* 62:244-278, 1990.

Salmon SE: Human tumor clonogenic assays: growth conditions and applications, *Cancer Genet Cytogenet* 19:21-28, 1986.

Schor SL: Cell proliferation and migration on collagen substrata *in vitro*. *J Cell Sci* 41: 159-175, 1980.

Schor SL, Schor AM, Winn B, Rushton G: The use of three-dimensional collagen gels for the study of tumour cell invasion *in vitro*: experimental parameters influencing cell migration into the gel matrix. *Int J Cancer* 29:57-62, 1982.

Smith R, Crocker J: Evaluation of nucleolar organizer region-associated proteins in breast malignancy. *Histopathology* 12:113, 1988.

Smith HS, Liotta LA, Hancock MC, Wolman SR, Hacket AJ: Invasiveness and ploidy of human mammary carcinomas in short-term culture. *Proc Natl Acad Sci USA* 82:1805-1809, 1985.

Soule HD, McGrath CM: A simplified method for passage and long-term growth of human mammary epithelial cells. *In Vitro Cell Dev Biol* 22:6-12, 1986.

Suen KC: Atlas and Text of Aspiration Biopsy Cytology. Baltimore, Williams & Wilkins, p 106, 1990.

Teasdale JJ, Cowen PN: Development and validation of a sensitive immunohistochemical oestrogen receptor assay for use on archival breast cancer tissue. *Histochemistry* 92:149-152, 1989.

Teasdale J: The use of streptavidin gold in breast cancer prognosis. *Life Sci* 3:5-6, 1990.

Tjandra JJ, McKenzie IFC: Murine monoclonal antibodies in breast cancer: an overview. *Br J Surg* 75:1067-1077, 1988.

Van Huyssteen GJ: The development of an *in vitro* chemotherapy predictive assay and its application on tumours of squamous epithelial origin, Ph.D. Thesis, University of the Orange Free State, Bloemfontein, South Africa, 1994.

Vasiliev JM, Gelfland IM: Neoplastic and Normal Cells in Culture. Cambridge, Cambridge University Press, 1981.

Vescio RA, Redfern CH, Nelson TJ, Ugoretz S, Stern PH, Hoffman RM: *In vivo*-like drug responses of human tumors growing in three-dimensional gel-supported primary culture. *Proc Natl Acad Sci USA* 84:5029-5033, 1987.

Willett W: The search for the causes of breast and colon cancer. *Nature* 338:389-394, 1989.

Yoshida Y, Hilborn V, Hasset C, Melfi P, Beyers MJ, Freeman AE: Characterization of mouse fetal lung cells cultured on a pigskin substrate. *In Vitro* 16:433-445, 1980.

APPENDIX

Figure 1

Figure 1
Single cells cultured in soft agar.

Figure 2
Fibroblastic overgrowth in a breast carcinoma culture on a plastic substrate.

Figure 2

Figure 1
Single cells cultured in soft agar.

Figure 2
Fibroblastic overgrowth in a breast carcinoma culture on a plastic substrate.

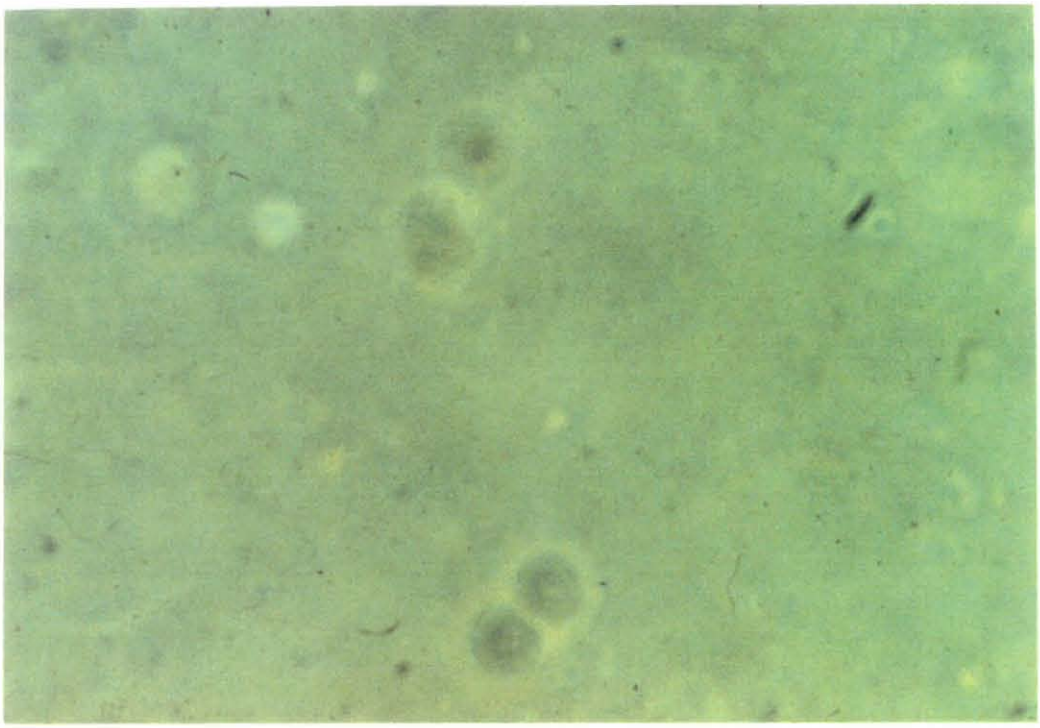
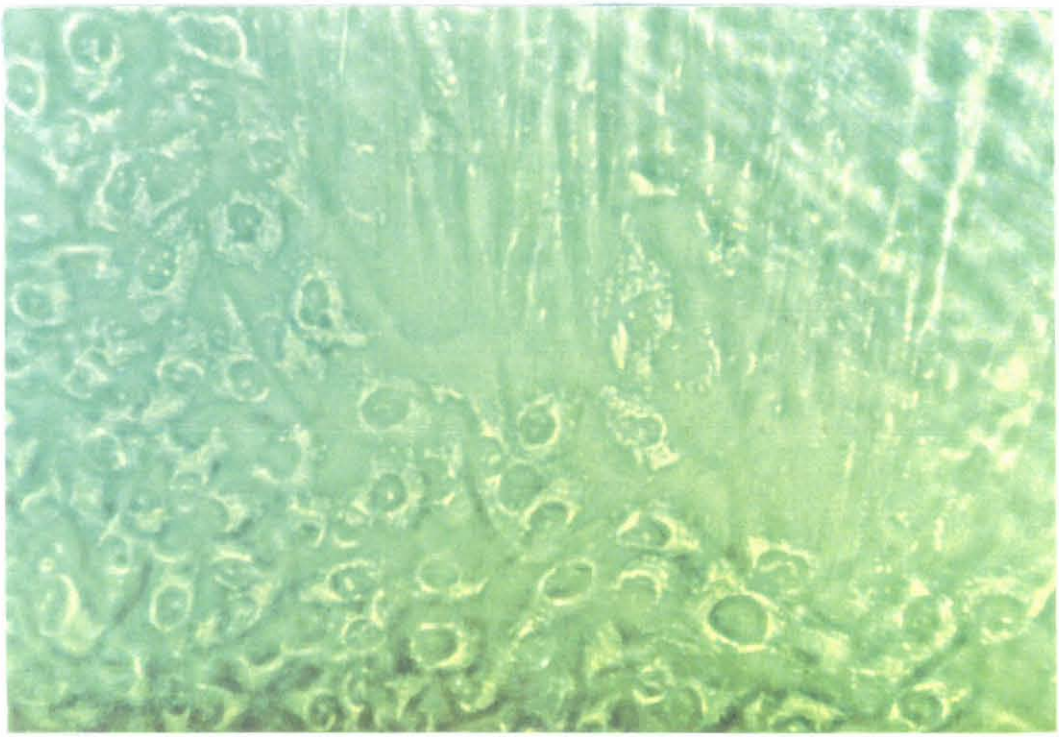


Figure 1



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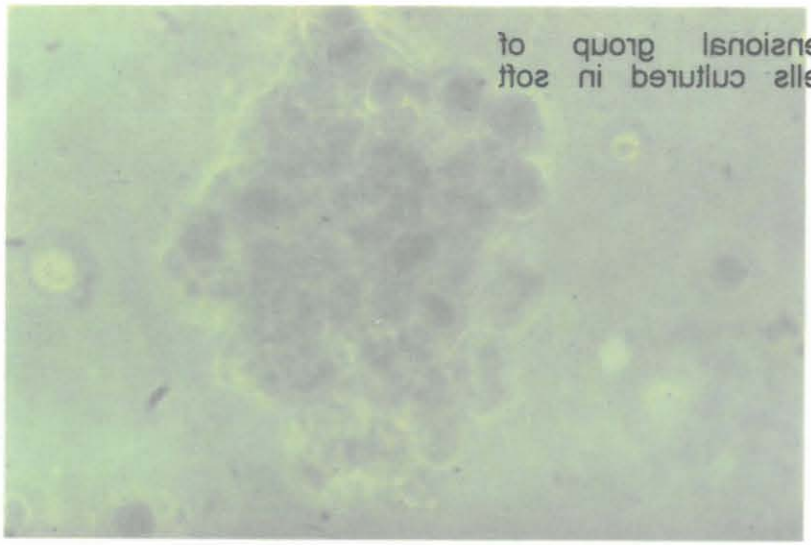


Figure 3

Figure 4

Figure 3
Three dimensional group of malignant cells cultured in soft agar.

Figure 4
Photograph of the organotypic culture system developed during this study. Note the re-usable stainless steel grid supporting alcohol sterilized pigskin blocks. The light yellow pieces of breast tumour placed on top of the pigskin are clearly visible. Growth medium (Ham's F12 with foetal calf serum) was added until the level was above the grid, touching the pigskin. Note the incisions in the pigskin to encourage tumour inflitration.

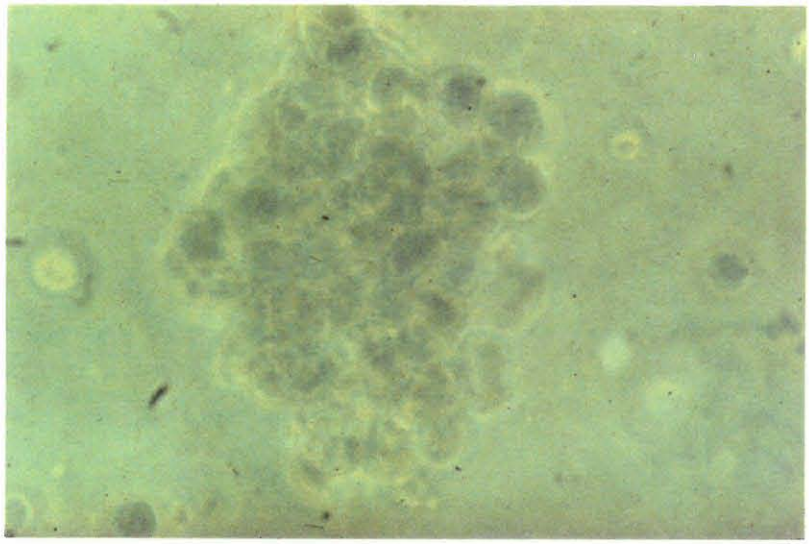


Figure 3

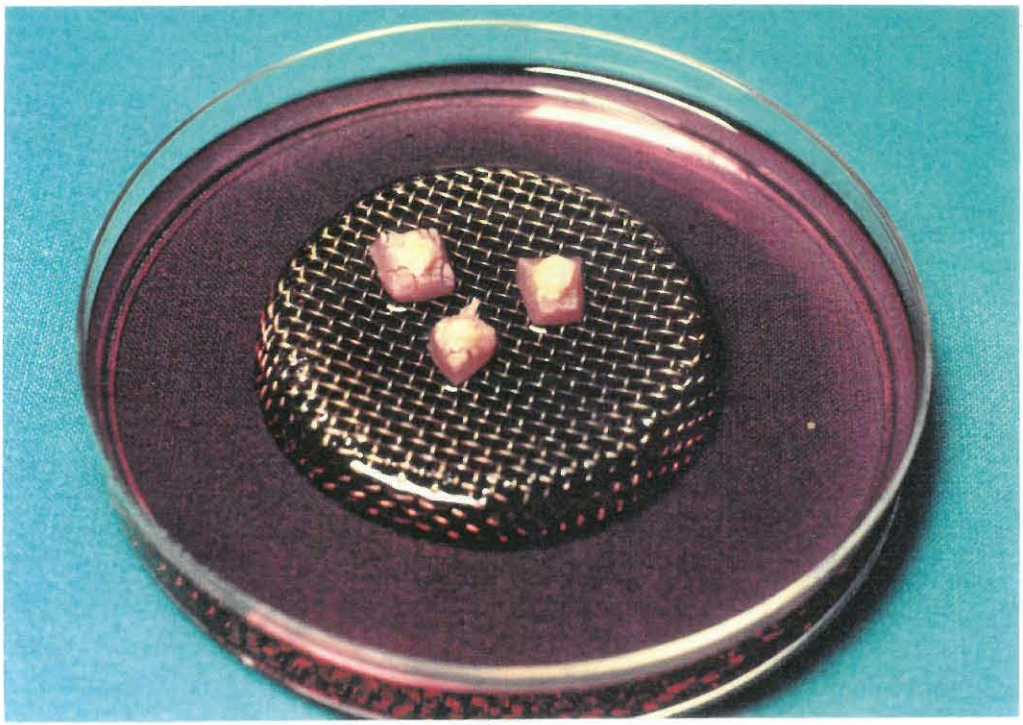


Figure 4

Figure 5
X400
Culture on boiled pigskin embedded in paraffin wax, sectioned and stained with haematoxylin and eosin stain (H&E). Spores of a fungal contaminant can be seen in centre and upper regions of the micrograph. Mycelia are in evidence in the upper right hand region.

Figure 6
x1000
High power micrograph of previous slide showing fungal spores and lightly stained hyphae in greater detail. Note the three larger darkly stained spores with granulated appearance.

Figure 7
X400
Culture on boiled pigskin embedded in paraffin wax, sectioned and stained with haematoxylin and eosin stain (H&E), illustrating smudged nuclei, reddish purple in colour. Capillaries are light pink and oval in shape. Diffuse collagen fibres are prevalent exhibiting a blue-grey hue.

Figure 8
X400
Autoclaved pigskin embedded in paraffin wax, sectioned and stained with haematoxylin and eosin.
Note the gel-like consistency of the autoclaved pigskin which often appeared translucent. The collagen stained light pink with darker folds. No nuclei or identifiable cells could be observed.

Figure 9
X400
Pigskin sterilized by alcohol and subsequent boiling, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. The numerous darkly stained smudged nuclei without chromatin pattern are clearly discernable. Collagen material is light pink in colour and translucent in places.

Figure 10
X400
By comparison, groups of malignant cells grown in the same material has clearly discernable chromatin pattern, irregular nuclear membranes and nucleoli.
The culture on pigskin was sterilized by alcohol and subsequent boiling, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin.

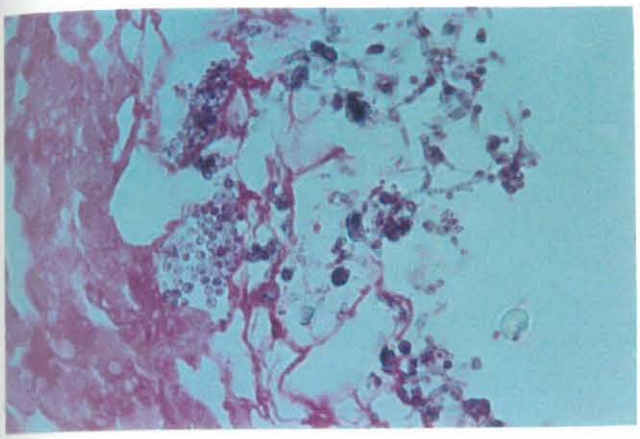


Figure 5

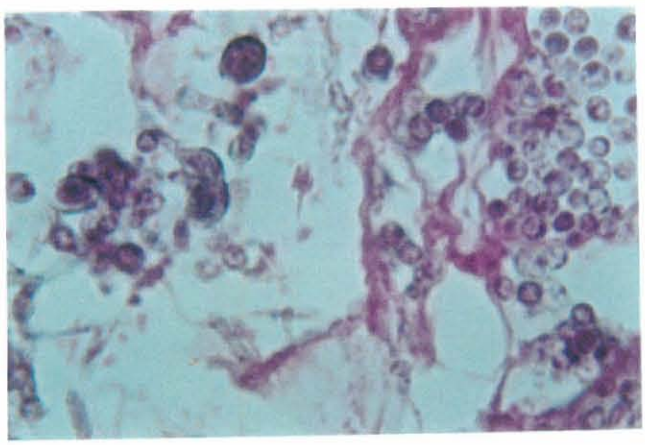


Figure 6

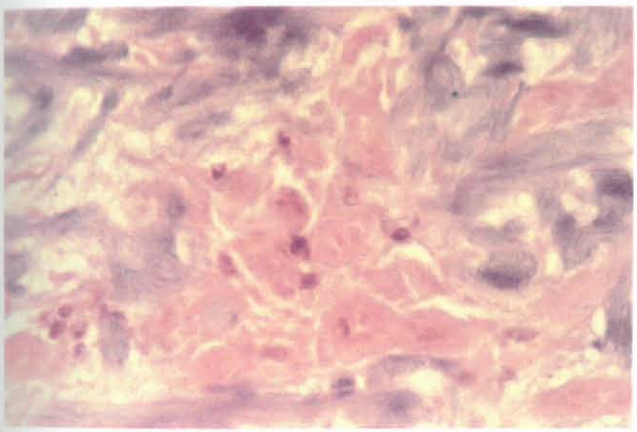


Figure 7

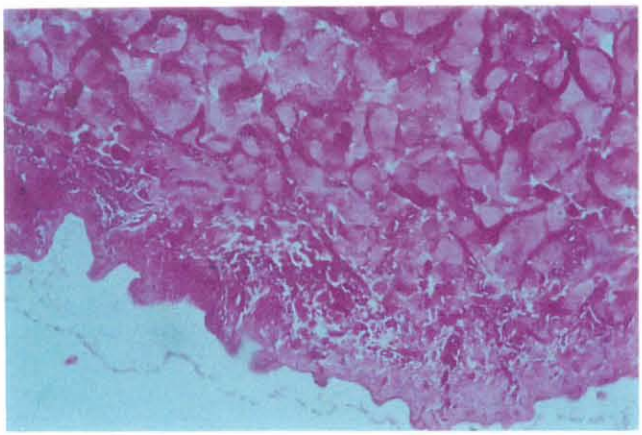


Figure 8

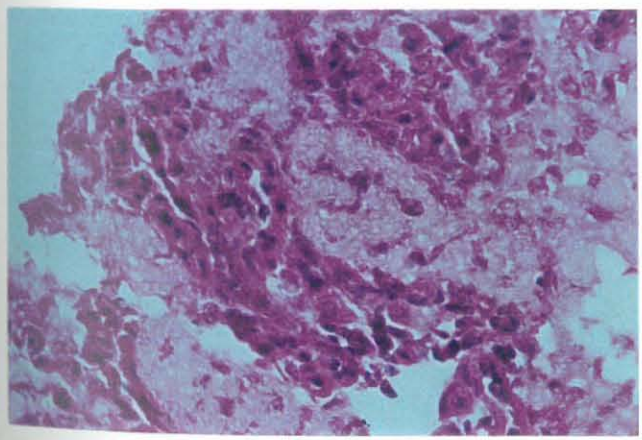


Figure 9

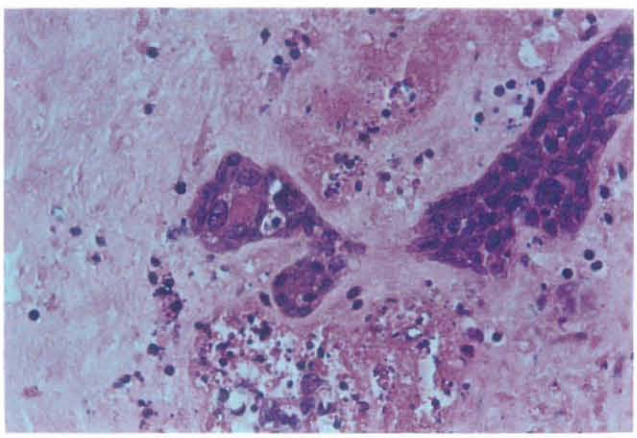


Figure 10

Figure 11
X100
Cultures of breast carcinomas grown on alcohol sterilized pig-skin. Paraffin wax embedding was followed by sectioning and staining with haematoxylin and eosin. Large accumulation of viable malignant cells on the left with pigskin on the right. In this case no clear invasion of the underlying pigskin by the malignant cell component is evident.

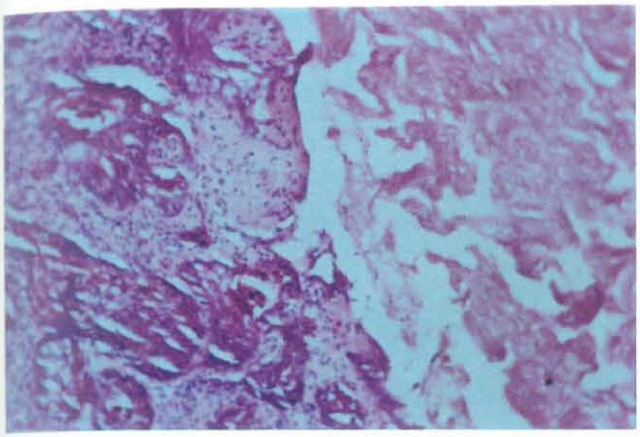
Figures 12
X400
Nests or clusters of malignant breast cells after infiltration into the pigskin. The darkly-stained nuclei exhibit obvious malignant characteristics. Note the circular or acinar arrangement of the malignant cells indicative of their glandular origin (adenocarcinoma). Collagen material appears diffuse and light pink in colour.

Figures 13
X400
Groups of malignant breast cells after infiltration into the pigskin. The nuclei exhibit malignant characteristics and the arrangement of the cells suggest a glandular origin. Collagen material appears diffuse and light pink in colour.

Figure 14
X400
Culture time 5 weeks
Nests of malignant breast cells after invasion of the underlying pigskin. The presence of macronucleoli indicates that the cells are still viable. Nevertheless the cluster in the centre shows signs of cytoplasmic degeneration. This was typical of the longer term (5 - 6 weeks) cultures.

Figure 15
X400
Kulzer resin embedded breast tumour biopsies. 3µ sections stained with haematoxylin and eosin. Note the crisp, clear detail of the nuclei and nuclear content of the malignant cells. The thinner sections provided a microscopic advantage over the paraffin wax sections (Fig 12 and 13).

Figure 16
X100
Kulzer resin embedded breast tumour biopsies, sectioned and stained with haematoxylin and eosin.
Purple nuclei and pink cytoplasm and collagen can be seen.



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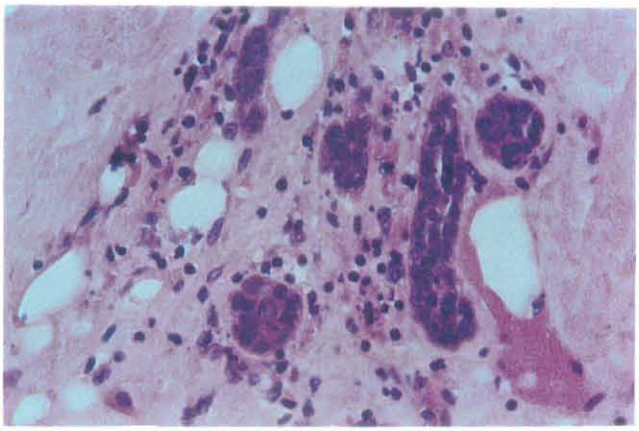

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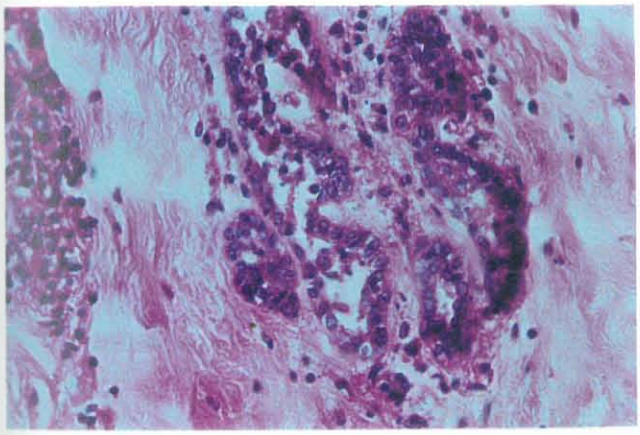

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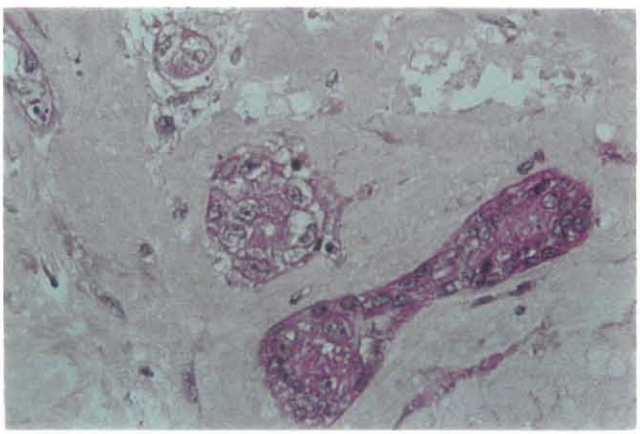
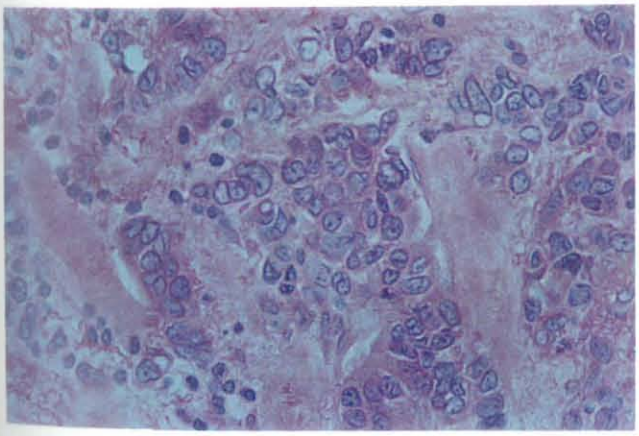


Figure 14



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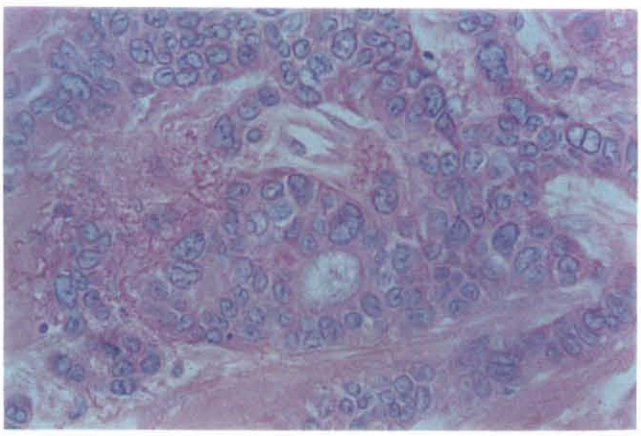


Figure 16

Figure 17
X100
Breast carcinoma biopsies, paraffin wax embedded, sectioned and stained with haematoxylin and eosin.
Purple nuclei and pink cytoplasm and collagen can be seen.

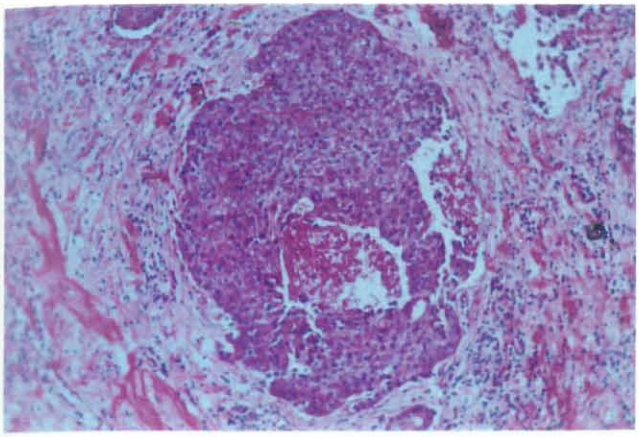
Figures 18
X400
AgNORs in paraffin wax sections of archival tissue of normal cervix stained with silver for nucleolar organizer regions. Silver deposits are small, scarce and single.

Figure 19
X400
Large, multiple AgNORs (dark brown/black silver deposits) in section of archival non-keratinizing squamous carcinoma tissue of the uterine cervix. The tissue was embedded in paraffin wax, sectioned and stained with silver for nucleolar organizer regions.

Figures 20
X400
Paraffin wax embedded and stained with silver for nucleolar organizer regions. Large, multiple AgNORs can be seen in this section of archival cervical adenocarcinoma tissue.

Figures 21
X400
Paraffin wax embedded sections stained with silver for nucleolar organizer regions. Nest of breast carcinoma cells can be seen in the pigskin with AgNORs appearing very large and occupying relatively large areas of the nucleus.

Figure 22
X400
Culture on plastic of breast carcinoma cell line MCF-7 stained directly with silver for nucleolar organizer regions. The presence of AgNORs is indicated by the dark brown/black silver deposits in the nuclei. Small and large AgNORs present.



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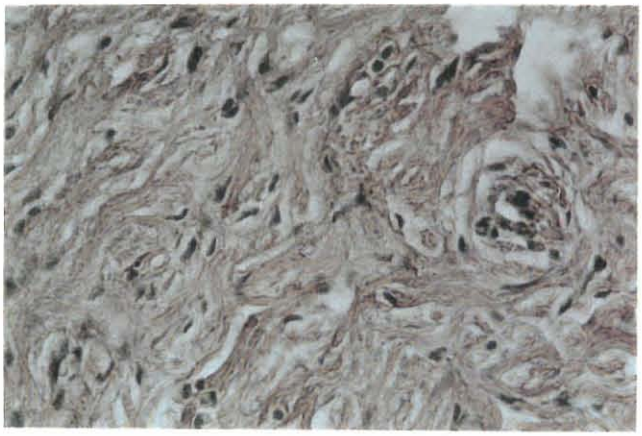

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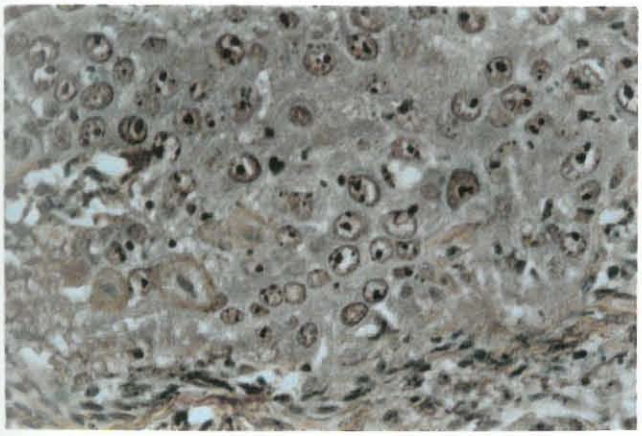
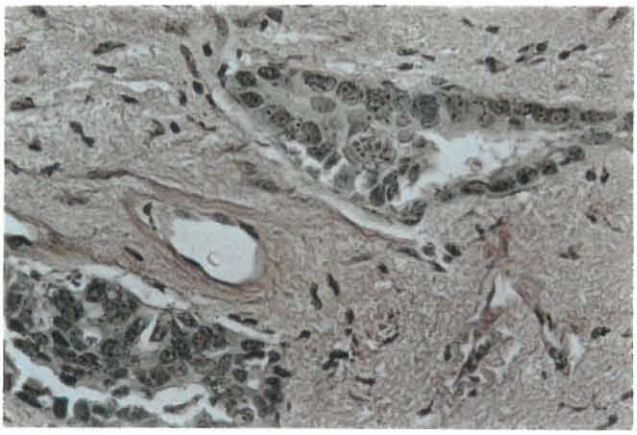


Figure 19



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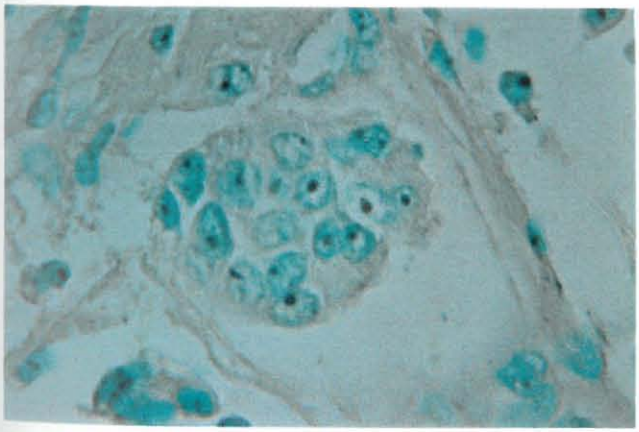


Figure 21

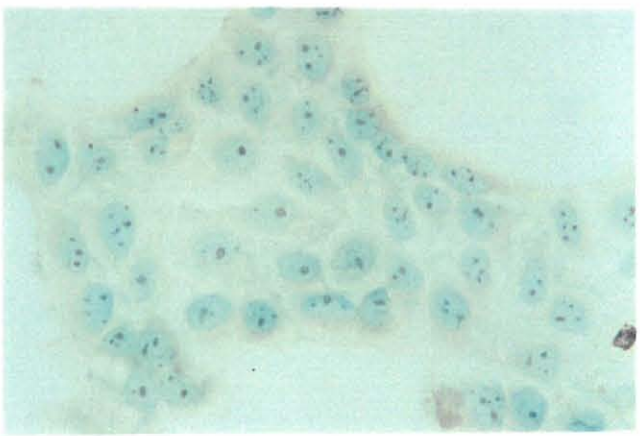


Figure 22

Figure 23
X400
Tumour imprint sprayfixed and stained with Papanicolaou. All cell components stained basophilic due to increased uptake of haematoxylin.

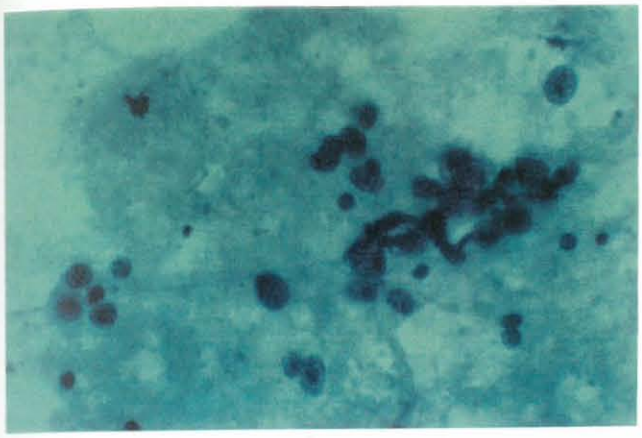
Figure 24
X400
Culture of breast carcinoma cell line MCF-7 sprayfixed and stained with Papanicolaou show no eosinophilic staining of nucleoli.

Figure 25
X100
Culture of breast carcinoma cell line MCF-7 sprayfixed and stained with Papanicolaou show no eosinophilic staining of nucleoli.

Figure 26
X400
Cell line T-47D sprayfixed and stained with Papanicolaou showing basophilic staining of all cell components.

Figure 27
X100
Breast carcinoma cell line MCF-7 sprayfixed and stained with immunogold-silver stain for oestrogen receptor. The slide shows positive staining reaction for oestrogen receptor through a dark brown to deep black reaction product in the cytoplasm of cells.

Figure 28
Negative control for breast carcinoma cell line MCF-7 sprayfixed and stained with immunogold-silver stain for oestrogen receptor.



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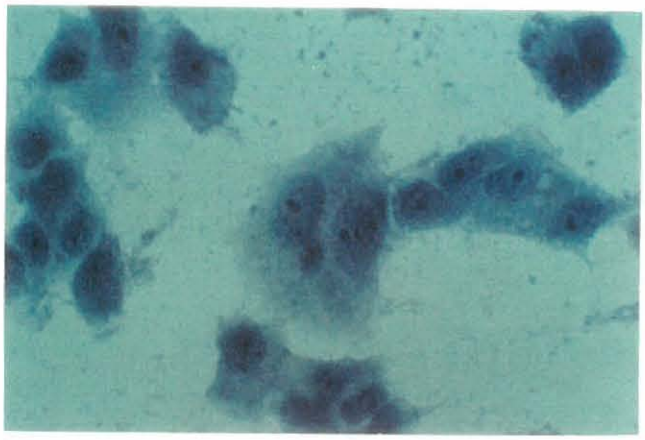

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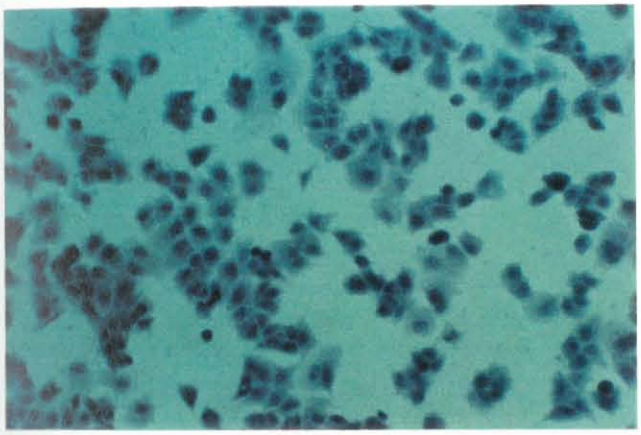

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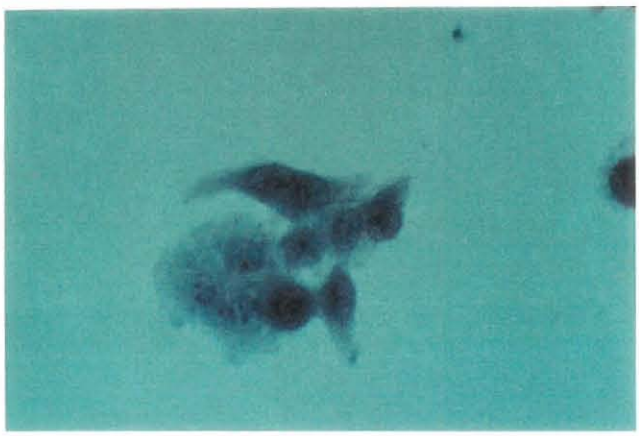

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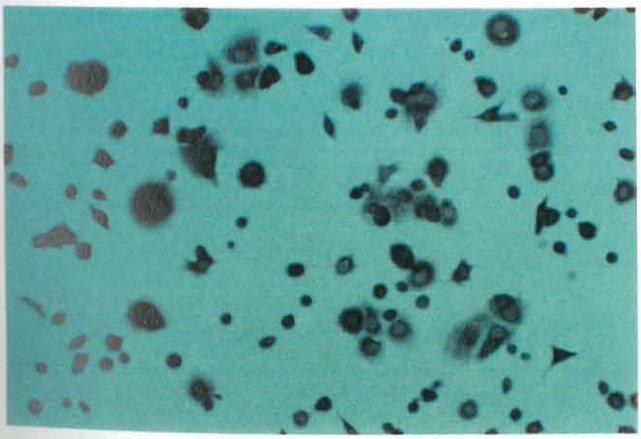
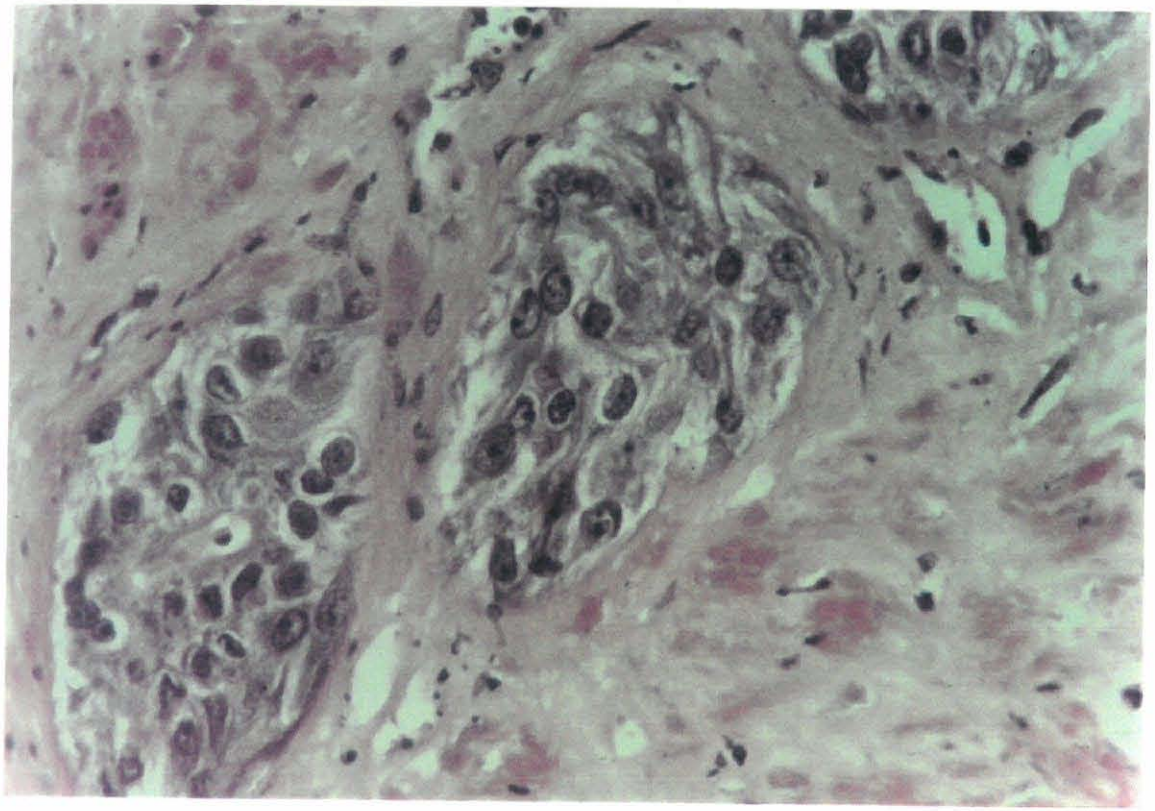


Figure 27

Figure 28

Figure 29
X400
Breast carcinoma biopsy T4c cultured on pigskin, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. Malignant cells with anisonucleosis, irregular nuclear membranes, irregular macro-nucleoli, chromatin clumping and parachromatin clearing is evident.

Figure 30
X1000
Breast carcinoma biopsy cultured on pigskin, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin.
Groups of viable malignant cells showing malignant criteria can be seen in the pigskin.



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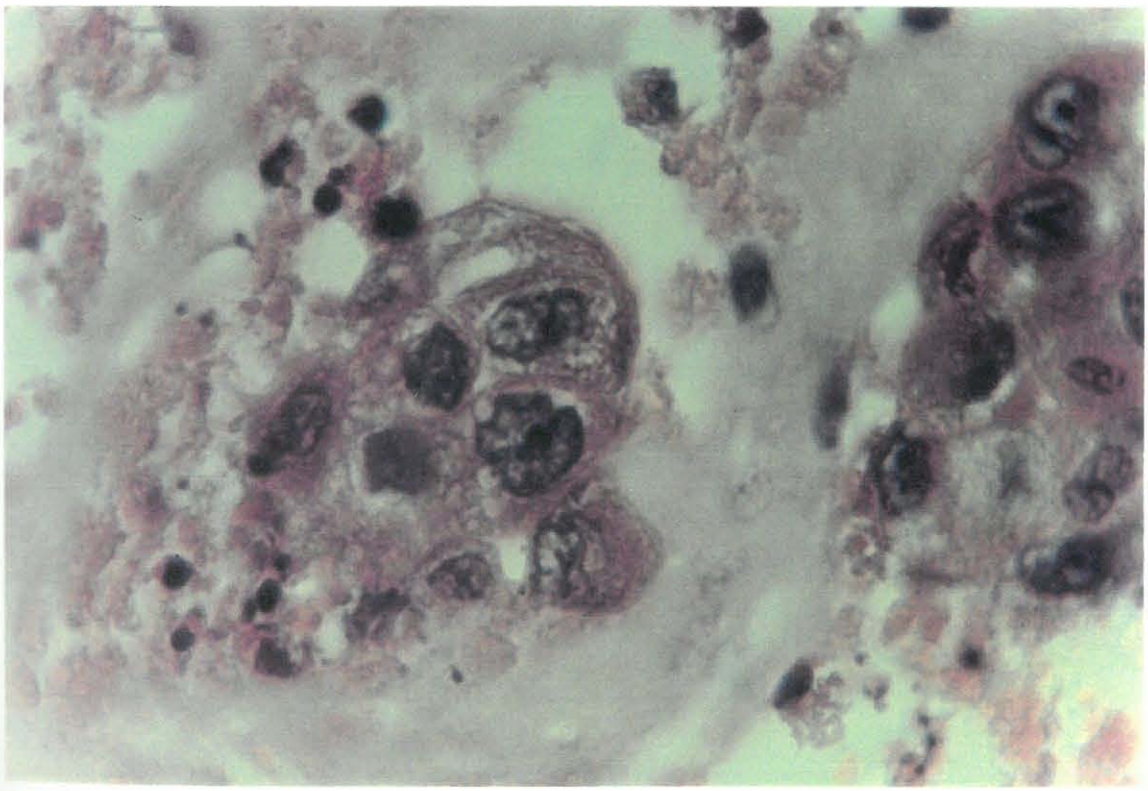
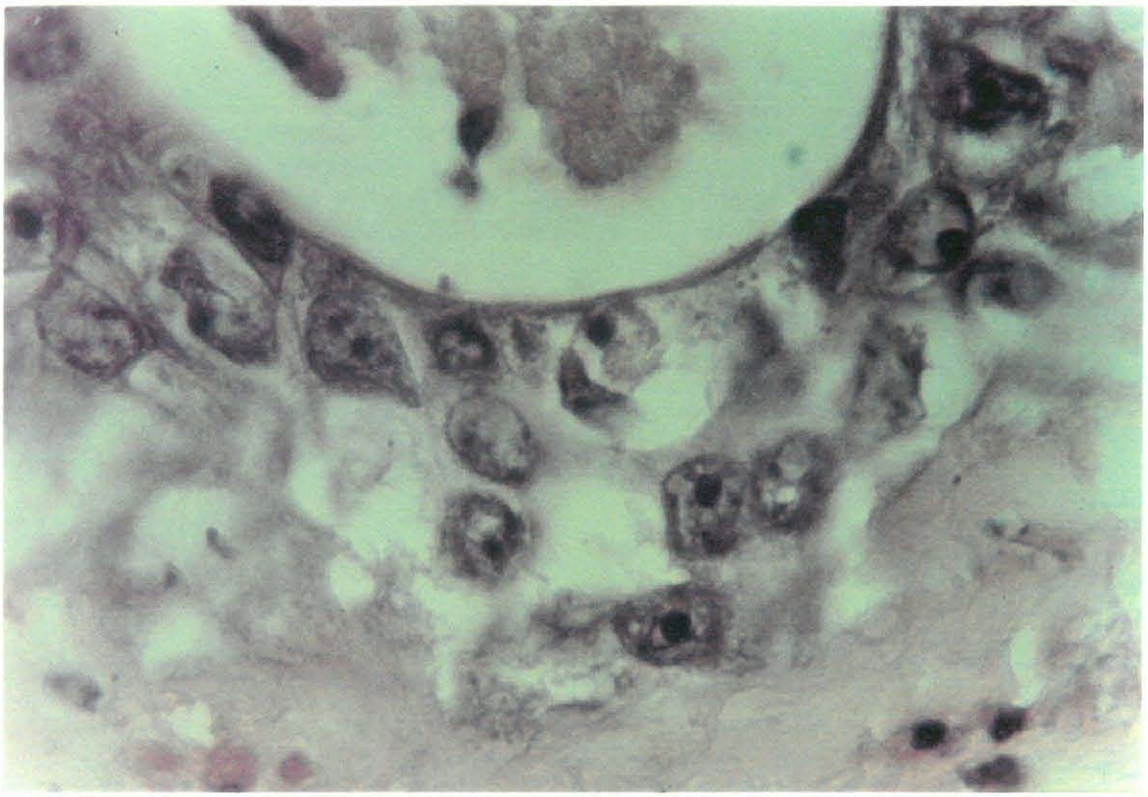


Figure 30

Figure 31
X1000
Breast carcinoma tumour T4c cultured on pigskin showing malignant cells with anisonucleosis, irregular nuclear membranes, irregular macronucleoli, chromatin clumping and parachromatin clearing.

Figure 32
X1000
Three dimensional group of malignant breast cells in pigskin.



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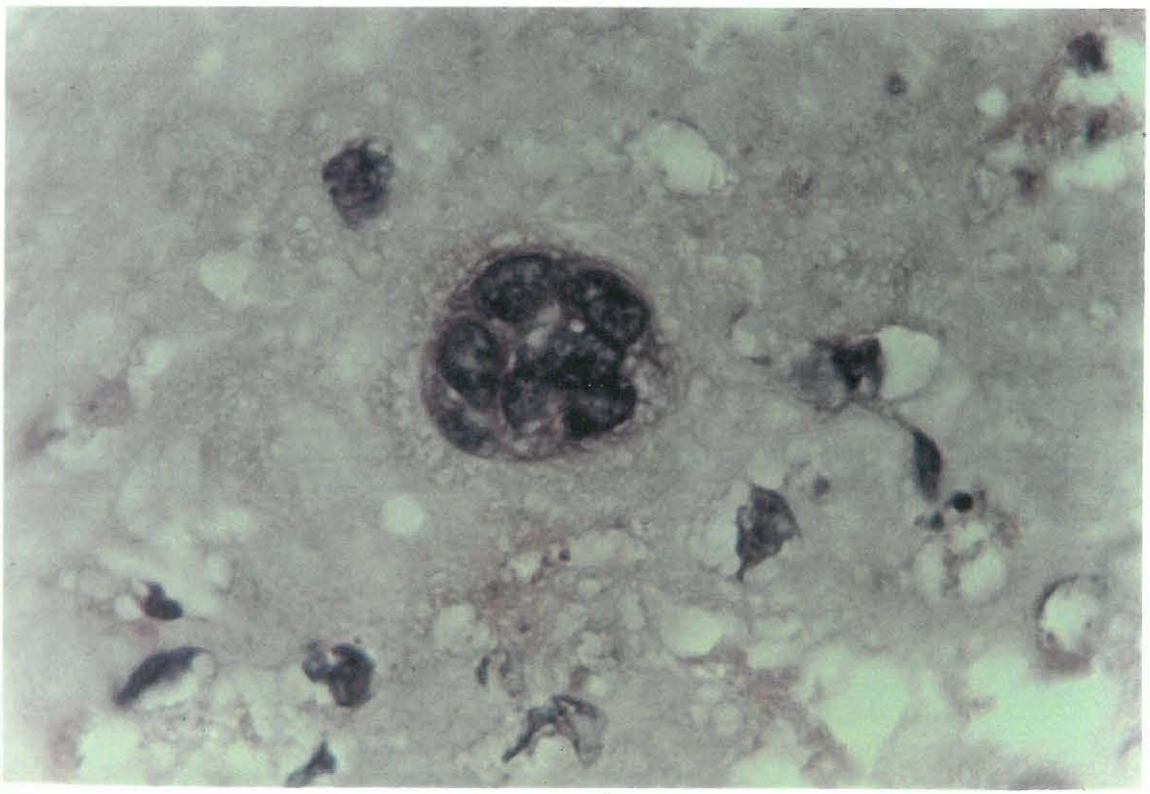


Figure 32

Figure 33
X 400
Paraffin wax section of tumour T20 stained with haematoxylin and eosin. Note irregular macronucleoli, anisonucleosis, irregular nuclear membranes, parachromatin clearing, nuclear beading and mitosis.

Figure 34
X400
Cell line MDA-MB-231 cultured on plastic and stained with Papanicolaou showing irregular nuclear membrane and irregular multiple macronucleoli.

Figure 35
X100
Cell line MDA-MB-231 cultured on plastic and stained Papanicolaou.

Figure 36
X400
Cell culture on plastic from tumour T20c, stained Papanicolaou and showing anisonucleosis, macronucleoli, irregular nuclear membranes and parachromatin clearing.

Figure 37
X400
Imprint of breast carcinoma tumour T20 stained Papanicolaou and showing anisonucleosis, macronucleoli and irregular nuclear membranes.

Figure 38
X100
Cell culture of tumour T20 on plastic subtrate, sprayfixed and stained for vimentin using DAKO PAP KIT. A reddish brown cytoplasmic stain indicates the presence of vimentin.

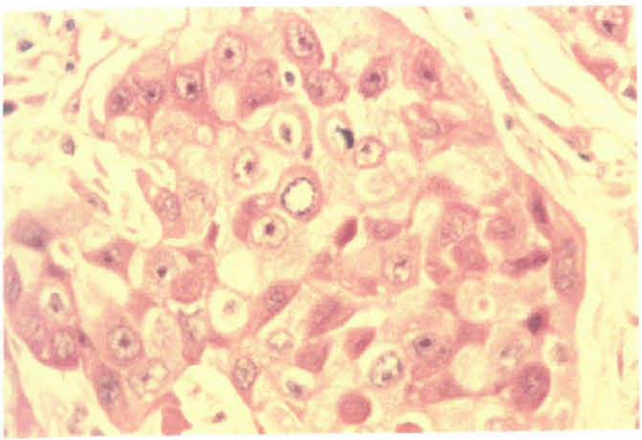
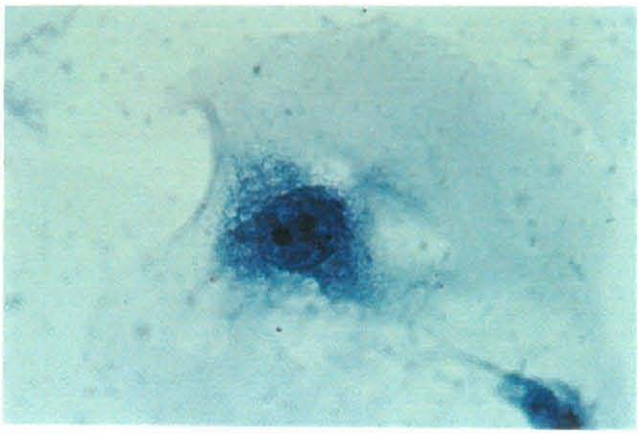


Figure 33



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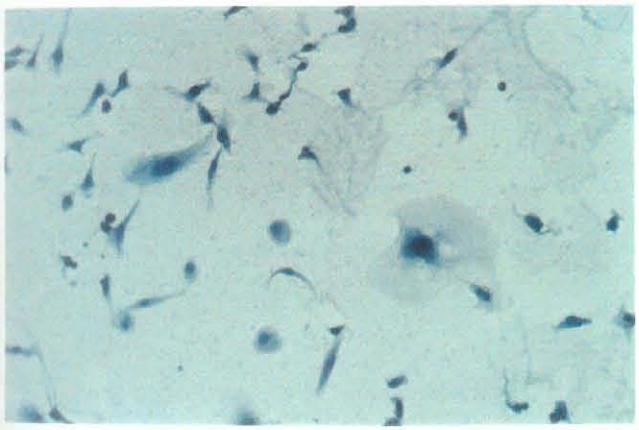

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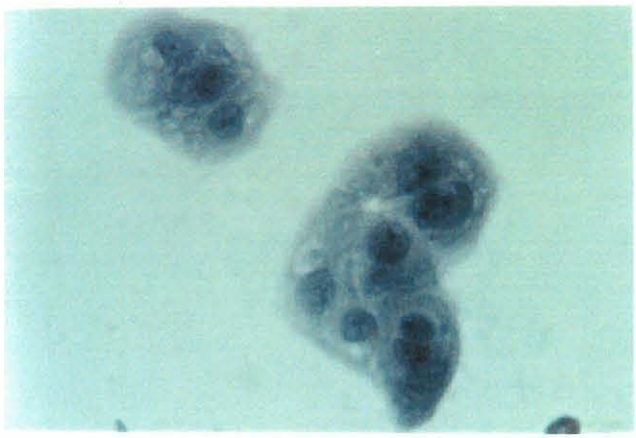

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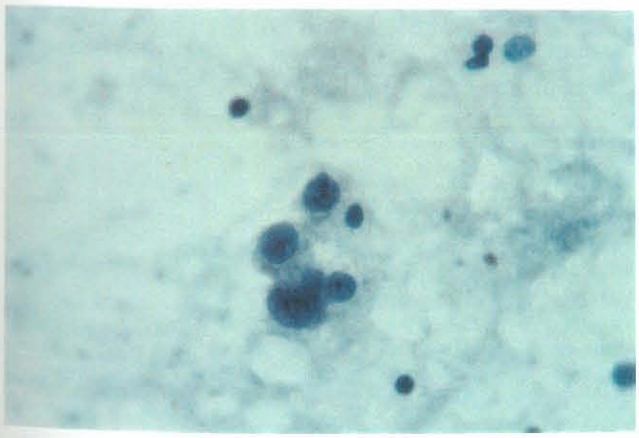

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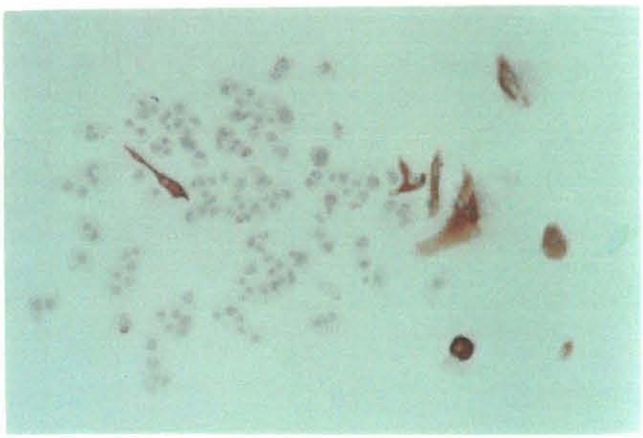

Figure 38

Figure 39
X100
Culture on plastic of cell line MDA-MB-231 showing positive staining reaction for keratin.

Figure 40
X100
Culture of cell line MDA-MB-231 used as negative control in staining for keratin.

Figure 41
X100
Positive vimentin staining in culture of cell line MDA-MB-231.

Figure 42
X100
Cell line MDA-MB-231 used as negative control for vimentin.

Figure 43
X100
Imprint of breast carcinoma tumour T20, sprayfixed and stained with DAKO PAP KIT for vimentin. The reddish brown stain was considered to be background staining.

Figure 44
X100
Imprint of tumour T20, sprayfixed and stained with DAKO PAP KIT used as negative control in staining for vimentin.

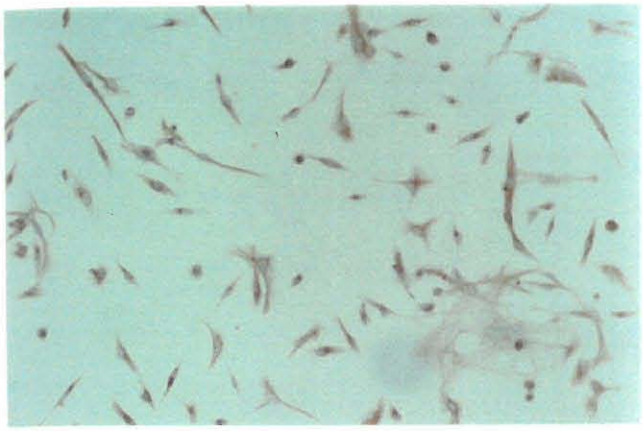

Figure 39

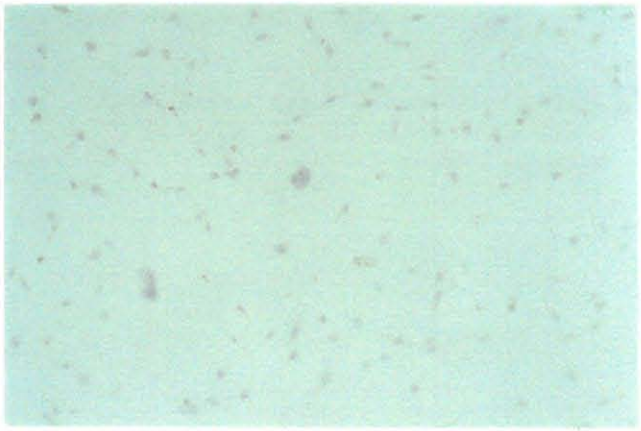

Figure 40

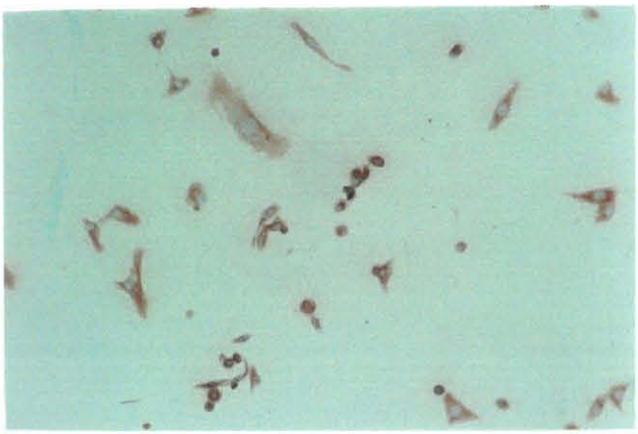

Figure 41

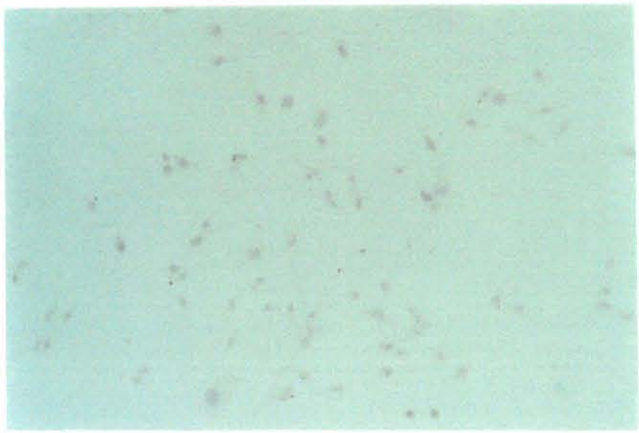

Figure 42

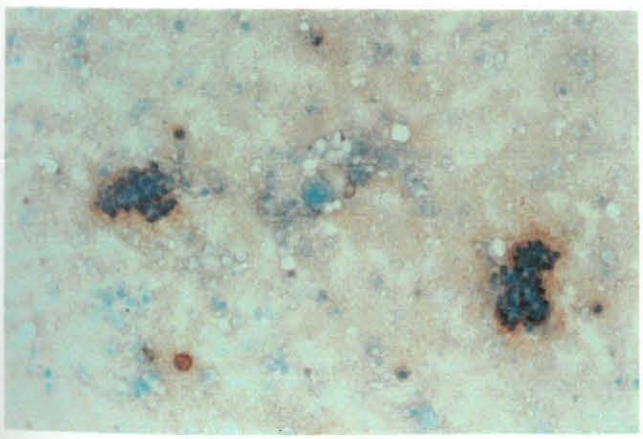

Figure 43

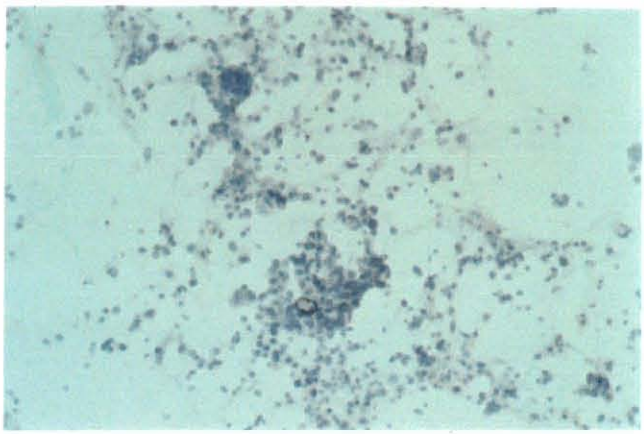

Figure 44

Figure 45
X100
Positive reddish brown staining for oestrogen receptor in imprint of tumour T20.

Figure 46
X100
Imprint of tumour T20 used as negative control in staining for oestrogen receptor.

Figure 47
X100
Cell culture on plastic of tumour T20c sprayfixed and stained for keratin using DAKO PAP KIT. A reddish brown cytoplasmic staining indicates a positive staining reaction for oestrogen receptor.

Figure 48
X100
No reddish brown staining observed in the negative control.

Figure 49
X100
Positve staining reaction for keratin in imprint of tumour T20 sprayfixed and stained for keratin using DAKO PAP KIT.

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Figure 50
X100
Tumour T20 imprint used as negative control showing no reddish brown staining.

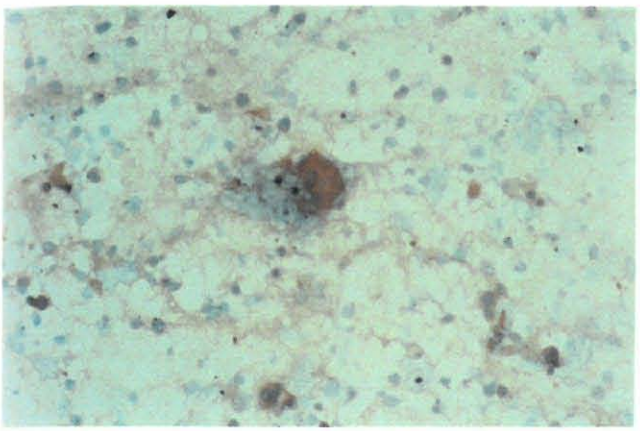

Figure 45

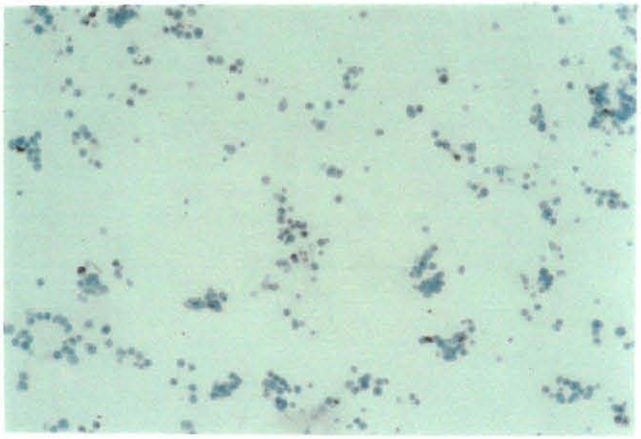

Figure 46

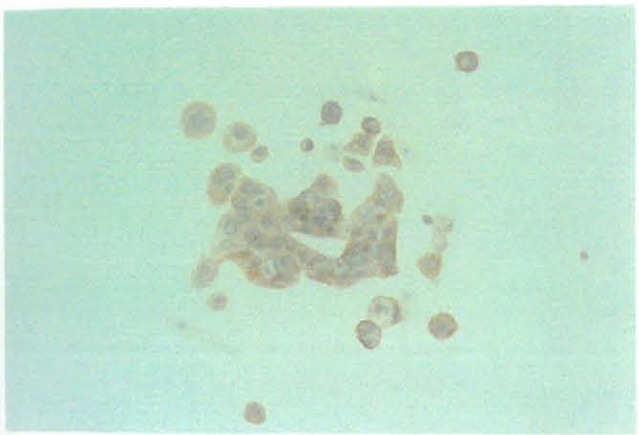

Figure 47

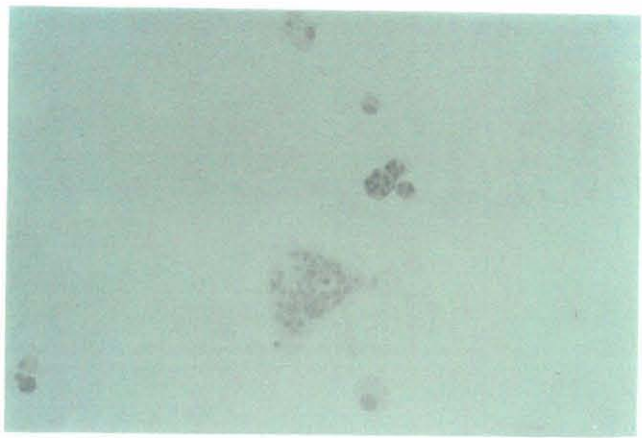

Figure 48

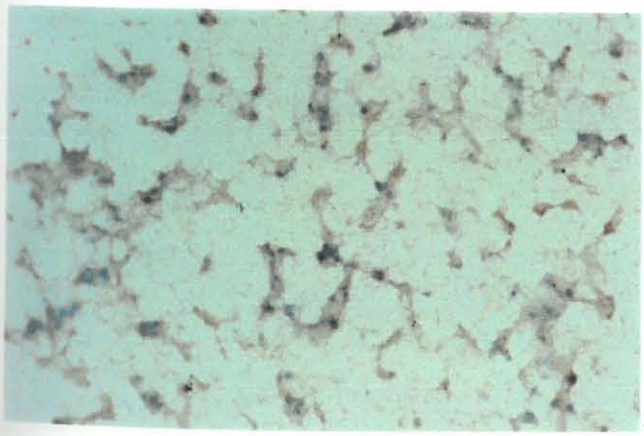

Figure 49

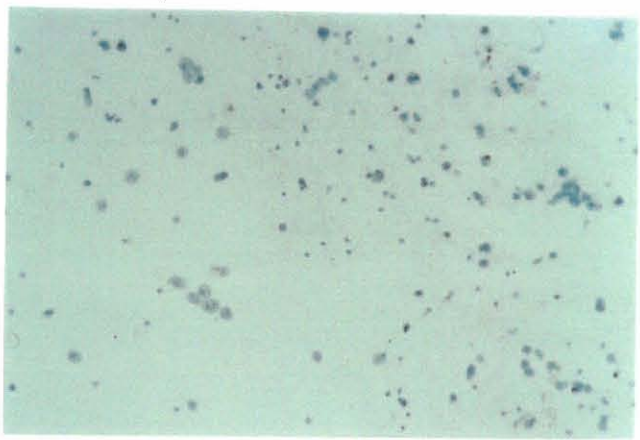

Figure 50

Figure 51
X100
Culture on plastic from tumour T21c, sprayfixed and stained for keratin using DAKO PAP KIT. Positive staining for keratin is indicated by reddish brown stain.

Figure 52
X100
Tumour T21c culture used as negative control in staining for keratin.

Figure 53
X100
Breast carcinoma tumour T21 imprint, sprayfixed and stained for keratin using DAKO PAP KIT and showing positive staining reaction for keratin.

Figure 54
X100
Negative control of tumour T21 imprint stained for keratin.

Figure 55

X100
Breast carcinoma tumour T21 imprint sprayfixed and stained for keratin using DAKO PAP KIT shows a positive reaction for vimentin.

Figure 56
X100
Imprint from tumour T21 used as negative control in staining for vimentin.

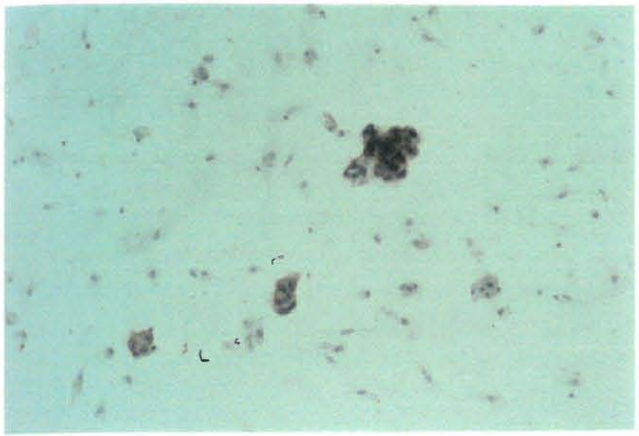

Figure 51

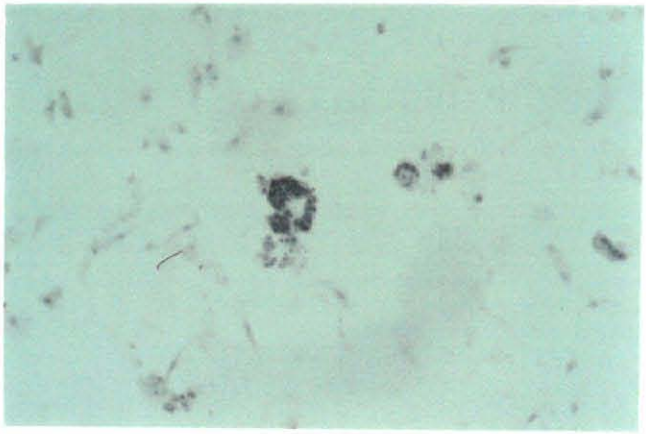

Figure 52

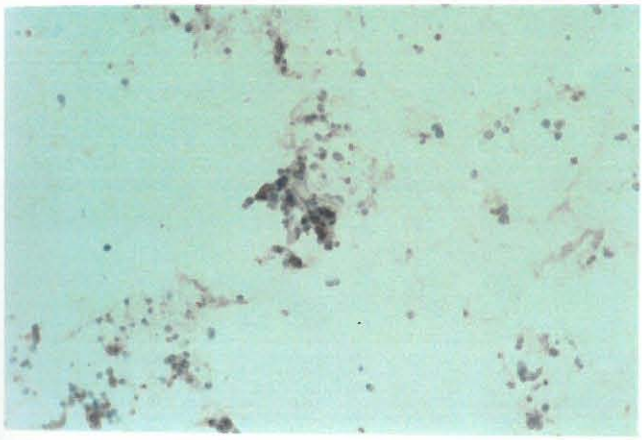

Figure 53

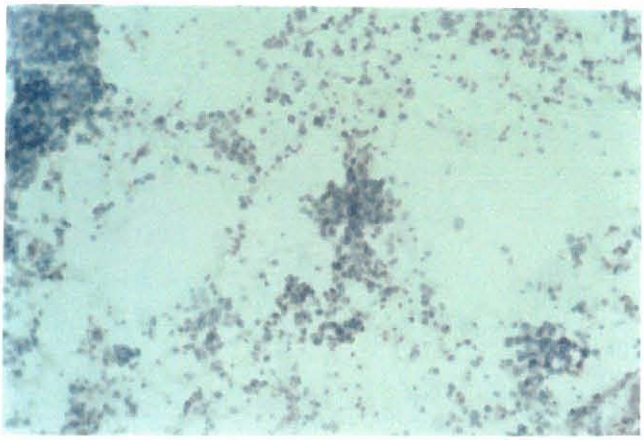

Figure 54

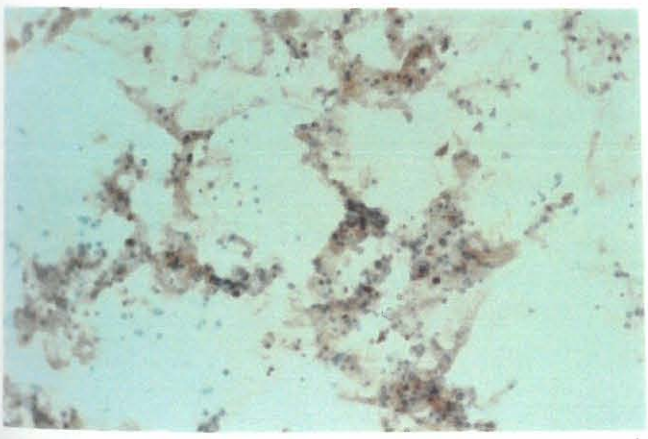

Figure 55

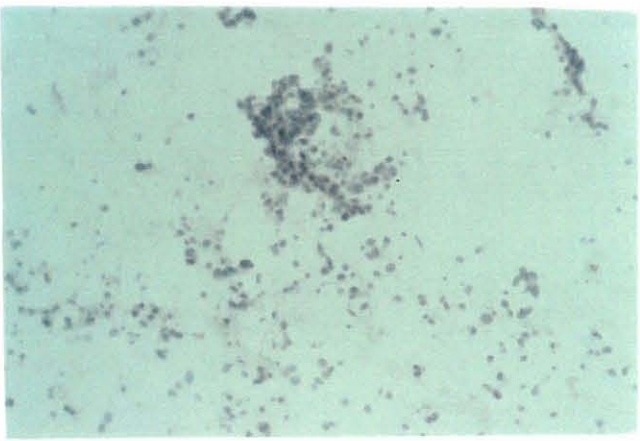

Figure 56

Figure 57
X100
Positive reddish brown staining of known ER positive section used as positive control in staining for oestrogen receptor using DAKO PAP KIT.

Figure 58
X100
Known ER positive section used as negative control.

Figure 59
X400
Positive reddish brown staining of known ER positive section used as positive control in staining for oestrogen receptor using DAKO PAP KIT.

Figure 60
X100
Universal positive EMA control stained using DAKO PAP KIT.

Figure 61
X100
DAKO positive keratin control used as positve control in staining for keratin using DAKO PAP KIT.

Figure 62
X100
Universal positive keratin control used as positive control in staining for keratin using DAKO PAP KIT.

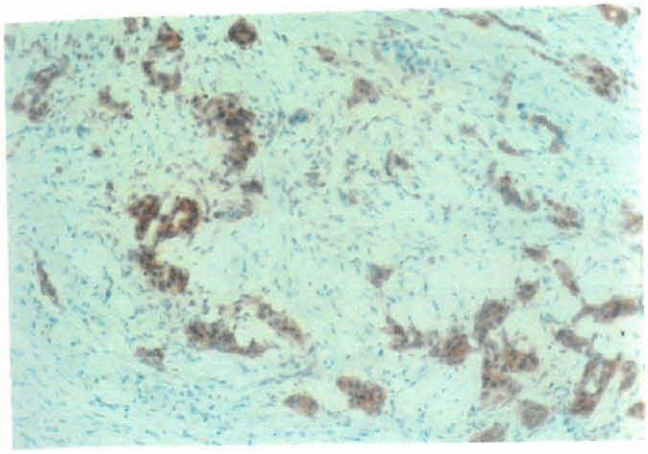

Figure 57

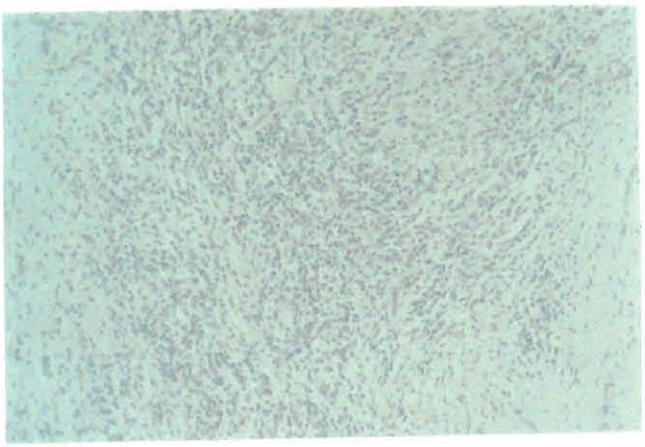

Figure 58

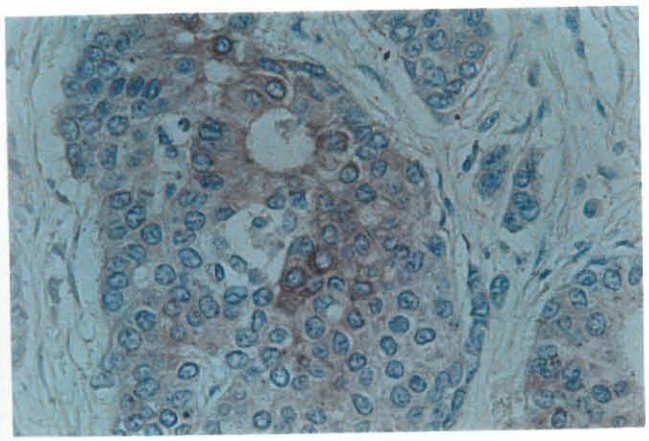

Figure 59

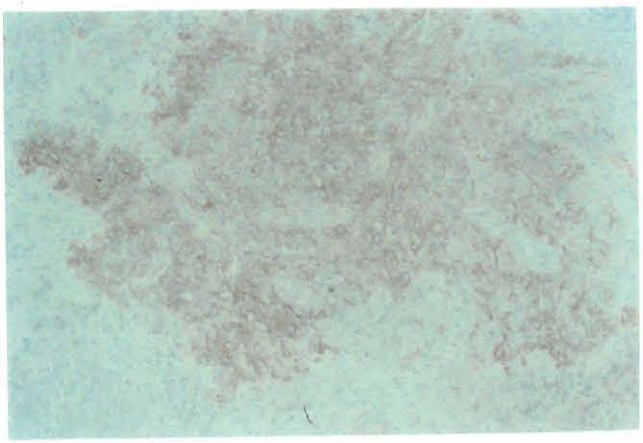

Figure 60

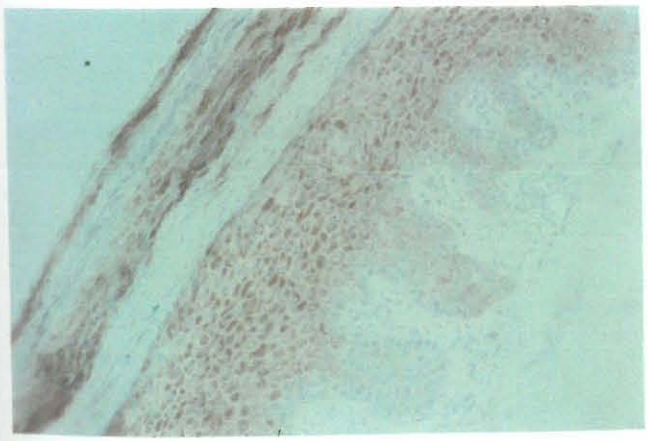

Figure 61

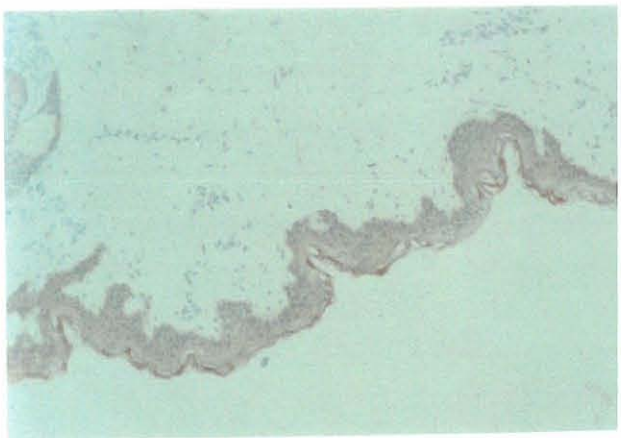

Figure 62

Figure 63
X100
MCF-7 breast carcinoma cell line cultured on plastic, fixed with sprayfix and stained with DAKO PAP KIT using appropriate monoclonal antibodies. This slide was used as a negative control in staining for EMA.

Figure 64
X100
MCF-7 breast carcinoma cell line cultured on plastic, fixed with sprayfix and stained with DAKO PAP KIT using appropriate monoclonal antibodies. This slide shows a positive staining reaction for EMA.

Figure 65
X100
MCF-7 breast carcinoma cell line cultured on plastic, fixed with sprayfix and stained with DAKO PAP KIT using appropriate monoclonal antibodies. This slide was used as a negative control in staining for oestrogen receptor.

Figure 66
X100
MCF-7 breast carcinoma cell line cultured on plastic, fixed with sprayfix and stained with DAKO PAP KIT using appropriate monoclonal antibodies. This slide shows a positive reddish brown staining reaction for oestrogen receptor.

Figure 67
X400
MCF-7 breast carcinoma cell line cultured on plastic, sprayfixed and stained with silver. AgNORs were demonstrated on this slide. Note multiple large and small black staining AgNORs in nuclei of cells.

Figure 68
X100
MCF-7 breast carcinoma cell line cultured on plastic, sprayfixed and stained with silver for demonstration of AgNORs.

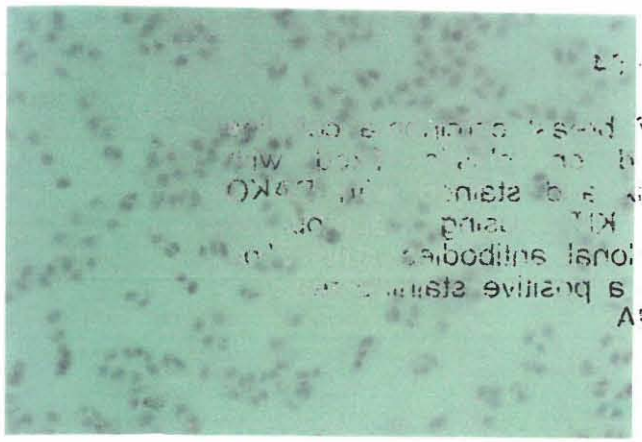

Figure 63

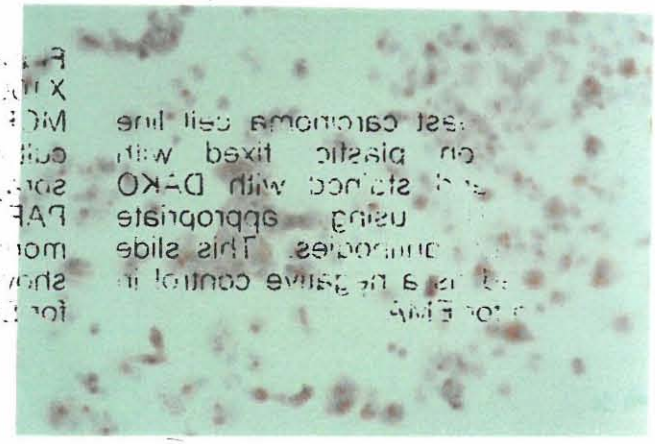

Figure 64

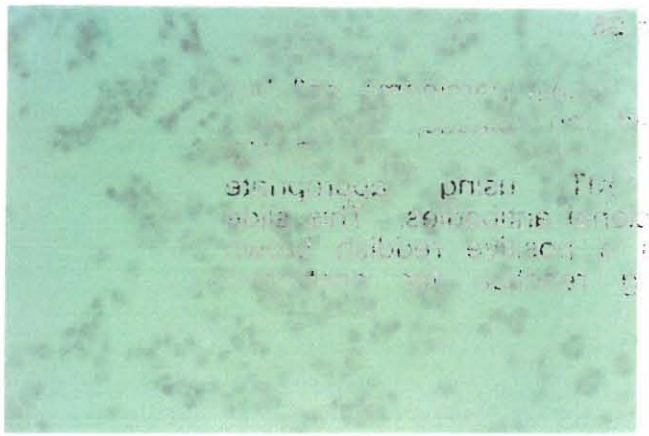

Figure 65

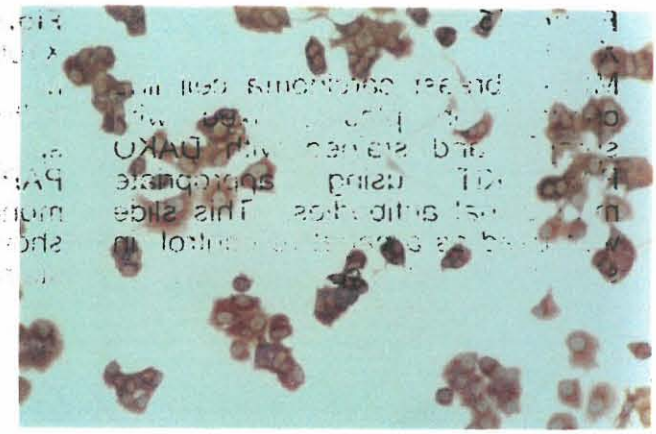

Figure 66

Figure 67

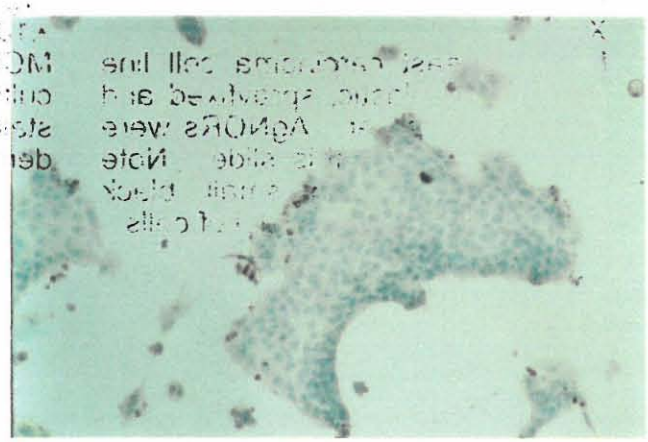

Figure 68