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THE PRIMATE MAMMARY EPITHELIAL CELL *IN VITRO*, GROWTH PROPERTIES, ANTIGEN EXPRESSION AND CELL SURVIVAL STUDIES.

by

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A thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg in fulfilment of the requirements for the Degree of Doctor of Philosophy.

Pretoria 1996.

ABSTRACT

Primary tissue cultures of primate mammary epithelial cells (PMEC) in tissue culture were evaluated to serve as a link between rodent models and *in vitro* human mammary epithelial cell (HMEC) systems used to study mammary carcinogenesis. PMEC growth *in vitro*, expression of cytokeratin and milk fat globule proteins (MFGP) and the effect of dimethylbenz(a)anthracene (DMBA) was investigated.

Organoids were isolated from primate (*Cercopithecus aethiops pygerythrus)* and classified according to the degree of lobular development namely less, moderately and well differentiated. Organoid attachment and growth in cell culture reflected mammary gland development. Rapid organoid attachment and growth in cell culture was associated with organoids derived from less and moderately differentiated tissue. A reduction in *in vitro* calcium extended the lifespan of PMEC and induced dramatic morphological changes. New cells shed into the medium were passaged.

Immunoblotting identified K8, K7 and K6 and/or K11 as the predominant keratins in primate mammary tissue. PMEC *in vitro* expressed the same keratins as *in vivo*, the expression of K8 was reduced and K7 and K6 and/or K11 was increased.

The milk fat globule protein (MFGP) fraction was isolated from human milk and lactating primate mammary tissue. Immunoblotting revealed that human anti-MFGP detected primate MFGP-70 while HMFG-2 did not detect primate sialomucins. Trypsinisation and subsequent thiol reduction resulted in the enrichment of MFGP-70 from human and primate MFGP fractions. MFGP-70 was not detected in cell lysates and tryptic digests of MCF-7 and PMEC cells following thiol reduction. A 53 kDa protein was detected in human and primate MFGP fractions, tryptic digests of the same fractions and MCF-7 and PMEC lysates under nonreducing conditions.

The effect of DMBA on the PMEC survival was determined by measuring succinate dehydrogenase inhibition and cell numbers *in vitro*. The use of Hoechst to determine

cellular DNA is limited due to nonspecific fluorescence caused by SDS which was effectively reduced by increasing the counterion concentration and adding cholate. A dose and time related sigmoidal decrease in cell survival was observed. Differences in cell survival and cell numbers *in vitro* occurs following 3 hours exposure to DMBA, while longer exposure times revealed that cell survival determined by all methods was the same.

PMEC *in vitro*, exhibit growth properties that reflect mammary gland development, express the same keratins as *in vivo*, and antigenic determinants associated with the primate MFGP fraction. PMEC growth *in vitro*, expression of antigens and response to the cytotoxic effects of DMBA is analogous to the HMEC *in vitro*. This indicates that the PMEC can contribute significantly to understanding the mechanisms of mammary carcinogenesis.

DECLARATION

I declare that this thesis is my own, unaided work. It is being submitted for the degree of Doctor of Physiology in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any other degree or examination in any other university.

M J Best

4th day of April, 1996.

I further declare that the experimental work for this dissertation was cleared by the Animal Ethics Committees of the University of the Witwatersrand
(Clearance certificate number 95/96/1)

M J Best

4th day of April, 1996.

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LIST OF ABBREVIATIONS

%	percentage
T	total
^0C	degrees Celcius
B(a)P	benza(a)pyrene
BSA	bovine serum albumin
C	crosslinkage
cAMP	cyclic adenosine-3',5'-monophosphate
CH	cholate
CI	counterion
cm^2	centimetre square
CTAB	cetyltrimethylammonium bromide
DAB	3,3'-diaminobenzidine
DMBA	7,12 dimethylbenz(a)anthracene
DMEM:F12	Dulbecco's modified Eagles medium: Ham's F12
DMEM:F12(RC)	DMEM:F12 with a reduced calcium content
dt	doubling time
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-(ß-amino-ethylether)N,N'-tetraacetic acid
Fig.	figure
GD	Glutamate dehydrogenase
GlyD	Gly-3-P dehydrogenase
H_2O_2	hydrogen peroxide
HMFG	human milk fat globule protein
K	keratin
kDa	kilodalton
Lob 1	Lobule type 1
Lob 2	Lobule type 2
Lob 3	Lobule type 3
Lob 1 and 2	Lobule type 1 and 2

M	molar
mA	milliampere
MFGP	milk fat globule protein
mg	milligram
ml	millilitre
M_R	relative molecular mass
mM	millimolar
MNNG	methyl-N-nitro-N-nitroguanidine
MX	milk mix
ng	nanogram
NMU	N-methyl-N-nitrosurea
OV	ovalbumin
P	pyruvate kinase
PBS	phosphate buffered saline
PIPES	piperazine-N,N'-bis(2-ethane sulphonic acid)
PMEC	primate mammary epithelial cells
PMEC-RC	PMEC cultured in medium with reduced calcium
SCID	Severe combined immunodeficient disease
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SV40	Simian virus 40
TBS	Tris buffered saline
TTBS	TBS containing Tween 20
TX100	Triton X100
µg	microgram
µM	micromolar

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CHAPTER 1:

LITERATURE SURVEY

1 INTRODUCTION

The incidence of breast cancer is increasing world wide and a marked increase has been observed in Western countries over the last 25 years (1-5). Environmental, socio-economic, dietary, hormonal and hereditary factors are associated with the aetiology of breast cancer (3-10). The biphasic nature of the incidence of breast cancer has led epidemiologists to postulate the presence of two types of breast cancer (1,4,8). The first type occurs in premenopausal years and shows similar worldwide incidence, while, the second type occurs in the post-menopausal years, where differences occur in high-, intermediate-, and low incidence countries. It is now believed that pre- and post-menopausal breast cancers may have different aetiologies. Intrinsic hormonal factors (1,2,7,10) may play an important role in the development of premenopausal breast cancer.

The role of hormonal contraceptive preparations as possible etiological agents in the development of breast cancer is still being strongly debated (7,8,10). Factors related to reproductive physiology appear to influence the risk of breast cancer; for example early menarche, late menopause, use of oral contraceptives before a full-term pregnancy, exogenous estrogen exposure and nulliparity increases the incidence for breast cancer. Although hormonal factors continue to play an important role, environmental factors such as diet and exposure to carcinogens are thought to enhance the incidence of post-menopausal breast cancer. Breast cancer shows both socioeconomic and geographic patterns in relation to high-fat and high caloric diets where high rates are associated with affluent countries (9).

A definite environmental etiological agent positively linked to the development of breast cancer is ionising radiation. Woman receiving repeated fluoroscopic examinations during therapeutic pneumothoraxes for the treatment of tuberculosis show a high incidence of breast cancer occurring at an early age, with a latent period of 10 to 15 years (10-12). Survivors of the Hiroshima and Nagasaki atomic bomb explosions, who mostly received a single dose of radiation also show a high incidence of breast cancer after 20 years (13). Radiation effects show an accumulative dose response and in addition adolescence seems to be a critical period when exposure leads to an increased incidence. While screening for breast cancer, repeated mammographic examinations pose another possible risk and this has led to a decrease to the number of examinations given to woman under the age of 50 (14).

In a small percentage (5%) of breast cancer cases, genetic predisposition to breast cancer appears to be strong (15). A family history of breast cancer increases the risk about 50-fold in woman whose mothers and sisters have breast cancer. In some high-risk families, susceptibility is inherited as an autosomal dominant gene (16-18). How this genetical susceptibility is expressed at the biochemical level is not known. Another predisposing factor is preexisting benign breast disease (1,19).

Structure of the mammary gland.

Although there are variations in the number, position and external physiology, the internal physiology of the mature female mammary gland is similar in all species from monotremes to man (20-22).

The non-human primates possess a single pair of mammary glands which contain 4 to 8 primary ducts, end buds and lobules (21). The mammary gland extends as a single plane of tissue several millimetres thick from the sternum to the anterior axillary line and from the clavicle to the lower margin of the ribs. As has been described for the human and rat mammary gland the lobularity of the primate mammary gland also increases with the number of reproductive cycles.

Very few species differences exist regarding the internal physiology of the mature female mammary gland and for this reason the structure of the human mammary (8,22-24) gland will be described. The human mammary gland consists of a branching network of glandular tissue embedded in a stromal matrix of adipose and connective tissue. Large ducts branch from the nipple area into smaller ducts which terminate in lobular-alveolar clusters, the production site of the milk components. During pregnancy there is a massive proliferation of these alveolar structures. Four different lobular types, lobules type 1-4 (Lob 1-4) representing different stages of lobule differentiation have been identified in the breast of postpubertal women. Furthermore the degree of lobule development has been identified as an important factor that may predetermine the type of tumours that develop in the human breast (23). Lobule type 1 (Lob 1), with clusters of 6-11 ductules per lobule, is the most undifferentiated type and are present in the immature female breast before menarche. Lobule type 2 (Lob 2) evolves from lobule type 1 while lobule type 3 (Lob 3), with 80 ductules per duct is the most differentiated. Lobule type 4 (Lob 4), considered to be the maximal expression of development and differentiation, is only found in the mammary gland during lactation (10,22,23,25).

The cells of the terminal ductule/lobule junction from which this proliferation occurs are thought to be the site of origin of most human carcinomas (24). There are basically two types of mammary epithelial cells (i) the commular epithelium responsible for secretory activity and the (ii) myoepithelial cells (26). The epithelial cells are present in a circular arrangement one or two cell layers thick, with the apical surfaces facing the central lumen and the basal surfaces resting on the myoepithelial cells or the basement membrane. These cells have a well-developed Golgi apparatus on the apical side of the nucleus, microvilli on the apical surface, tight junctions and desmosomal connections with their lateral neighbours. The myoepithelial cells rest between the epithelial cells and form a network of cells that provides structural support to the glandular tissue (26). These cells have prominent myofilaments, form desmosomal junctions (27,28) with overlying epithelial cells and contain hemidesmosomes on their basal surface. The contraction of the myoepithelial cells in response to appropriate

neurohormonal stimuli, causes ejection of milk. The basement membrane which surrounds the epithelial cells provides a "biomatrix" ground support as well as a barrier of selective permeability separating the epithelial cells from the stroma and the vascular system.

The cultivation of normal mammary epithelial cells.

Since the mammary epithelial cell has been identified as the cell population from which mammary carcinomas originate, greater insight would be gained in the mechanisms of mammary carcinogenesis with the development of human mammary epithelial cell (HMEC) culture systems.

The main objective in the cultivation of normal mammary epithelial cells is to obtain a pure epithelial cell population, with an extended lifespan *in vitro*, that retains many of the characteristics of the mammary epithelial cell *in vivo* and reflects the heterogeneity of the human population (23,25).

Until recently only two kinds of human mammary epithelial cells were available; Cell lines derived from breast tumours (29,30) and primary cultures (31,32), derived mainly from lactation fluids. The development of better tissue dissociation techniques, media formulations (33-35), the identification and isolation of growth factors, hormones (36-45) and other mitogens (34,35,46) has assisted the establishment of cultures HMEC. Cultures of mammary epithelial cells can be now be established from lactation fluids and mammary tissue derived from non-tumour areas of mastectomies, benign tumours and reduction mammoplasties (31,35,44,47).

Characterisation of human mammary epithelial cells.

The expression of the structural cytokeratin proteins and the membrane associated milk fat globule proteins are used to characterise mammary epithelial cells *in vivo* and *in vitro*.

(i) The cytokeratins.

Intermediate filaments (43-50) of which there are 5 distinct types are found in a wide variety of cells and are composed of 210-400 kDa proteins assembled into complex 8-12 nm cytoskeleton fibres. Keratin intermediate filaments are a multigene family of polypeptides that are characteristic of epithelial cells. These intermediate filaments form a loose three dimensional network concentrated around the nuclei while the peripheral parts radiate towards the plasma membrane. The expression of keratins is highly conserved between different tissue types and species. Specific sets of cytokeratin polypeptides that vary in molecular mass (40-68 kDa) and isoelectric points (5-8.5) are characteristic of normal (51,52) or tumour derived (53-56) cells. Cooper *et al* (49) proposed that the expression of cytokeratins followed well-defined rules that are mainly related to three factors (a) epithelial cell type (simple vs stratified) and among stratified epithelium (b) the differentiation program (functional and terminal differentiation) and (c) state of cellular growth (normal versus hyperproliferative).

Immunohistochemical analysis of keratin expression can distinguish between the luminal and the myoepithelial cells of the duct of the mammary gland. The basal cells express keratins typical of stratified epithelia (K14 and K5) while luminal cells express keratins typical of simple epithelia (K7, K8 and K18) K19 is expressed by both simple and stratified epithelial cells (56-60). Decreased K5 expression is correlated with increased tumorigenic progression (59). Cytokeratins, K13 and K16 are expressed by a large subpopulation of luminal epithelial cells in normal and hyperplastic tissue and benign tumours of the mammary gland (50,55). Mammary carcinomas and derived cell lines usually express cytokeratins associated with the cell population of origin within the mammary gland (54,58,60).

Immunocytochemical analysis of keratins expressed by monolayers of normal human HMEC (60-62) have shown that these cultures are a heterogenous mixture of myoepithelial, luminal epithelial cells and possibly a cell type with both myoepithelial and epithelial cell properties. These authors, have proposed that the expression of both

basal and luminal keratins by HMEC *in vitro* implies that the basal layer may contain a stem cell type which can develop along the luminal lineage.

Furthermore the expression of specific keratins by HMEC *in vitro* is dependent on cell morphology, culture substrate, tissue culture media, growth factors as well as the source of the cells (54,57,60-62).

(ii) The milk fat globule proteins.

The milk fat globule protein (MFGP) is representative of the apical membrane of the mammary epithelial cell. The milk fat globule is synthesised in the endoplasmic reticulum in the basal region of the mammary epithelial cell, it is enveloped in the plasma membrane and secreted into the lumen of the mammary gland (63-67). The unique characteristics of the MFGP membrane makes it the best choice for the procurement of mammary cell surface antigens.

SDS-polyacrylamide gel electrophoresis reveals the presence of some nine major components with relative molecular masses (M_R) of 15-240 kDa. The distribution of a 70 kDa (68,69), a 155 kDa (70,71) and a 400 kDa (72-75) MFGP has been studied in the normal tissue and tumours of the human mammary gland as well as mammary epithelial cells *in vitro* and tumour cell lines.

The 70 kDa MFGP (68,69) is located on the apical surface of normal HMEC and the perimeter or the cytoplasm of tumour cells. While, the 155 kDa MFGP (70,71) is expressed predominantly on the apical membrane of luminal epithelial cells lining the lobules and terminal ducts in the breast. Of the three major classes of mammary carcinomas, malignant cells of lobular carcinomas exhibit more intense staining than those of infiltrating ductal and medullary carcinomas. In poorly differentiated tumour cells at primary and metastatic sites the antibodies were shown to localize components mainly in the cytoplasm. Corcoran *et al* (73) observed that alterations in the cellular

localisation of the MFGP is a common feature of mammary carcinomas and is related to the degree of differentiation.

Taylor-Papadimitriou *et al* (76) produced monoclonal antibodies (HMFG-1 and HMFG-2) to a single determinant, a mucin (sialomucin) molecule with a molecular mass of greater than 400 kDa. This antigen is expressed strongly by the lactating mammary gland and weakly by resting mammary gland and on a number of primary and metastatic carcinomas. Although similar patterns of expression are observed for HMFG-1 and HMFG-2, the HMFG-1 antigen appears to be expressed more strongly on metastatic ductal carcinomas whereas HMFG-2 reacts more strongly with lactating breast (74,76) and tumour cell lines (75-77). Removal of the peripheral oligosaccharides reveals a polypeptide, the mucin core protein with a molecular mass of 68 kDa (74). Monoclonal antibodies, SM-3 raised against the mucin core protein reacts with most carcinomas of the mammary gland and tumour derived cell lines (75,77). No reactivity was observed with benign tumours, normal resting and lactating breast (75). The epitope that is usually masked by oligosaccharides moieties in normal cells may become exposed due to adherent glycosylation in malignant cells. However the immortalised, diploid, cell line, MCF-10 derived from fibrocystic mammary tissue expressed the HMFG-1, HMFG-2 and SM-3 antigens (77).

Calcium and the immortalization of human mammary epithelial cells.

The extracellular calcium concentration is a critical regulator of growth and differentiation of epithelial cells from various tissue and many different species (78-87).

At normal levels of calcium used in tissue culture the lifespan of epithelial cells is limited especially when compared normal fibroblasts *in vitro*. Epithelial cells undergo a process known as terminal differentiation (78,80,82). This process is characterised by a decrease in proliferation, stratification and cornification of the monolayer and detachment of the cells from the tissue culture surface.

McGrath and Soule (34) observed that cortisol and insulin stimulated the exponential growth of HMEC in short-term monolayer cultures. The response of the cells depended on their organisation into growth units on the surface of the dish. Label studies indicated that in the first two days the cells organized themselves into growth units, followed by a period of active linear growth until cessation of growth within each of the growth units, in a process defined as renewal inhibition (34). Growth of these stationary cultures can be restored by disruption and transfer or by reducing the calcium content of the media to less than 0.08 mM (35). A reduction of the *in vitro* calcium concentration not only extends the lifespan of HMEC but also has a dramatic effect on the morphology of these cells (61,80).

The effect of calcium on growth and differentiation was specific and reversible. The mechanisms whereby calcium triggers the process of terminal differentiation (78,80,82) and the cellular mediators that are involved are not known. Ochieng *et al* (83) observed that an increase in the extracelluar calcium concentration precipitated terminal differentiation in HMEC via the inositol trisphospate pathway. An important early effect that has also been identified in HMEC is the effect of calcium on desmosome formation (80,83,86,87).

Utilization of a cell culture medium with a reduced calcium content for the cultivation of HMEC has extended the lifespan of these cells *in vitro* and has led to the establishment of cell lines, MCF-10 and MCF-12 (77,88). MCF-10 and MCF-12 are derived from fibrocystic mammary tissue of a premenopausal and postmenopausal women respectively. Both cell lines express luminal cytokeratins K7, K8, K18 and basal cytokeratin K14 as well as MFGP antigens.

SV40 immortalization of human mammary epithelial cells.

Cellular senescence occurs in normal cell cultures from many species of animal cells and the stringency with which it occurs depends upon the species of origin. Senescence is particularly stringent in human cells and is virtually complete and

irreversible whereas for rodent cells the decline in proliferation can in some instances give rise at low frequencies of immortal cell lines. Immortalization differs from *in vitro* transformation which is a heritable change, that occurs either intrinsically or following exposure to exogenous agents resulting in altered morphology, antigen expression and neoplastic properties (85). Therefor immortalized cells may express only some of the characteristics of the transformed phenotype and a subsequent event/s are required for complete cellular transformation.

The SV40, DNA tumour virus is frequently used to extend the lifespan of normal HMEC in culture (89-97). Primate cells are permissive for SV40 infection by supporting the full expression of the viral genome and release of progeny viral particles whereas mouse cells are nonpermissive for SV40 resulting in immortalization without release of progeny viral particles. Human cells are semipermissive, infected cultures of HMEC experience a crisis period, during which there is a reduction in cell proliferation, detachment of cells from the culture substrate and the formation of multinucleated or giant cells. Sometimes a rare focus of dividing cells appears and an established mammary epithelial cell line which retains many of the characteristics of the cultures of origin is established (89,91). SV40 immortalized mammary epithelial cell lines derived from milk epithelial cultures express luminal epithelial cytokeratins (89,92,95) while luminal and myoepithelial associated cytokeratins are expressed by cultures derived from mammary reduction tissue (90-92,94). Both express epithelial MFGP antigens. These cell lines are not tumorigenic in nude mice (89-91,93). Garcia *et al* (96) observed that immortalization of HMEC with SV40 is associated with a deletion on chromosome 11 which includes the c-Ha-Ras and beta-globulin genes. A tumorigenic cell line has established following the transfection of a SV40 immortalized cell line with the v-Ha-ras oncogene (92).

Chemical transformation and oncogenes in the transformation of mammary epithelial cells.

Stampfer and Bartley (98) were the first describe the establishment of an immortalized

immortalized cell line following the exposure of HMEC to a chemical carcinogen, benz(a)pyrene (B(a)P). A tumorigenic cell line was only established following transfection with the T-antigen and the v-Ha-ras oncogene (98). Russo *et al* (99, 100) investigated the effect of carcinogens dimethylbenz(a)anthracene (DMBA) and N-methyl-N-nitrosurea (NMU) on the HMEC cells derived from mammary tissue with varying degrees of lobular development. Cell cultures derived from less differentiated mammary tissue exhibited increased survival, colony formation efficiency, alterations in karyotype and amplification of the ras proto-oncogene (101). Although these cells do not give rise to continuous cell lines or form tumours in nude mice, these cells are considered phenotypically altered or partially transformed. Exposure of the immortalized HMEC line, MCF-10 (102-104) to DMBA, methyl-N-nitrso-N-nitrosoguanidine (MNNG), NMU, and B(a)P resulted in the establishment of cell lines with an altered morphology, increased growth rate, anchorage independent growth and an increased chemo-invasiveness index. Only one B(a)P subline induced tumour formation in SCID mice. The most frequently observed mutation occurred in these cells was in the first portions of codon 12 and 61 of the c-Ha-Ras gene (105). Transfection of the B(a)P subline with the c-Ha-Ras oncogene enhanced the transformed phenotype indicating that ras gene activation is essential for the maintenance of the transformed phenotype in HMEC (105,106).

Cellular transformation of normal HMEC is a complex multistage process (107-110) when compared to the simple model of two-stage model (111) of carcinogenesis involving initiation and promotion. Initiation by a chemical carcinogen is dependent on carcinogen dosage, metabolism and carcinogen-DNA interactions. This may result in proto-oncogene activation or the inactivation of suppressor genes (107-111). Other factors such as early genetic alterations occurring within the tissue of the donor (29,30,61,88), degree of tissue differentiation (6,10,23,99,100), and/or extended growth *in vitro* may result in the destabilisation of genetic material (77,88). Non-genotoxic effects (112) of growth factors, hormones and ions over an extended period may also contribute to the immortalization of mammary epithelial cells *in vitro*. Further exposure

SV40 infection will result in full expression of transformed phenotype (93-106,113).

Study objective.

The development of rodent model systems (20,22,114) to study the process of breast carcinogenesis has increased our knowledge of mammary gland development, differentiation (23,115,116) and mammary carcinogenesis (6,113). Experimentation within a genetically homogenous rodent population (117-119), under controlled conditions, including age, enviroment, and diet is the foundation to many hypotheses in the eatiology of breast cancer. *In vitro* studies within a normal human mammary epithelial cell, has revealed that human mammary carcinogenesis is a complex multistage process (6,10, 25,31,98,99,101-106). Aspects such as the role of repressor genes, proto-oncogene activation must still be addressed. The development of a primate mammary model can serve as a link between models already established for the rat and the human. This model would reflect the genetical heterogeneity of the human population and similarities in carcinogen metabolism (121). Natural tumour formation in the primate is rare and only occurs in the second decade of life (122). Furthermore, the primate is non-permissive for SV40 viral infection, implying that cellular transformation would be the direct result of carcinogenic insult. The development of cell cultures of normal primate mammary epithelial cells (PMEC) could therefore assist in addressing some of the differences observed in the aetiology of breast cancer in the rat and human.

The main aim of this investigation was to establish primary cultures of normal vervet mammary epithelial cells and to determine whether these cells exhibit characteristics comparable to those described for normal HMEC.

Once the PMEC cultures have been established and the growth conditions optimized, these cells will be used to :

(i) investigate the lobular development and the effect of cellular differentiation on the growth kinetics of PMEC cultures;

(ii) study the modulatory effect of the calcium content of the growth medium on the morphology and the lifespan of PMEC in culture;

(iii) investigate the expression and the phenotype of cytokeratins, characteristic of epithelial cells by PMEC;

(iv) determine the properties of isolated milk fat globule proteins (MFGP) from human milk and lactating primate mammary tissue and compare them to those expressed by HMEC and PMEC in culture;

(v) investigate the toxic effects of the pre-carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA) on the growth of PMEC in culture.

CHAPTER 2:

MAMMARY GLAND DIFFERENTIATION AND CALCIUM DETERMINES THE GROWTH POTENTIAL OF PRIMATE MAMMARY EPITHELIAL CELLS *IN VITRO*.

2.1 INTRODUCTION

Cell cultures of normal mammary epithelial cells that have retained many of the characteristics of the tissue of origin would broaden our comprehension of the processes involved in mammary gland differentiation, development (10,22,23,115,116) and neoplastic transformation (6,10,22,23,123). Normal mammary epithelial cells should reflect the heterogeneity of the normal population and must express biological markers associated with normal mammary tissue. Furthermore, tissue culture conditions which extend the longevity of normal mammary epithelial cells *in vitro* would permit more detailed studies within a single cell population.

Mammary gland differentiation has been identified as an important physiological factor that influences the cultivation of HMEC. Mammary tissue can be classified into three major groups: less differentiated, composed of predominantly lobule type 1, moderately differentiated, a mixture of lobule type 1 and 2 and lobule type 2 and most differentiated lobule type 3 (10,23,124-126). Characteristic of cultures of HMEC derived from less and moderately differentiated tissue are rapid organoid attachment, high population doublings, increased DNA-labelling index and many cells in the S-phase of the cell cycle (124-126).

The mitogenic effect of cholera toxin (46), epidermal growth factor (EGF) (127), insulin and hydrocortisone (34) promotes epithelial cell growth while pituitary gland extract (31,44) and a decrease in calcium content (34,35,61,88,123-126) of the medium increases the longevity of HMEC in *vitro*. HMEC maintained in a medium with a reduced calcium content have retained ultrastructural properties and possess growth properties *in vitro* associated with the normal phenotype of HMEC (23,61,77). *In vitro* these cells

express the same cytokeratins and milk fat globule membrane antigens as *in vivo* (23,61,77).

The purpose of this investigation is to establish the PMEC *in vitro* and to determine whether these cells exhibit some of the characteristics of the normal HMEC *in vitro*. The effect of primate mammary gland lobular development on the growth kinetics of PMEC in culture will be determined. Furthermore the modulatory effect of the calcium content of the tissue culture medium on cell growth, morphology and lifespan in PMEC *in vitro* will be established.

2.2 MATERIALS

All media, custom made DMEM:Hams F12 (1:1)(DMEM:F12) with a reduced calcium content (DMEM:F12(RC)) and antibiotics were supplied by Highveld Biologicals (Sandton, South Africa). Enzymes, growth factors, hormones were tissue culture grade and were obtained from the Sigma Chemical Company (St. Louis, USA). Heat inactivated horse serum was from ICN Biomedicals (Thame, England). The ion exchanger, Chelex 100 was purchased from Bio Rad Laboratories (Richmond, USA). Disposable tissue culture ware was from Sterilin (Stone, England).

2.3 METHODS

Animals and tissue

Vervet monkey (*Cercopithecus pygerythrus*) female mammary glands were obtained from the H.A. Grové Research Centre. The mammary tissue was aseptically removed from the skin, dissected into small pieces and washed in RPMI medium containing a mixture of 100 µg/ml Penicillin G, 100 µg/ml Streptomycin and 250 ng/ml Fungizone for 60 minutes at room temperature on a rotary mixer.

The isolation of organoids.

The tissue was collected by centrifugation at 350 *xg* digested in 10 volumes DMEM:F12 containing 200 IU/ml collagenase (type 1A) and 100 IU/ml hyaluronidase at 37^0 C on a rotary mixer.

After 6 hours the tissue was collected by centrifugation and resuspended in fresh enzyme solution. Digestion was complete once the lobular structure of the mammary tree was recognizable by light microscopy. These tissue fragments known as organoids were repeatedly (5 times) allowed to sediment at room temperature before being resuspended in supplemented DMEM:F12 medium.

Primary cultures of primate mammary epithelial cells.

The organoids were plated in DMEM:F12 medium supplemented with 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 5% heat inactivated horse serum and antibiotics (100 µg/ml Penicillin G, 100 µg/ml Streptomycin and 250 ng/ml Fungizone) (33,34). All media, supplemented media and enzyme solutions were sterilized by filtration using 0.2 µm filters (Satorius, Zeiss, Randburg). All cultures unless specified were maintained in an humidified environment with 5% CO_2/air at 37^0C.

Lobular structure, organoid attachment and growth.

The number of organoids plated and the lobular development of each organoid sample was determined by light microscopic examination of each culture. The lobular structure of each organoid sample was classified according to the method described for human mammary glands (22,116,124-126).

Attachment was defined as the susceptibility of the organoids to adhere to the surface of T25 flasks when plated in supplemented DMEM:F12. Percentage attachment is

defined as the number of organoids attached divided by the total number of organoids plated expressed as a percentage. Organoid growth, the beginning of spreading of epithelial cells from the organoids that attached was also evaluated every 24 hours for 10 days and expressed as the percentage of the organoids that attached.

Primate mammary epithelial cells in tissue culture medium with a reduced calcium content.

The tissue culture medium of primary cultures of PMEC was changed to supplemented DMEM:F12(RC) when no increase in organoid growth was observed. DMEM:F12(RC) was supplemented with insulin, hydrocortisone, EGF, cholera toxin and calcium free heat inactivated horse serum at the same concentrations as for PMEC in primary culture. Divalent ions were removed from the heat inactivated horse serum by using an ion exchanger, Chelex 100 (128). The calcium content of the medium was determined by flame ionization spectroscopy (129) and adjusted to 0.04 mM with a 4 mM stock $CaCl_2$ solution. The cells were passaged by trypsinization (130) or the direct replating of new cells that were shed into the medium (35,88). The number of cells were counted using a haemocytometer and the viability of the cells were determined by the trypan blue exclusion assay (130).

Cell number determinations with the Crystal Violet assay.

In all experiments where the number of adherent cells were too few to determine by the usual methods of cell enumeration, the number of cells were determined by the Crystal Violet assay (131). PMEC growing in cell culture 24 well dishes (1.9 cm^2) were fixed by adding 20 µl of a 11% glutaraldehyde solution to 200 µl medium in each well. The microwell plate was shaken at room temperature for 15 minutes. Each well was rinsed three times with H_2O and airdried. All wells representing different time intervals were fixed individually and stained later as a batch. A 200 µl volume of the staining solution; 200 mM formic acid buffer pH 3.5 containing 0.1% Crystal Violet was added to each well. The Multidishes were shaken for 20 minutes at room temperature. The excess dye

was removed by extensive washing with H_2O and the dishes were airdried before the solubilization of the bound dye in 200 µl 10% acetic acid. The optical density of the dye was measured spectrometrically 590 nm. A standard curve was prepared plotting the number of cells in each well following trypsinization against the absorbance at 590 nm of the same number of cells determined by the Crystal Violet assay. The standard curve represents the average of 4 experiments using different cultures and each point was assayed in triplicate.

Primate mammary epithelial cell growth curves.

PMEC were collected from the supernatant of primary cultures 2(1), 3(1), 4(1), 8(1) and 10(1). These cells were plated in conditioned, supplemented DMEM:F12(RC) at a cell density of 2×10^4 cells/well (1.06×10^3 cells/cm^2) and maintained in the same medium and conditions described, previously. The number of cells present at each time interval was determined using the Crystal Violet staining method. Each point represents, the average of 8 wells. Doubling times of PMEC cells in culture was determined from the gradient of the growth curve of each sample in tissue culture.

The total yield of free floating cells was determined for cultures 2(1), 3(1), and 4(1) by collecting the free floaters from the tissue culture medium by centrifugation at 300 *xg*. The number of cells in primary culture that gave rise to the free floaters was determined following trypsinization of the adherent cells on completion of each experiment.

Light microscopy.

Lobular structure of the organoids and the growth of PMEC in supplemented DMEM:F12 and DMEM.F12(RC) was monitored using a Leitz Labovert microscope and photographs were taken using a Contax camera coupled to a Zeiss IM inverted microscope.

Scanning electron microscopy.

PMEC from 2(1) were plated in culture dishes at a cell density of 7 x 10^3 cells/cm^2 in supplemented DMEM:F12(RC). After 48 hours in culture the dishes were split into two groups and maintained for a further 48 hours in the following medium supplemented (i) DMEM:F12 and (ii) DMEM:F12(RC). The medium was removed and the monolayer was washed with a phosphate buffered saline (PBS) solution. The monolayer was fixed with 2.5% glutaraldehyde in a 0.1 M phosphate buffer pH 7.4 for 90 minutes and postfixed in a 0.5% aqueous osmium tetroxide solution for 60 minutes. The dishes were inverted and floated on water and discs of plastic were removed using a heated metal bore. The samples were dried by the CO_2 critical point procedure and sputtercoated with gold.

Transmission electron microscopy.

Free floating PMEC from primary cultures were collected by centrifugation at 350 *x g* and washed thrice in PBS. The pellet was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 90 minutes, postfixed in a 0.5% aqueous osmium tetroxide solution for 60 minutes. The samples were dehydrated in a graded series of ethanol and embedded in quetol. Sections were contrasted with uranyl acetate and lead citrate.

2.4 RESULTS

Assessment of mammary gland development, organoid attachment and growth.

The body mass of the primates used is this study varied from 2.1-4.2 kg. Although no record was kept by the animal facility an indication of the approximate age of each animal could be inferred from its physical appearance and mass.

Selective collagenase and hyaluronidase digestion of primate mammary tissue resulted in the isolation of organoids, which are aggregates of epithelial and myoepithelial cells which have retained much of the lobular structure of the mammary gland of origin.

Three types of lobules were identified: (i) lobule type 1 (Lob 1), the least differentiated structure is composed of a few alveolar buds clustered around a terminal duct (Fig. 2.1a), (ii) an intermediate lobule type 2 (Lob 2) (Fig. 2.1b) and lobular type 3 (Lob 3) (Fig. 2.1c), the most differentiated lobule type.

It was observed that lower lobular development such as Lob 1 is associated with very young or very old animals, a mixture of Lob 1 and 2 and Lob 2 with young mature animals while Lob 3 was usually present only in old animals.

Organoids isolated and established in culture were either only one lobule type, Lob 1 - Lob 3 or a mixture of two types of lobules, Lob 1 and 2.

ganoid attachment and growth in supplemented DMEM:Hams F12.

The attachment and the growth responsiveness of primate mammary organoids originating from 18 animals with different degrees of lobular development was investigated. Three groups were identified, less differentiated with Lob 1, moderately differentiated with Lob 1 and 2 and Lob 2, and well differentiated Lob 3. For all groups organoid attachment was observed within 24 hours and reaching a maximum by day 4 (Fig. 2.2.1-4)(Table 1). Of the total number of organoids seeded the percentage of organoids that had attached by the third day *in vitro* was 80% for Lob 1, 70% for Lob 1 and 2, 55% for Lob 2 and 50% for Lob 3.

Light microscopic examination of PMEC in primary culture revealed that the first outgrowths from the organoids were observed in cultures with Lob 1 from Day 2, Lob 1 and 2 from Day 3, Lob 2 from Day 4 and only from Day 5 for Lob 3 (Fig. 2.2.1-4)(Table 1). The number of organoids that gave rise to outgrowths of viable epithelial cells (Fig. 2.3) divided by the total number of attached organoids expressed as a percentage measured the growth responsiveness of the organoids originating from tissue with different types of lobule development. After 5 days *in vitro*, growth in cultures with Lob 1 development had reached a maximum of 90% (Fig. 2.2.1), while for Lob 1

and 2 (Fig. 2.2.2), 50%, Lob 2 (Fig. 2.2.3), 42% and Lob 3 only 5% growth was observed (Fig. 2.2.4).

The PMEC grew as a flat monolayer of cuboidal cells from the organoids in definite closed colonies of cells (Fig. 2.3). If retained in this medium, PMEC undergo renewal inhibition (34)(Fig. 2.4.a) and eventual cellular senescence.

Once the growth curve of each organoid sample in culture reached a plateau the calcium content of the medium was reduced to 0.04 mM. In subconfluent cultures the PMEC continued to grow as a monolayer of typically cuboidal cells with decreased cell-cell contact.

At confluency (Fig. 2.4.b) no contact inhibition occurred but rather new cells known as the free floating cells (94 $\pm$ 4% viable) are shed into the medium (Fig. 2.4.b). These cells could be collected by centrifugation and be replated to give rise to further cultures of PMEC.

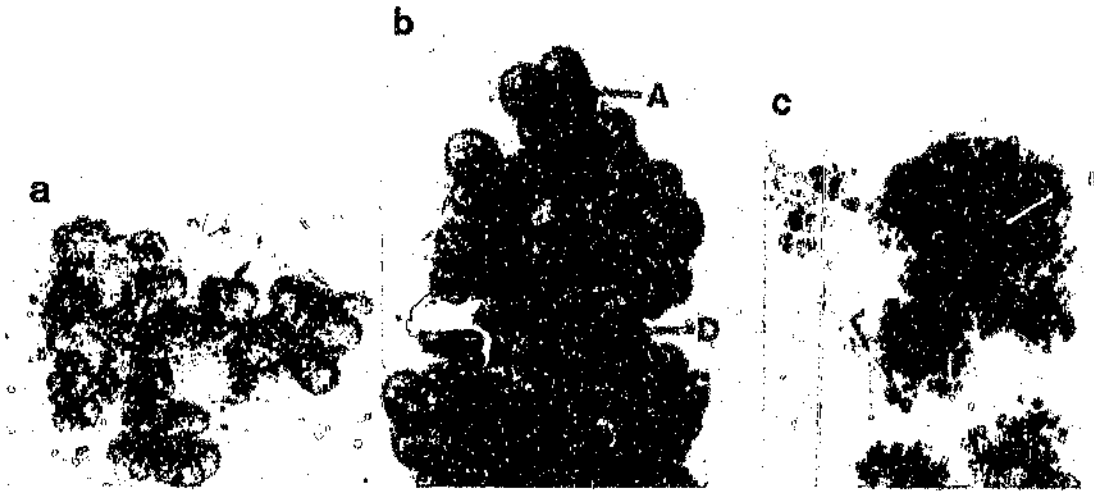


Figure. 2.1. The lobular development of the primate mammary gland. Organoids isolated from the mammary gland of *Cercopithecus pygerythrus* retains much of the lobular structure of the mammary gland of origin. a) Lob 1 (Magnification x200), b) Lob 2 (Magnification x200) c) Lob 3 development (Magnification x100). Alveolar buds (A) and ducts (D).

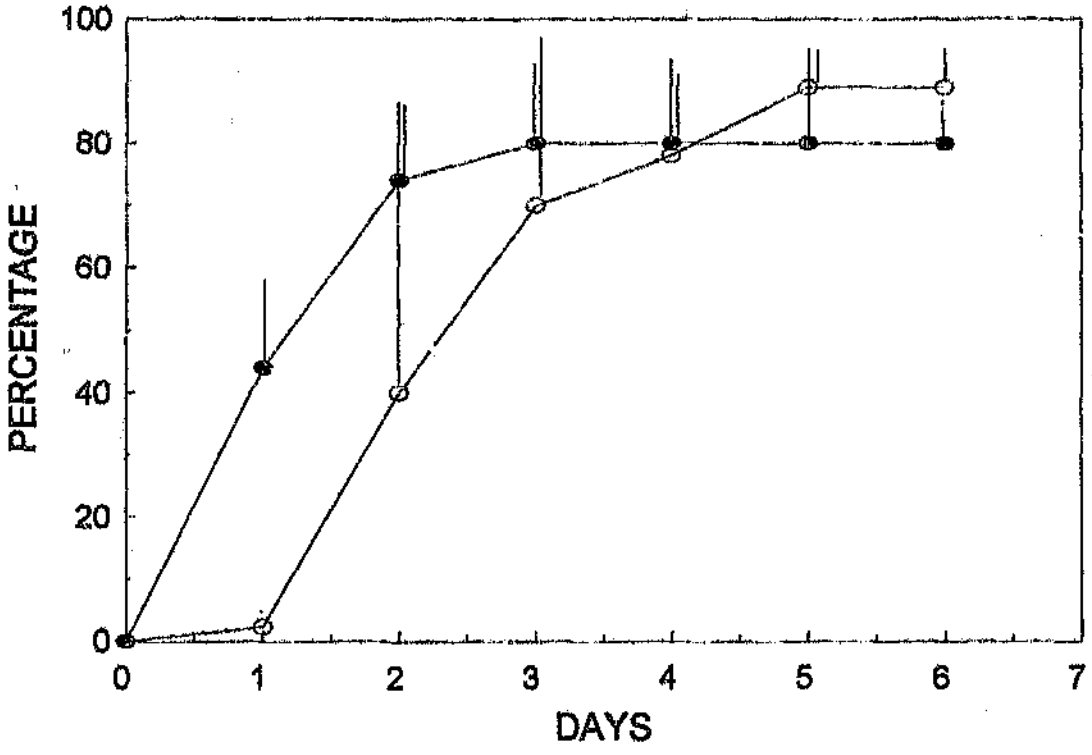


Figure 2.2.1. Percentage organoid attachment (–●–) and growth (–o–). Organoids isolated from primates with Lob 1 development were plated and cultured in supplemented DMEM:F12 (n=3). Bars indicate SEM.

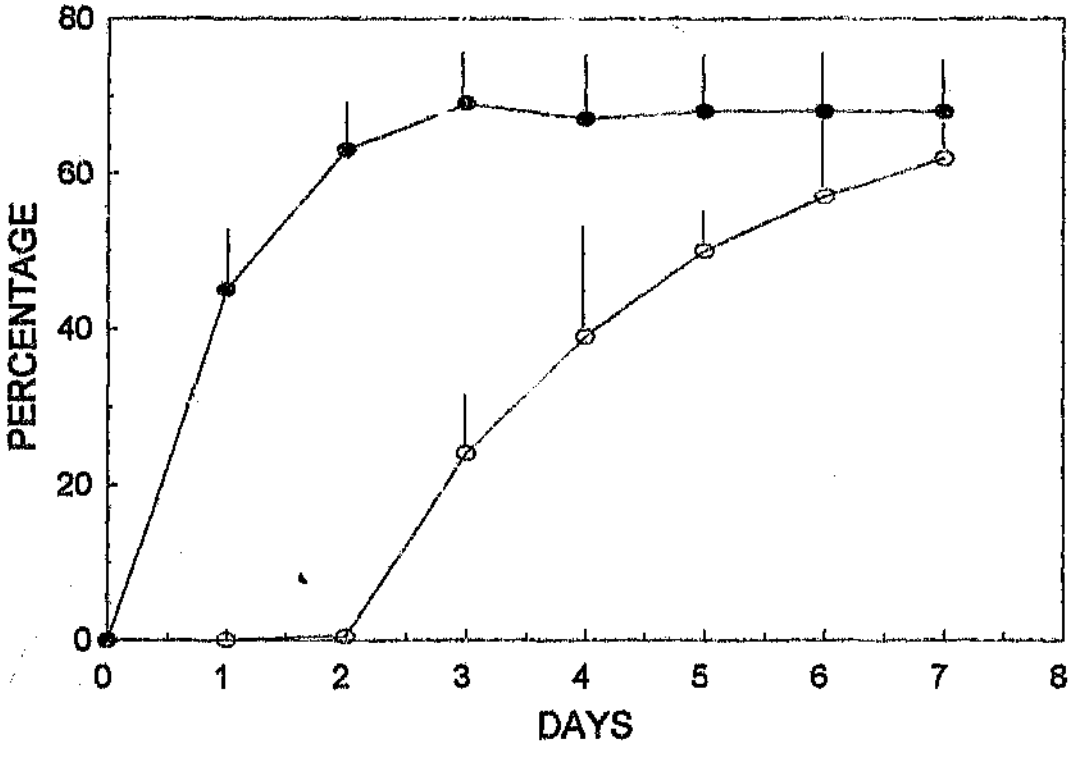


Figure 2.2.2. Percentage organoid attachment (--●--) and growth (--o--). Organoids isolated from primates with Lob 1 and 2 development were plated and cultured in supplemented DMEM:F12 (n=5) Bars indicate SEM.

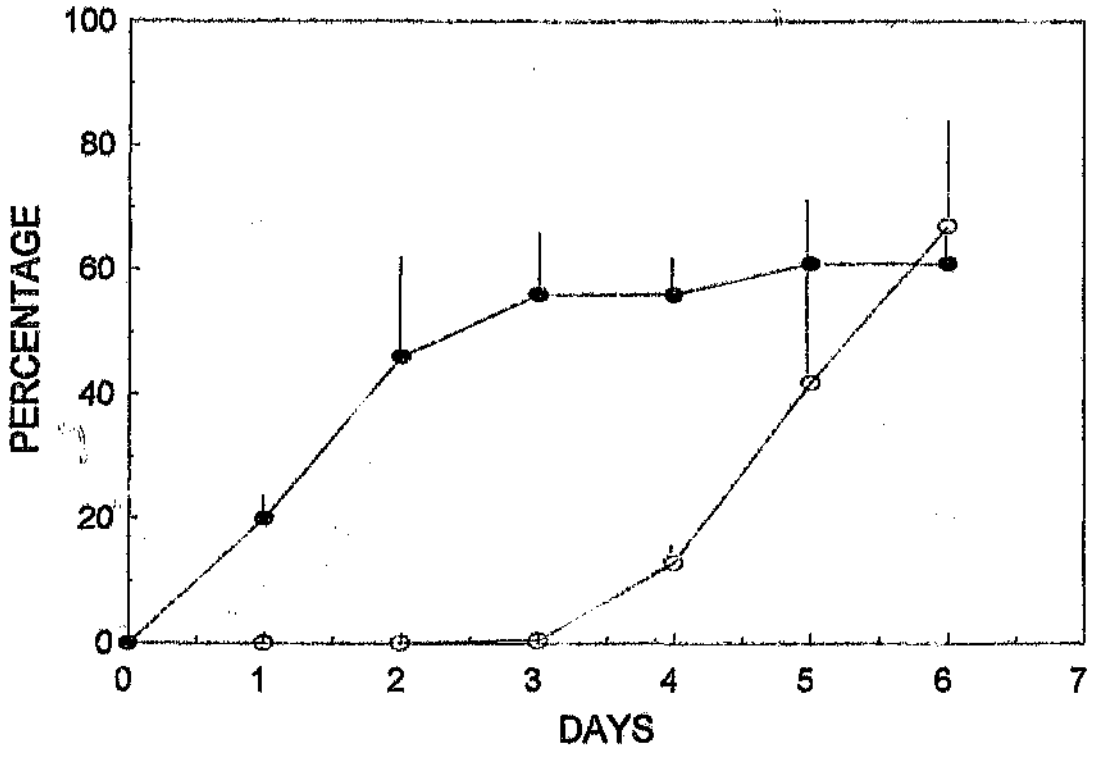


Figure 2.2.3. Percentage organoid attachment (--●--) and growth (--o--). Organoids isolated from primates with Lob 2 development were plated and cultured in supplemented DMEM:F12 (n=7), Bars indicate SEM.

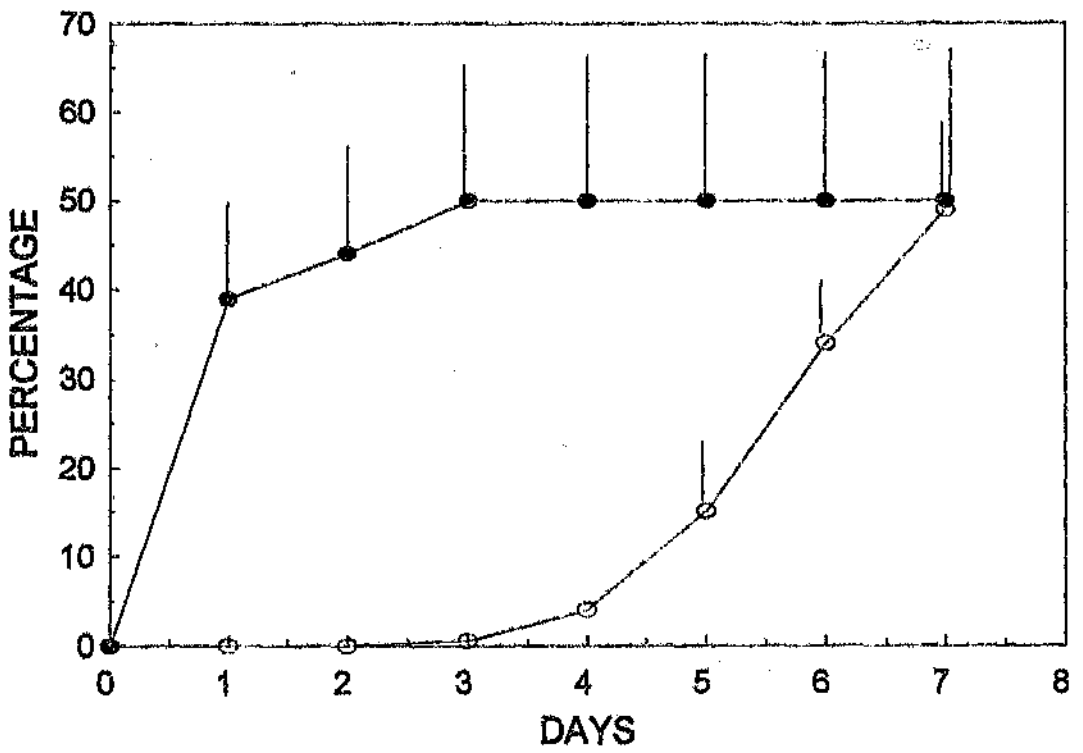


Figure 2.2.4. Percentage organoid attachment (–●–) and growth (–○–). Organoids isolated from primates with Lob 3 development were plated and cultured in supplemented DMEM:F12 (n=3) Bars indicate SEM.

TABLE 1: RELATIONSHIP BETWEEN LOBULE DEVELOPMENT, ORGANOID ATTACHMENT AND GROWTH.

DIFFERENTIATION	LOBULE TYPE	n	ATTACHMENT		GROWTH	
			Days[a]	Percent[b]	Days[c]	Percent[d]
Less	1	3	3	80	≤2	90
Moderate	1 and 2	5	3	70	3	50
	2	7	3	55	4	42
Well	3	3	3	50	5	5

n = number of cultures established.
a = number of days *in vitro* before maximal organoid attachment is observed.
b = % attachment where no further increase in attachment is observed.
c = number of days *in vitro* before epithelial cell growth is observed.
d = % growth from organoids observed on day 5.

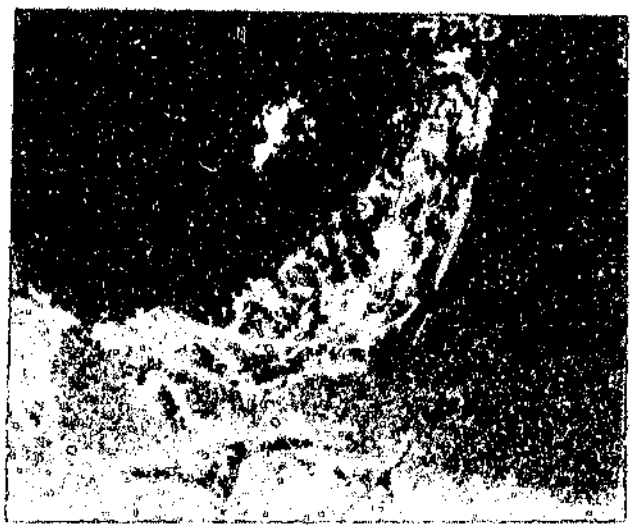


Figure 2.3. Outgrowths of PMEC from attached organoids plated in supplemented DMEM:F12 (Magnification x100).

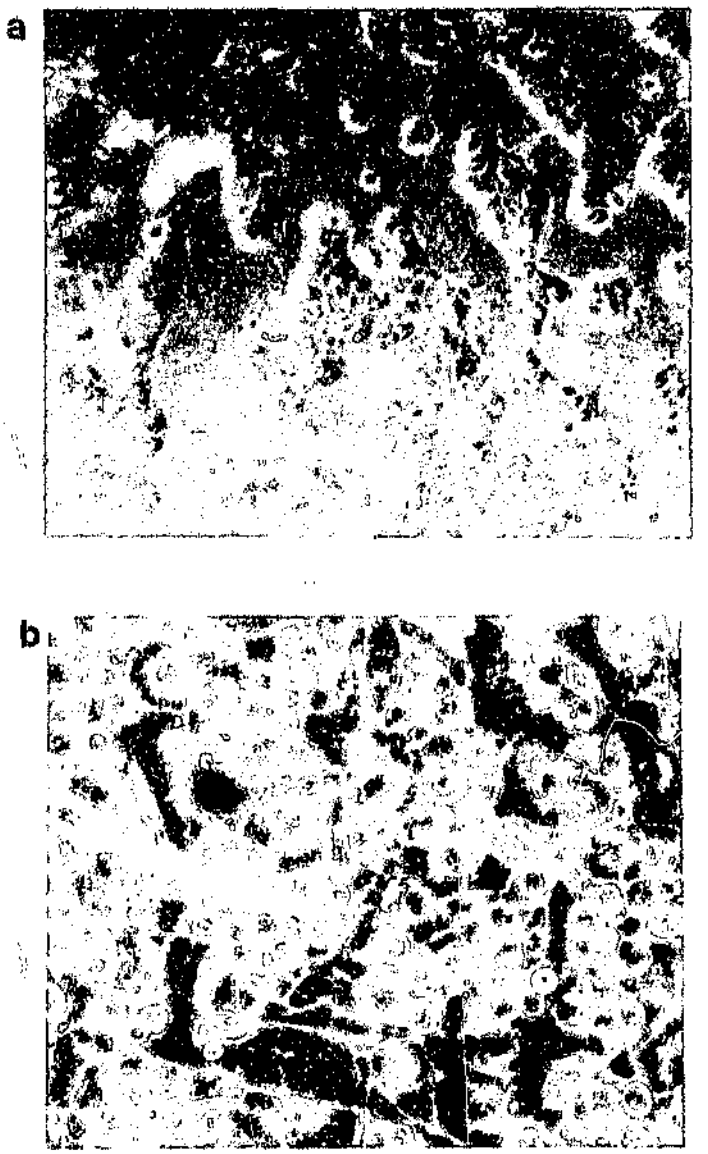


Figure 2.4. Effect of calcium on the morphology of PMEC *in vitro*. PMEC were cultures in supplemented a) DMEM:F12 (Magnification x100) and b) DMEM:F12(RC) (Magnification x200), ↑ showing free floaters.

Doubling times and growth curves.

The doubling times of PMEC in tissue culture was determined using the crystal violet staining procedure. A standard curve (Fig. 2.5) was prepared by measuring the enhancement of staining as measured at 595 nm of a known number of PMEC grown in supplemented DMEM:F12(RC). A typical growth curve for PMEC is depicted in Fig. 2.6. for culture 8(1). The doubling times of 5 cultures of PMEC grown in DMEM:F12 (RC) were 41 hrs for 2(1), 64 hrs for 3(1), 87 hrs for 4(1), 19.5 hrs for 8(1) and 65.5 hrs for 10(1).

A biphasic curve is observed for the total number of free floating cells that are harvested from primary cultures 2(1), 3(1) and 2(1) of PMEC (Fig. 2.7) in supplemented DMEM:F12(RC). Initially, over a period of 9 days there is a linear increase in the total yield of free floating cells harvested from the tissue culture medium. A decrease in the number of free floaters that are shed in to the tissue culture medium is sometimes associated with a new type of cell in culture. This cell is large, slower growing, multinucleated and does not divide (Fig. 2.8). Within a couple of days most of the PMEC in culture undergo this change, the tissue culture medium becomes acidic, the cells detach and the culture deteriorates rapidly

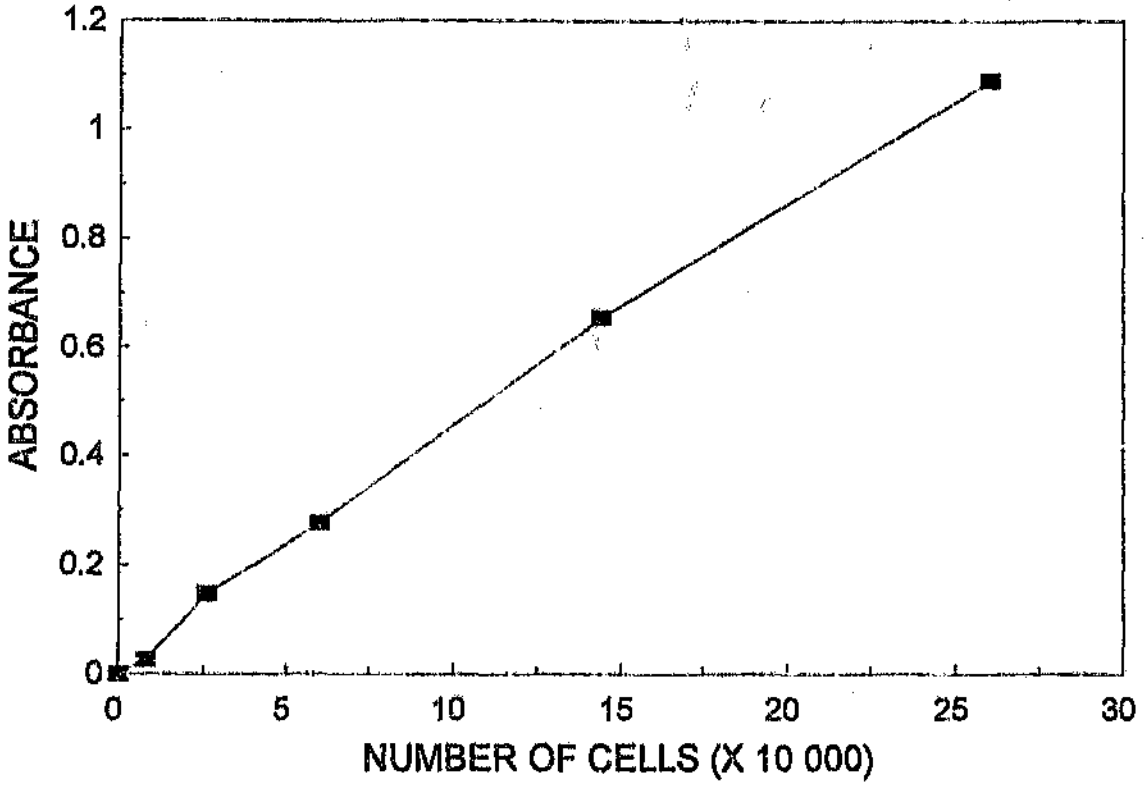


Figure 2.5. Standard curve for the determination of the number of cells in tissue culture. A known number of PMEC were plated and retained supplemented DMEM:F12(RC) for 48 hours. The number of cells were counted following trypsinisation and the increase in absorbance due to Crystal Violet uptake was measured at 590 nm in wells containing the same number of cells.

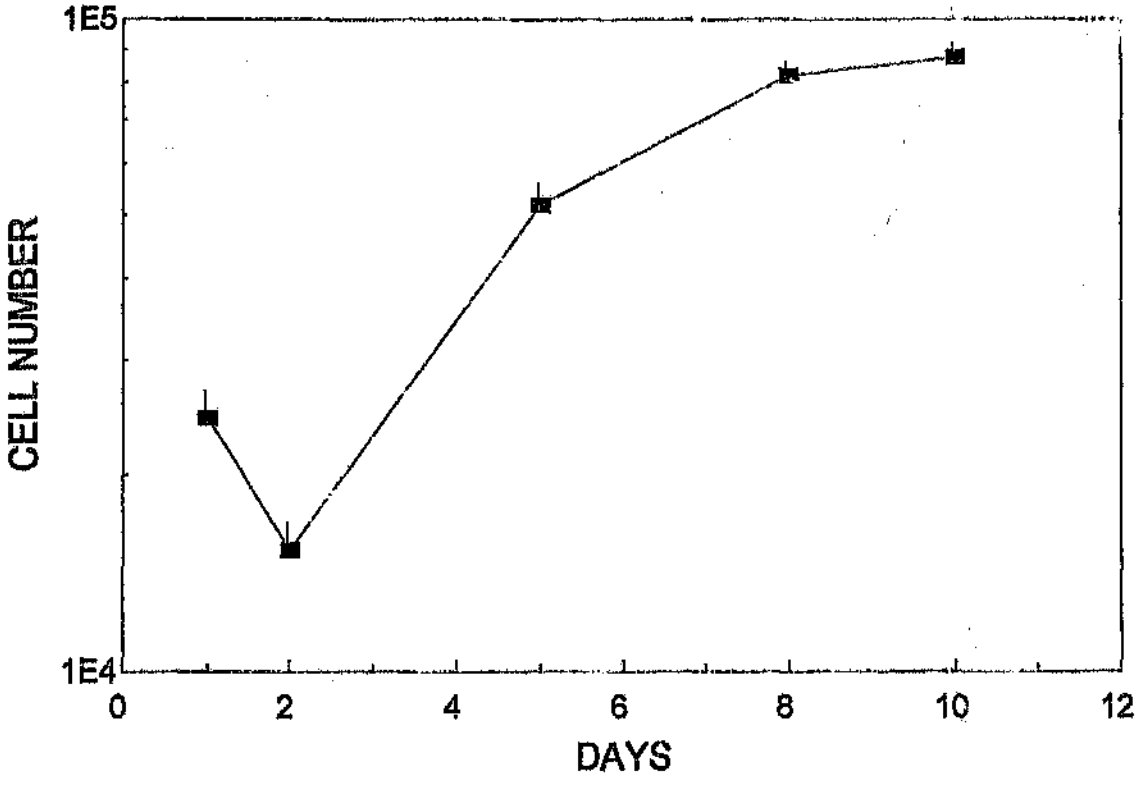


Figure 2.6. A typical PMEC growth curve. PMEC (1) were plated at density of 1.06 x 10^3 cells/cm^2 in supplemented DMEM:F12(RC). The number of cells was determined by Crystal Violet staining. Bars indicate SEM.

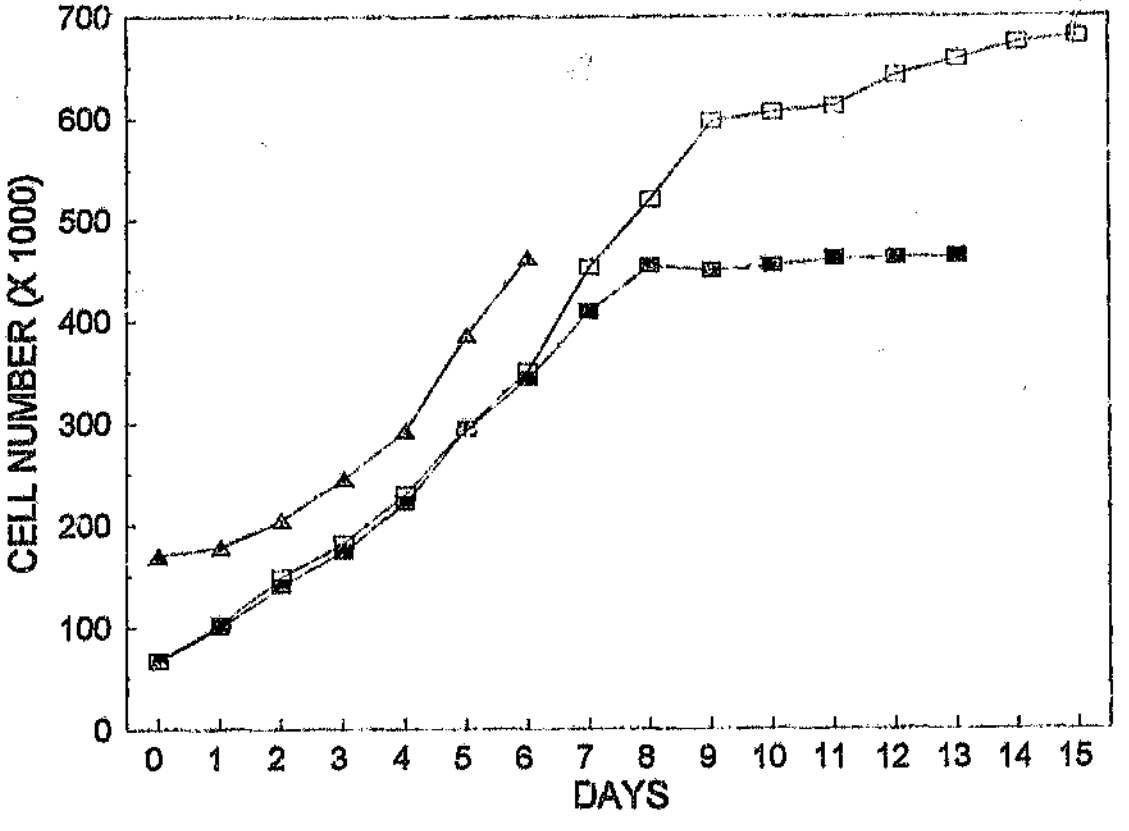


Figure 2.7. The total yield of free floating cells harvested from cultures 2(1)(–□–), 3(1)(–▲–), and 4(1)(–■–) of PMEC. The free floating cells in the tissue culture medium were collected by centrifugation. The adherent cells giving rise to the free floaters were determined following trypsinisation of monolayers on completetion of each



Figure 2.8. A decrease in the number of free floaters that are shed into the medium is sometimes associated with the occurrence of large, non-dividing multinucleated cells (Magnification x200)



Figure 2.9.a and b Scanning electron micrograph of PMEC cultured in suppiemented DMEM:F12(RC) showing decreased cell-cell contact, almost no desmosomes and

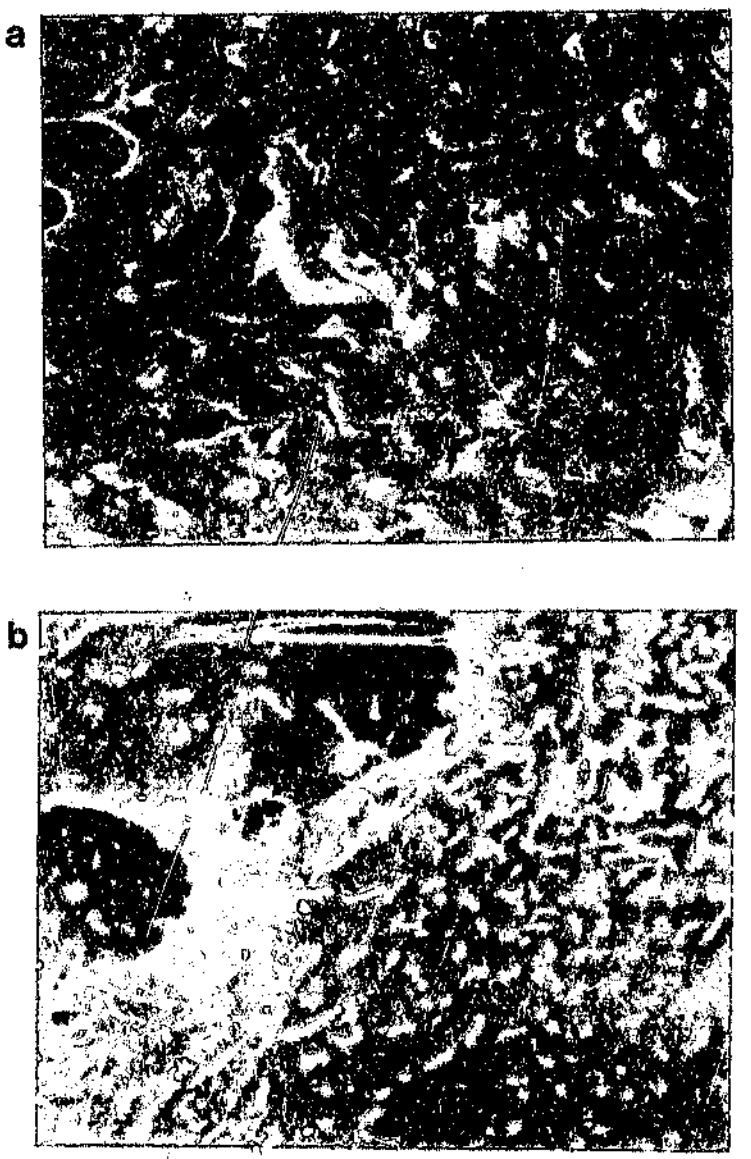


Figure 2.10a and b. Scanning electron micrograph of PMEC cultured in supplemented DMEM:F12 showing increased cell-cell contact, few microvilli and desmosomes (Bars = 2µm)

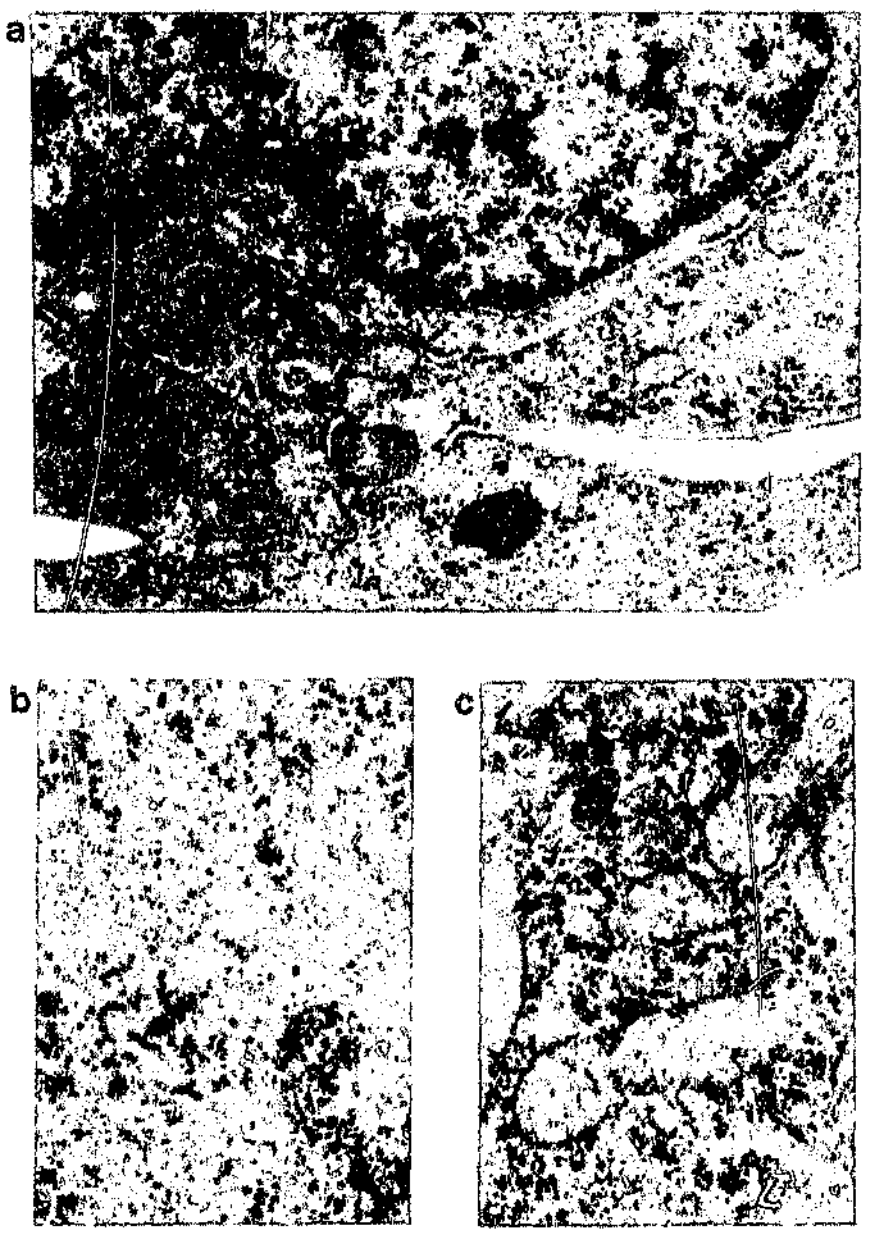


Figure 2.11. Transmission electron micrograph of PMEC cultured in DMEM:F12(RC) (Magnification x6450), (a) showing distended endoplasmic reticulum,(b)

Ultrastructure of primate mammary epithelial cells in cell culture.

Ultrastructurally PMEC maintained in supplemented medium with a reduced calcium content appeared rounded with very little cell-cell contact, while broad lamellae and many microvilli were observed on the outer membrane of the cell (Fig. 2.9a and 2.9b). By increasing the calcium content of the tissue culture medium to 1.05 mM, the morphology of the cells changed dramatically. The cells flattened, cell-cell contact increased and desmosomes formed and the number of microvilli on the outer membrane decreased (Fig. 2.10a and 2.10b). Transmission electron microscopy revealed that the PMEC maintained in a tissue culture medium with a reduced calcium content (Fig 2.11a) had a regular nucleus, a prominent nucleolus, a large number of polisomes and mitochondria (Fig. 2.11c) and a granular and distended endoplasmic reticulum indicating a high level of metabolic activity. Bundles of fine cytoplasmic filaments or intermediate filaments appeared within the cytoplasm (Fig. 2.11b), perinuclearly.

2.5 DISCUSSION

McPherson and Montagna (132) observed that the mammary gland of non-human primates and the human breast differed mainly in their gross structure while the glandular components are essentially identical. A system (10,22,23,126) that is used to classify the lobular structure of the mammary gland of the rat and the human can also be used for the classification of the lobular development of the nonhuman primate *Cercopithecus pygerythrus*. Collagenase and hyaluronidase digestion of Vervet mammary tissue produced organoids which contained epithelial, myoepithelial and stromal elements, whole retaining much of the lobular structure of the mammary tissue of origin (31). Lobules types, Lob 1-3 were identified with Lob 1 usually associated with younger animals and Lob 3 with old animals.

important determinant of lobular development. However, there were no records kept by the H.A. Grové Research Centre of each animal's age and reproductive history. It was not possible to determine whether there was any relationship between age, reproductive history, lobular development and growth in tissue culture as has been described for human mammary tissue (124-126).

The degree of organoid attachment was determined for cultures derived from primate mammary tissue with different degrees of lobular development. Attachment of primate mammary organoids was more rapid than has been reported for human mammary organoids. The total percentage of organoids that attached to the tissue culture substratum, was the highest for organoids derived from mammary tissue with less and moderately differentiated lobule types. Organoids derived from well differentiated human mammary tissue, Lob 3 exhibited delayed and decreased organoid attachment (126).

Russo *et al* (126) observed that human mammary organoids in culture reflect certain properties of the intact tissue, where less differentiated lobular structures with a greater proliferative rate attached and grew more efficiently than the more differentiated and less proliferating lobules. The rate of primate organoid growth *in vitro* reflected to a greater degree the differences in growth responsiveness of organoids derived from mammary tissue with different degrees of lobular development. Outgrowths of epithelial cells were observed immediately following attachment of organoids from less differentiated, Lob 1, while growth from organoids derived from moderate and well differentiated mammary tissue was lower. Furthermore, Russo *et al* (126) observed that the highest yield of HMEC was obtained from primary cultures of organoids originating from less differentiated lobule types.

As primate tissue with Lob 1, Lob 1 and 2 and Lob 2 development was the most responsive *in vitro* and gave rise to cultures of PMEC with high yields of free floating

A decrease in *in vitro* calcium, has been observed to alter cell morphology and extend the lifespan of mouse epidermal cells (78), rat esophageal cells (133) and HMEC (34,35,61). The lifespan of PMEC is limited when the medium contains normal (1.05 mM) Ca^{2+} concentrations, although normal levels of calcium do promote the attachment of organoids. By decreasing the calcium content of the tissue culture medium it is possible to extend the lifespan of mammary epithelial cells in tissue culture. Calcium has a dramatic effect and reversible effect on the morphology of PMEC *in vitro*. At a calcium concentration of 1.05 mM, PMEC *in vitro*, are flattened, with extensive contact between individual cells, desmosomes and only a few microvilli are present. In contrast, with a reduction in the calcium content to 0.04 mM, the cells become rounded, with little or no contact between cells, broad lamellae and abundant microvilli are present. New cells are shed into the tissue culture medium. These free floating cells can be replated and were used in subsequent experiments. The doubling times for cultures of PMEC derived from less and moderately differentiated mammary tissue differed considerably. A similar variation in the number of population doublings within cultures derived from human mammary tissue with moderate development has been described (126). Other unknown heritable properties may also influence mammary epithelial growth *in vitro*.

PMEC have a finite lifespan in tissue culture, where cellular senescence is associated with a decrease in the number of free floating cells and the appearance of large flat cells which do not undergo any cell divisions. Hammond *et al* (44) described that in cultures of normal HMEC that these slowly growing larger cells are diluted by the more rapid multiplication of the smaller cells thus reestablishing a morphologically uniform population by later passages. It was also observed that it was essential to include cAMP increasing agents that maintains an actively growing population during this "selective period". In cultures of PMEC such selection was not observed to occur but rather that these larger cells soon dominated the cultures, the medium became acidic as these cells detached and the cultures deteriorated rapidly.

population, with an extended lifespan *in vitro*, which reflects mammary gland differentiation *in vivo* and retains many of the characteristics of the mammary epithelial cell *in vivo* (26).

The attachment and growth of organoids in *vitro* reflects the growth potential of the tissue of origin. PMEC, *in vitro* are morphologically typically epithelial, and respond to a decrease in extracellular calcium similarly to other epithelial cell types. The establishment of cultures of PMEC with an extended lifespan, facilitates studies within a single cell population that retains the grow ı properties of the tissue of orig ı. PMEC were further characterised, by immunodetection of keratin and milk fat globule proteins. These results are presented in Chapters 3 and 4.

CHAPTER 3:

PRIMATE MAMMARY EPITHELIAL CELLS *IN VITRO* EXPRESS KERATINS ASSOCIATED WITH THE LUMINAL PHENOTYPE.

3.1 INTRODUCTION

The cytokeratins are a family of water insoluble proteins of 40-70 kDa, that form intermediate filaments that are present in almost all vertebrate epithelial cells (48-59,134). The cytokeratins are invaluable in defining cell phenotype due to their specificity, abundance and their high degree of antigenicity. Analysis of cytokeratin patterns of epithelial cells i.e. expression of a particular keratin or lack of it can be used to characterise a specific cell type. Its specific phenotype, degree of differentiation and proliferative capability *in vivo* and *in vitro* can be determined (48,56,58,60,134).

In the normal resting mammary gland the epithelial cell population can be topographically divided in two groups, the luminal (54,62) and basal epithelium (25) found between the luminal cells and the basement membrane. Immunocytochemically it is possible to distinguish between the luminal and basal epithelium by the cytokeratins that are expressed (54,56,62,135). Myoepithelial cells express keratins K5, K14, K15 and K17, lumenal epithelial cells express keratins K7, K8, K18 and K19, while an intermediate "stem" cell type expresses both types of keratins (60,66). Cellular transformation leads the decreased regulation in the expression of some of these keratins, however those that are expressed are still representative of the tissue of origin (52,53,135). Expression of keratins proteins by HMEC *in vitro* is highly conserved and is representative of the human mammary gland (54,58,60-62). Keratin expression is modulated by the origin of the cells (54,60), cell morphology (60), culture substrate (60), tissue culture media (54,61) and growth factors (54,58).

whether keratins found in primate mammary tissue are retained *in vitro* and are associated with the luminal and/or myoepithelial phenotype.

3.2 MATERIALS

All reagents used for electrophoretic procedures were analytical grade and were purchased from either Merck (Darmstadt, Germany) or BDH Laboratory (Poole, England). Immobilin PVDF was a Millipore product (Bedford, USA). The primary antibody, anti-Cytokeratin 8.13 (mouse IgG 2a) and antimouse IgG (whole molecule) peroxidase conjugate was purchased from the Sigma Chemical Company (St. Louis, USA).

3.3 METHODS

The cultivation of MCF-7 cells.

The tumour cell line, MCF-7 cell line was obtained from Highveld Biologicals (Kelvin). The cells were maintained in DMEM containing 10 % fetal calf serum at 37^{0}C (52,136).

Isolation of the cytokeratins from organoids, MCF-7 and PMEC.

MCF-7 cells and PMEC cultured either in medium with a reduced calcium content were collected by trypsinisation. Organoids and the cell pellets were washed with PBS. The pellet was suspended in 5 mM piperazine-N,N'-bis(2-ethane sulphonic acid)(PIPES) pH 6,1 containing 0.1% Triton X100, 2 mM ethyleneglycol-bis-(ß-amino-ethylether)N,N'-tetra acetic acid (EGTA) and 2 mM $MgCl_2$ for 2 hours. The insoluble material was collected by centrifugation at 6 000 *xg* and was resuspended in two volumes of 0.025 M imidazole pH 9.2 buffer containing 8-9.5 M urea and 5% mercaptoetnanol (136,137). The samples were mixed for 12 hours at room temperature.

SDS polyacrylamide electrophoresis of the cytokeratin proteins.

SDS polyacrylamide electrophoresis (SDS-PAGE) was carried out using a Hoeffer Mighty Small Slab Gel Electrophoresis unit (SE 250). Protein samples were separated on a gel with 10%T and 2.5%C in 1.5 M Tris/HCl, 0.1% SDS pH 8.8 and a stacking gel with 4%T and 2.7%C in a 0.5 M Tris/HCl, 0.1% SDS, pH 6.8.

Samples were prepared with a 1:1 dilution with 2 x treatment buffer, 0.125 M Tris/HCl pH 6.8 containing 4% SDS, 20% glycerol and 2% 2-mercaptoethanol. Molecular mass standards were treated similarly. Two additional proteins namely pyruvate kinase (M_R 58 kDa) and L-glutamate dehydrogenase (M_R 53 kDa) were included for the more accurate molecular mass determination.

The protein samples were separated at 20 mA per gel, with a 0.025 M Tris pH 8.3 tank buffer containing 0.192 M glycine and 0.1% SDS at 10^0C.

The gels were stained with a 0.125% Coomassie Blue R-250 solution prepared in 50% methanol and 10% acetic acid and destained in 50% methanol, 10% acetic acid in H_2O.

Protein transfer.

The cytokeratin proteins were blotted onto a Immobilon PVDF membrane using a LKB semi dry transfer system. A transfer buffer system was used with two anode buffers (0.3 M Tris, pH 10.4 and 25 mM Tris, pH 10.4) and one cathode buffer (25 mM Tris, 40 mM glycine and 40 mM 6-amino-n-hexanoic acid, pH 9.4) all containing 10% methanol (138). The gels were equilibrated for 5 minutes in the cathodic buffer. The separated protein bands were blotted onto a Immobilon PVDF transfer membrane. The membranes were prepared according to the manufactures directions which involved

transferred for 60 minutes at 2.5 mA/cm^2. The membranes were either stained for protein or were used for immunodetection of the cytokeratin proteins. The gels were always stained with Commassie Blue to verify that transfer was complete.

Immunodetection of the cytokeratin proteins.

The membrane was placed in blocking buffer, TBS, (20 mM Tris/HCl pH 7.5 containing 150 mM NaCl) containing 0.2% gelatin for 90 minutes at room temperature. The blot was transferred to TBS containing 0.2% gelatin and primary antibody diluted 1:500 and incubated again for 12 hours at room temperature. The membrane was washed four times for 10 minutes with TBS containing 0.1% Tween 20 and was then incubated with the secondary antibody for 1 hour at room temperature. The secondary antibody, antimouse IgG (whole molecule) peroxidase conjugate was diluted 1:2000 in TBS containing 0.2% gelatin. The blot was washed twice with TBS containing 0.1% Tween 20 and twice in TBS. The substrate, 3,3'-diaminobenzidine (DAB) was prepared as follows; 0.03 g DAB in 50 ml TBS containing 3 mg $NiCl_2$. Before use 10 µl H_2O_2 was added. The membranes were incubated in the substrate solution until the bands appeared. The reaction was terminated by washing the blots into TBS. The membranes were air dried and stored.

3.4 RESULTS

A cytokeratin enriched fraction was prepared from MCF-7 cells, primate mammary organoids and PMEC cultured in supplemented DMEM:F12(RC)(Fig. 3.1a). The protein bands separated by SDS-PAGE were blotted onto a Immobilon PVDF membrane. Immunodetection of cytokeratins was accomplished using a mixture of antibodies (C8.13) specific for K1, K5, K6, K7, K8, K10, K11 and K18.

The presence of the K8, K18 and K19 keratins in the MCF-7 cell line was substantiated by the presence of three proteins with a M_R of 52.5, 45 and 40 kDa (Fig. 3.1a) and the

samples studied. Cytokeratins K8, K7 and K6/K11 (indistinguishable due to similarities in M_R) were expressed by organoids prepared from primate mammary tissue and by PMEC in culture. Although organoids and PMEC have the same cytokeratin profile, the amounts of the different cytokeratins expressed differ (Fig. 3.2 and 3.3). PMEC *in vitro* show a decrease in the expression of K8 and an increase in the relative expression of K7 and K6/K11 when compared to primate mammary tissue *in vivo*.

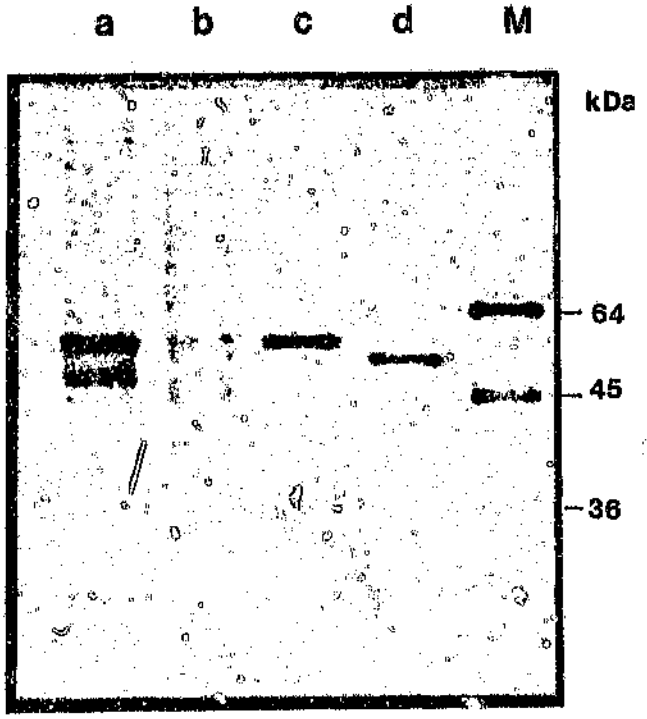


Figure 3.1a. SDS PAGE of cytokeratin enriched fraction isolated from a) MCF-7 cells (3.2 µg), b) PMEC (RC)(2.5µg) and c) pyruvate kinase(58kDa)(1µg), d) glutamate dehydrogenase (53 kDa) (1µg) and molecular mass standards (M) (i)bovine serum albumin, (64 kDa), (ii) ovalbumin (45 kDa) and (iii) gly-3-P dehydrogenase (36 kDa). Commassie blue stalning.

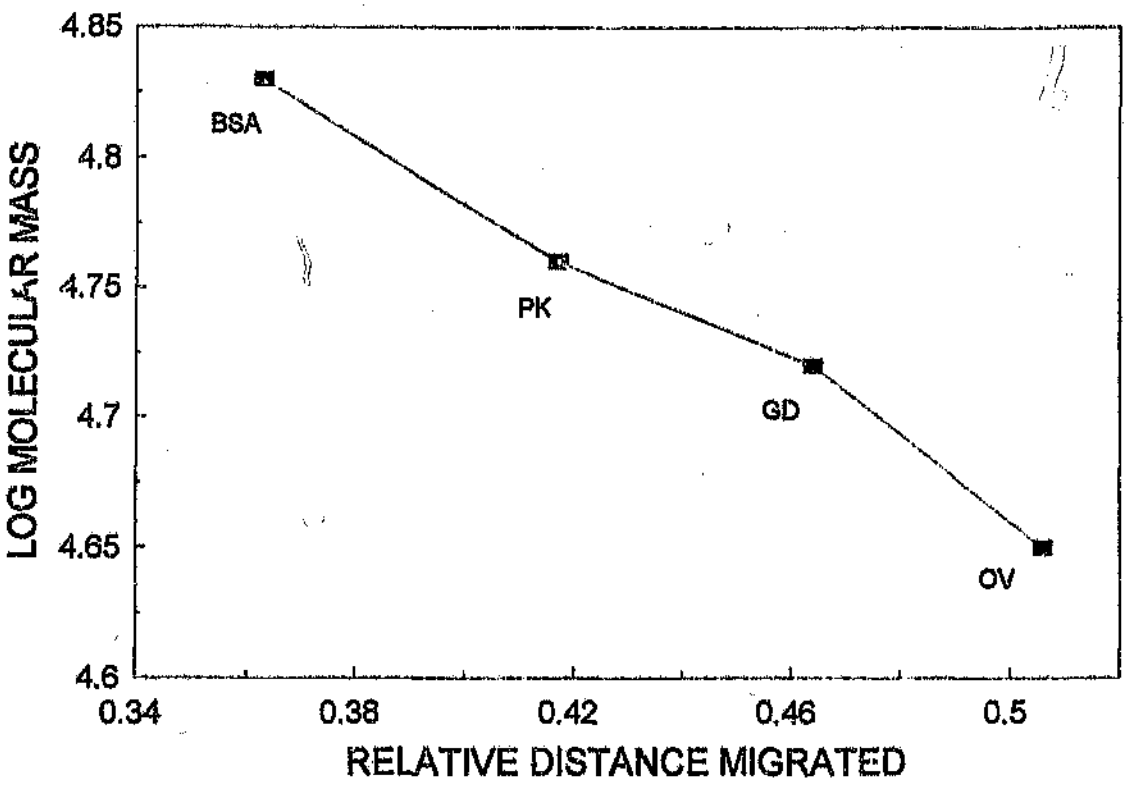


Figure 3.1b. Calibration curve for the determination of the relative molecular masses of the cytokeratin proteins isolated from MCF-7, PMEC-RC cells and organoid tissue. Molecular weight standard proteins are bovine serum albumin (BSA), pyruvate kinase (PK), glutamate dehydrogenase (GD) and ovalbumin (OV).

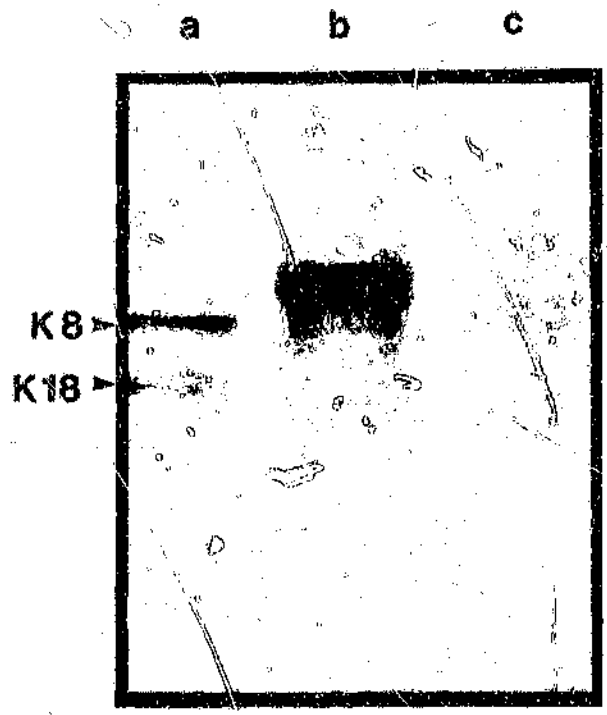


Figure 3.2. Immunodetection of cytokeratins present in (a) MCF-7 (3.2 µg), (b)PMEC-RC (2.5 µg) and (c) organoid tissue (3.3 µg).

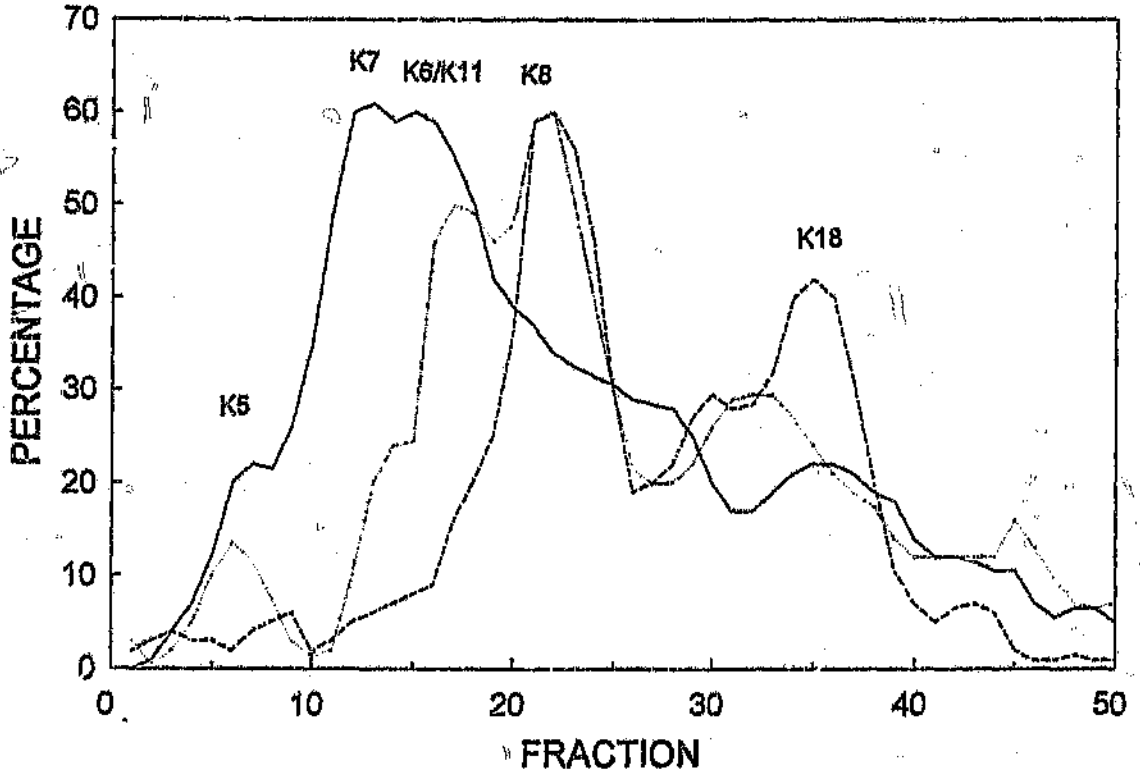


Figure 3.3. Keratin profile of cytokeratins expressed by (a) MCF-7 cells (– –), (b) organoids isolated from primate mammary tissue (...) and (c) PMEC *in vitro* (—).

3.5 DISCUSSION

Immunohistochemical studies undertaken by Nagle *et al* (56) revealed that the antibody mixture K 8.13 identified the luminal epithelium of ducts and acini of normal and lactating mammary tissue. Positive staining was also observed for luminal cells of the terminal ducts as well as epithelial cells from invasive lobular and ductal carcinoma. No staining was observed for any myoepithelial cell types. For this purpose this antibody was used to determine whether PMEC *in vitro* expresses cytokeratins associated with luminal epithelial cells.

Organoids isolated by selective digestion of primate mammary tissue contain both luminal and basal cells derived from the primate mammary gland. K8, K6/11 and K7 were identified as the predominant keratins expressed in primate mammary tissue. K8 is a specific marker for luminal epithelial cells while K7 is expressed by luminal and myoepithelial cells. K18 is present in primate organoids and PMEC *in vitro* only as a minor component, while K5, a myoepithelial associated cytokeratin is expressed by both primate mammary organoids and PMEC *in vitro*. The keratin profile of separated luminal and myoepithilial cells from normal human mammary tissue was studied by Dairkee *et al* (62). K7 followed by K8 was the predominant keratins expressed by lumenal epithelial cells. In myoepithelial cells K5 followed by K14 and K17 were the principal keratins identified. Dairkee *et al* (62) also observed that K6 was absent in the myoepithelial and luminal fraction derived from normal mammary tissue, K6 was the predominant keratin expressed by HMEC *in vitro* while K18 a luminal keratin was identified as a minor component HMEC cultures. Myoepithelial keratins K5 and K14 were the other major keratins expressed *in vitro*. Trask *et al* (58) also observed that K6 is absent in normal human mammary tissue and is usually only elevated during growth in cell culture and therefor is indicative of a hyperproliferative state. K6/K11 is expressed by primate mammary organoids and increased expression is observed by PMEC *in vitro*. K11 is a specific cytokeratin that is expressed by mouse luminal epithelial cells (10). However this will need to be verified by using a more specific antibody.

The proliferation of specific cellular phenotypes is selected for in different tissue culture medias (54). In milk mix (MX) developed for growth of luminal cells from milk, the mammary epithelial cells expressed mostly K7, K8, K18 and K19 associated with the luminal cell phenotype. In medium MCDB 170 containing bovine pituitary extract which was developed for the long-term growth of HMEC from reduction mammoplasty organoids, K7 and the myoepithelial keratin, K14 is expressed but not K19 while in later passage an increase is observed in K18. The HMEC lines, MCF-10 and MCF-12 and subsequent sublines developed from fibrocystic mammoplasty tissue expressed predominantly K7, K8, K15, K16 (77). The presence of K14 and K19 was variable and expression of K19 was lost in later passage

Immunocytochemical analysis of monolayers of normal HMEC (54,60,77) have shown that these cultures are a heterogenous mixture of myoepithelial, lumenal epithelial cells and possibly a cell type with both myoepithelial and epithelial cell properties implying that the basal layer may contain a stem cell type which can develop along the luminal linage. Furthermore, Rudland *et al* (60) observed that the expression of luminal, myoepithelial and both luminal and myoepithelial keratins in primary cultures of HMEC are associated with morphologically different cell types.

The expression of a specific cellular phenotype, is determined by the origin of the cells (54,60), cellular substrate (60), cell culture media (54,61) and the effect of growth factors (54,58). Furthermore, mammary gland lobular development has been identified as an important physiological factor that predetermines the growth of human and primate mammary epithelial cells *in vitro*. However the manner in which lobular development may predetermine the dominant cellular phenotype *in vitro* must still be addressed.

The expression or lack of expression of a particular keratin can be used to characterise a specific cell type. In this study the expression of cytokeratin proteins by PMEC in culture is used to verify that the cells maintained in culture are epithelial. Increased expression of K6 is associated with most mammary epithelial cells *in vitro* while K7 is

present in HMEC derived from milk and reduction mammoplasties and is also present in HMEC cell lines. Although both K8 and K5 are present as minor components it is not possible to conclusively determine the predominant phenotype. Whether K11 a mouse luminal keratin marker is present in primate mammary tissue and PMEC *in vitro* can only be determined with a monospecific antibody. To conclusively determine the exact nature of the PMEC *in vitro* it is necessary to determine the presence of two other important cytokeratins namely K19 (luminal epithelial) and K14 (myoepithelial).

CHAPTER 4:

THE PRIMATE MILK FAT GLOBULE PROTEINS AND EXPRESSION IN PRIMATE MAMMARY EPITHELIAL CELLS *IN VITRO*.

4.1 INTRODUCTION

The milk fat globule proteins (MFGP) are representative of the apical membrane of the mammary epithelial cell (62,63,64). Immunohistochemical studies reveals that antibodies raised against delipidated MFG membrane proteins binds specifically to the outer surface of milk fat globule, the luminal epithelial cell *in vivo* (61) normal HMEC derived from breast milk (65,139), carcinomas (50,65) and cell lines derived from breast carcinomas (50,74,141).

Immunocytochemistry reveals that MFGP-70 is expressed on the apical membrane of cells lining the ducts (66-69) and the MFGP-155 (69,140) is preferially expressed on the surface of luminal epithelial cells of the terminal ductules (70). MFGP-70 has been identified as a protein bond by disulphide bonds to a larger protein (142) analogous to a family of cervical and gastrin mucin proteins (143-146). Thiol reduction of proteolytic digests results in the release of this protein.

HMFG1 and HMFG2 antibodies raised against MFGP-400 (72-74) differ with respect to specificity; HMFG1 for metastatic ductal carcinomas and derived cell lines while HMFG2 for lactating breast and normal HMEC cells derived from milk (73). Antibodies SM-3 raised against the core protein of MFGP-400 are highly specific for mammary carcinomas (73). This epitope usually becomes exposed when adherent glycosylation results in the exposure this epitope. Antibodies HMFG1, HMFG2, and SM-3, recognized epitopes of the MFGP in the HMEC lines, MCF-10 and MCF-12 (77) The expression of MFGP antigens in primary cultures of HMEC (60) was associated with morphologically distinct cell types. Positive peripheral staining of the cuboidal/apical and large flat cells identified these cells as luminal epithelial cells, while negative

staining was observed for the large epithelioid/elongated cells thereby confirming the myoepithelial origin of these cells.

Butyrophilin (147), a 67 kDa component of the MFGP fraction of cow, goat, sheep, guinea pig and rat is unrelated to human MFGP-70. Butyrophilin is however, detected in the apical secretory epithelial cells of large and small ducts but not myepithelial cells.

The main aim of this investigation was to isolate the milk fat globule proteins (MFGP) from human milk and primate lactating mammary tissue. To determine whether primate MFGP are structurally and/or immunologically related to human MFGP. Furthermore it was necessary to establish whether PMEC, *in vitro,* express proteins associated with the human and/or primate MFGP fraction. Antibodies raised against delipidated MFGP, MFGP-70, MFGP-150 and HMFG2 are used in immunocytochemical studies to identify the lumenal mammary epithelial cell population *in vitro.* However very few studies have been undertaken to determine exactly which MFGP are expressed by mammary epithelial cells *in vitro.*

4.2 MATERIALS

All reagents used for electrophoretic procedures were analytical grade and were purchased from either Merck Chemicals (Darmstadt, Germany) or BDH Laboratory (Poole, England).

Rabbit polyclonal against HMFG protein fraction and HMFG-2 was a Serotec product (Oxford, England). The secondary antibodies used were antirabbit IG (whole molecule) peroxidase conjugate (Product number A-0545) or antimouse IG (whole molecule) peroxidase conjugate was purchased from the Sigma Chemical Company (St Louis, USA).

4.3 METHODS

Isolation of the milk fat globule proteins from human milk and primate mammary tissue.

Human breast milk was extracted twice with two volumes of chloroform and twice with two volumes of ether (139). Any excess organic solvent was removed under N_2.

Mammary tissue obtained from a primate that was lactating was lacerated, washed in RPMI medium for 30 minutes at 37°C. The tissue was pelleted at 500 *xg* for 15 minutes and the supernatant was retained for the extraction of the milk fat globule membrane proteins. The supernatant was extracted twice with two volumes of chloroform and twice with two volume of ether and any organic solvent remaining in the H_20 fraction was removed as described above. The protein content of each sample was determined using the Bio-Rad Protein Kit.

Preparation of MCF-7 and PMEC cell lysates.

Monolayers of MCF-7 and PMEC were washed twice with PBS and were exposed for 12 hrs to 0.125 M Tris/HCl pH 6.8 containing 4% SDS.

Preparation of tryptic fragments of milk fat globule proteins and cell lystates.

Human and primate MFGP fractions were diluted 1:1 with a 0.25% trypsin solution prepared in PBS. Trypsin digested fragments of MCF-7 and PMEC cell lystates were prepared by incubating washed monolayers with 0.25% trypsin in PBS at 37°C for 24 hours.

SDS-PAGE of the milk fat globule proteins.

Electrophoresis was carried out using a Hoeffer Mighty Small Slab Gel Electrophoresis unit (SE 250). SDS polyacrylamide electrophoresis was undertaken with a resolving gel

acrylamide concentration of 10%T and 1%C with a 1.5 M Tris/HCl pH 8.8 gel buffer and a stacking gel acrylamide concentration of 4%T and 1%C with a 0.5 M Tris/HCl pH 6.8 gel buffer. For the separation of high molecular mass components of the human and primate MFGP fraction a 3-12%T and 1%C gel was prepared with a 2 mm, stacking gel prepared as described above.

Samples were prepared with a 1:1 dilution of 2 x treatment buffer, 0.125 M Tris/HCl pH 6.8 containing 4% SDS, 20% glycerol and with/without 2% 2-mercaptoethanol, and were heated at 100^{0}C for three minutes.

The protein samples were separated at 20 mA per gel, with a 0.025 M Tris pH 8.3 tank buffer containing 0.192 M glycine and 0.1% SDS at 10^{0}C.

Protein staining.

The gels were stained with a 0.125% Commassie Blue R-250 solution prepared in 50% methanol and 10% acetic acid and destained in 50% methanol, 10% acetic acid in H_2O.

Periodic acid-silver staining of glycoproteins.

The periodic-silver staining procedure of Dubray and Bezard (148) is a more sensitive staining procedure for the detection of glycoproteins. Following SDS-PAGE the gels are fixed overnight in 25% isopropanol and 10% acetic acid and then soaked for 30 minutes in 7.5% acetic acid. Following extensive washing with double distilled water for 3 hours, a freshly prepared ammonical silver solution was added. To prepare 100 ml, 1.4 ml of fresh NH_4OH were added to 21 ml of 0.36% NaOH. To this solution, 4 ml of a 19.6% $AgNO_3$ solution was added slowly while agitating vigorously. The gel was exposed to this solution for 30 minutes before being washed extensively. The gel was transferred to a freshly prepared solution of 0.05% citric acid and 0.019% formaldehyde

in 10% methanol. As the carbohydrate bands become more visible, the reaction was terminated by removing the gel from the developer solution placing in a 10% acetic acid solution.

Protein transfer.

Following separation of the MFGP by SDS-PAGE, the proteins of unstained gels were blotted onto a Immobilon PVDF membrane using a LKB semi dry transfer system as described for the detection of cytokeratins in Chapter 3.

Immunodetection of milk fat globule proteins.

The membrane blots were placed in blocking buffer, TTBS, ((20 mM Tris/HCl pH 7.5 containing 150 mM NaCl (TBS)) and 0.1% Tween 20) containing 4% bovine serum albumin for 2 hours at 4°C. The membranes were washed four times for 15 minutes with TTBS. The primary antibody (rabbit polyclonal raised against human milk fat globule protein) was prepared at a dilution of 1:1000 in TTBS containing 4% BSA. The membranes were incubated with the primary antibody solution overnight at 4°C. The above washing step was repeated and the blots were incubated with the secondary antibody for 90 minutes at room temperature. The secondary antibody, antirabbit IG (whole molecule) peroxidase conjugate (Product number A-0545) was diluted 1:4000 in TTBS containing 4% BSA. The membranes were again washed extensively in order to reduce nonspecific binding. The membranes were rinsed in TBS. The substrate, 3,3'-diaminobenzidine (DAB) was prepared as follows a 0.03g DAB in 50 ml TBS containing 3 mg $NiCl_2$. Before use 10 µl H_2O_2 was added. The membranes were incubated in the substrate solution until the bands appeared. The reaction was terminated by washing the blots into TBS. The membranes were air dried and stored.

Detection of human milk fat globule protein, MFGP-400.

The membrane blots were placed in blocking buffer, TTBS, (20 mM Tris/HCl pH 7.5 containing 150 mM NaCl (TBS) containing 0.2% gelatin for 90 minutes at room temperature. The membranes were washed four times for 15 minutes with TBS. The primary antibody (mouse monoclonal, HMFG-2 antibody) was prepared at a dilution of 1:250 in TBS containing 0.2% gelatin. The membranes were incubated with the primary antibody solution overnight at 4°C. The above washing step was repeated and the blots were incubated with the secondary antibody for 3 hours at room temperature. The secondary antibody, antimouse IG (whole molecule) peroxidase conjugate diluted 1:500 in TBS containing 0.2% gelatin. The blots were stained as described above.

4.4 RESULTS

The primate milk fat globule proteins.

Chloroform/ether extraction of the milk fat globule proteins from the milk of the primate resulted in the isolation of two major glycoproteins with a M_R of 70 and 66 kDa (Fig. 4.1.1 A (b). Glycoprotein staining revealed the presence of a number of high molecular mass glycoproteins (Fig. 4.1.1B(b)) which were not detectable by Commassie Blue staining. The anomalous behaviour of some membrane glycoproteins on SDS-PAGE has questioned the use of the usual protein markers for the determination of the molecular mass of glycoproteins (148). For this purpose the human MFGPs with well documented molecular masses were used to determine the M_R of the MFGPs isolated from lactating primate mammary gland (Fig. 4.1.3). SDS-PAGE under reducing conditions (with thiol reduction (+))revealed the presence of six major human MFGP (Fig. 4.1.1A(a) and Fig. 4.1.1B(b)) with the following molecular masses 70, 58, 52, 42, 39 and less than 10 kDa as described by Imam *et al* (71). The greater sensitivity of the periodic acid-silver staining method for glycoproteins assisted in the classification of the primate milk fat globule proteins according to the PAS nomenclature for human MFGPs (65,148).

The 66 kDa protein present in the primate MFGP fraction is only present at very low concentrations in the human MFGP fraction (Fig. 4.1.1A(a) and Fig. 4.1.1B(a)). Immunoprobing revealed that the polyclonal antibody against the whole MFGP fraction of human (anti-MFGP) was specific for all human milk fat globule proteins (Fig. 4.1.2 (1)(a,b)) and only the 70 kDa component of primate MFGP (MFGP-70) (Fig.4.1.2 (2)(c-f)). Trypsinisation of human (Fig. 4.2.1(a)) and primate (Fig. 4.2.1.(c)) results in the enrichment of MFGP-70 (Fig. 4.2.1.b and d) which stains positively with anti-HMFG (Fig. 4.3.2(a) and Fig. 4.3.2 (c) respectively). HMFG2 detected the MFGP-400 component in human milk, no cross reactivity was observed for any component of the MFGP fraction isolated from lactating primate mammary tissue (results not shown).

SDS-PAGE (Fig. 4.2.2) under nonreducing conditions (without thiol reduction (-)) of human and primary fractions before and following trypsin digestion revealed the absence of human (Fig. 4.2.2.a and b) and primate (Fig. 4.2.2.c and d) MFGP-70. Immunostaining verified the absence of human (Fig. 4.3.1.(b) and Fig. 4.3.2.(b)) and primate (Fig. 4.3.1.(d) and Fig. 4.3.2.(d)) MFGP-70 before and after trypsin digestion. Increased expression of a 53 kDa protein (MFGP-53) was observed in MFGP fractions derived from human and primate milk before (Fig. 4.3.1.(b) and (d)) and following trypsin digestion (Fig. 4.3.2.(b) and (d)).

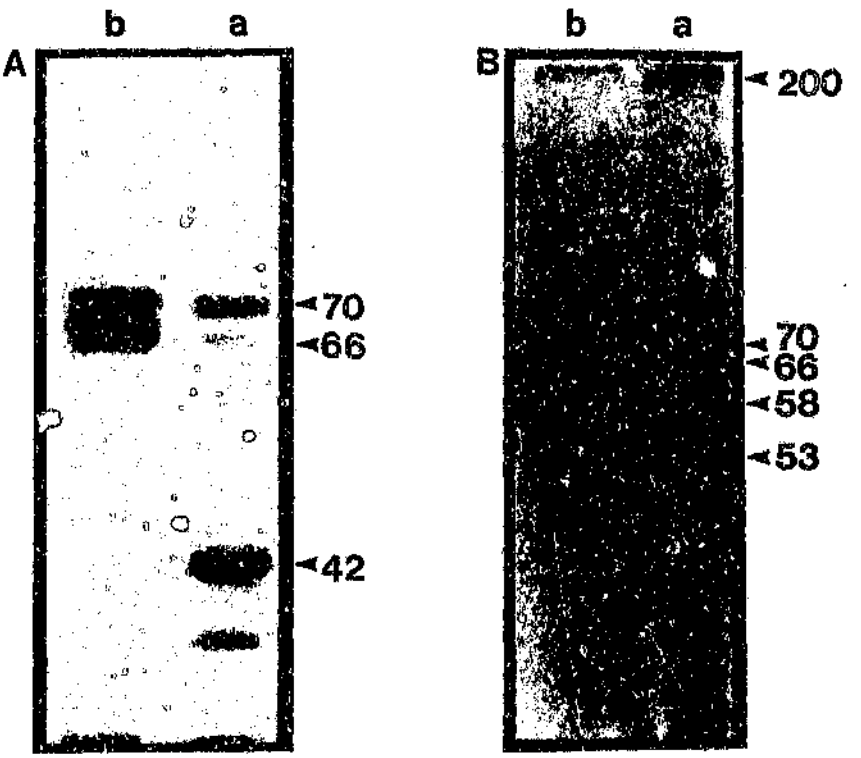


Figure 4.1.1. SDS-PAGE of MFGP isolated from human milk and primate mammary tissue. A) protein staining of a) human milk (5 µg) and b) lactating primate mammary tissue (5 µg) and B) glycoprotein staining of a) human milk (1 µg) and b) lactating primate mammary tissue (1 µg). ➢Showing the positions of MFGP-200, MFGP-70, MFGP-66, MFGP-58, MFGP-42 and MFGP-39.

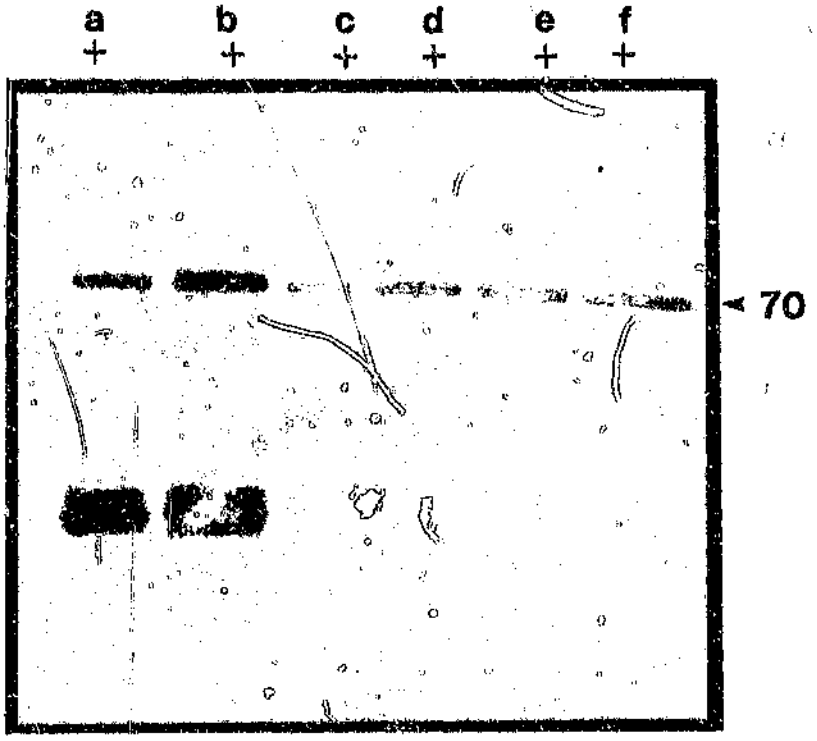


Figure 4.1.2. Immunodetection of MFGP isolated from (1) human milk a)(0.5 µg) and b)(1 µg) and (2) lactating primate mammary tissue c)(0.5 µg), d)(1 µg), e)(2 µg) and f)(4 µg) with anti-HMFG. ➤Indicates MFGP-70.

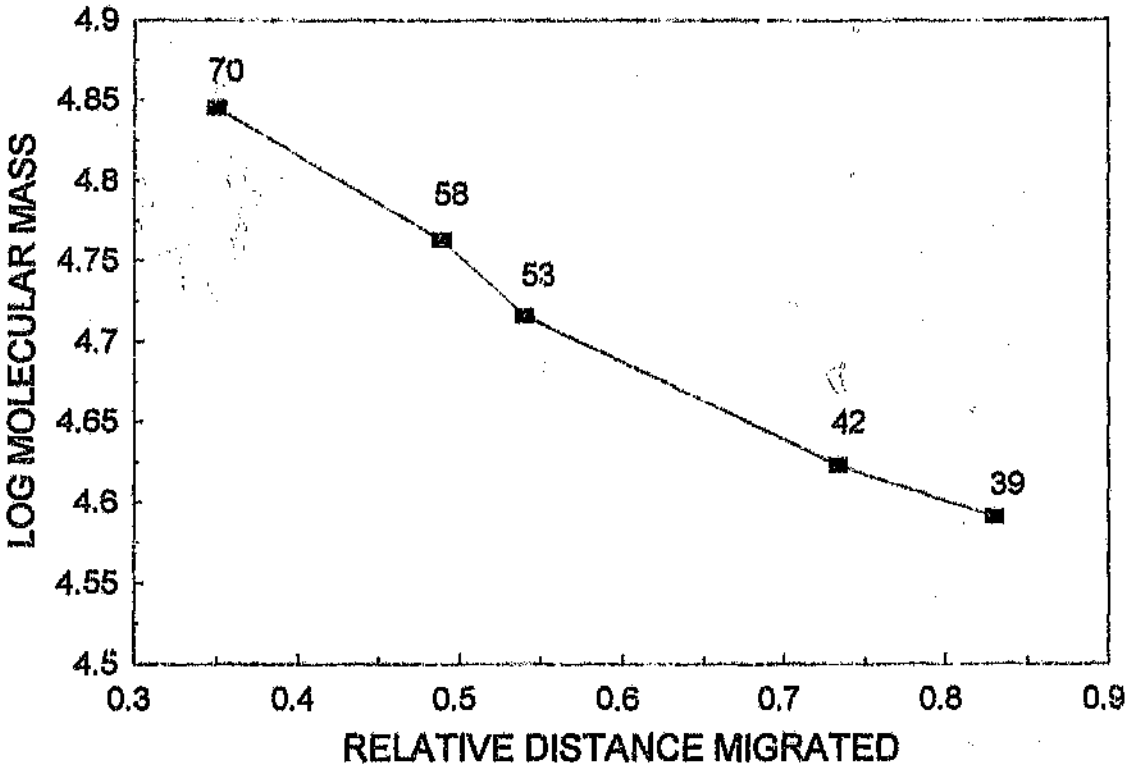


Figure 4.1.3. Calibration curve for the determination of the molecular mass of the MFGP isolated from human milk and lactating primate mammary tissue with human MFGP as standards i) 70, ii) 58, iii) 53, iv) 42 and v) 39 kDa.

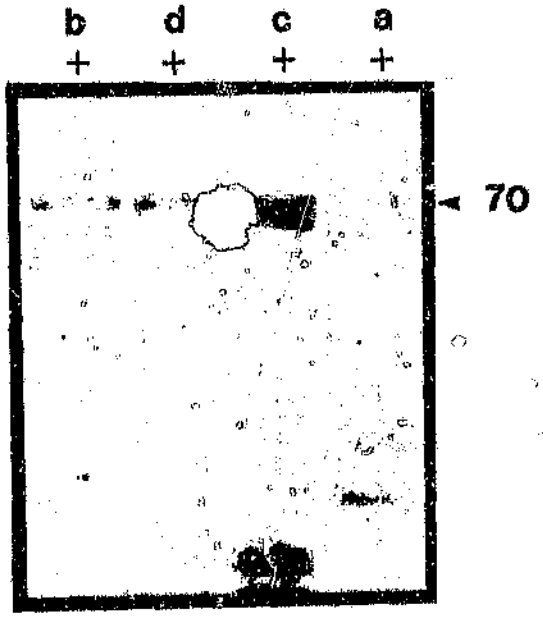


Figure 4.2.1. SDS-PAGE of MFGP in human milk and primate mammary tissue with thiol group reduction (+). a) human milk (5 µg), b) tryptic digest of human milk (3.8 µg human milk + 1.2 µg trypsin), c) lactating primate mammary tissue (5 µg) and d)tryptic digests of primate mammary tissue (3.8 µg lactating primate mammary tissue + 1.2 µg trypsin). Commassie blue staining. ➢Indicates the position of MFGP-70.



Figure 4.2.2. SDS-PAGE of MFGP in human milk and primate mammary tissue without thiol group reduction (-). a) human milk (5 µg), b) tryptic digests of human milk (3.8 µg human milk + 1.2 µg trypsin), c) lactating primate mammary tissue (5 µg) and d) tryptic digests of lactating primate mammary tissue (3.8 µg lactating primate mammary tissue + 1.2 µg trypsin), e) trypsin (1 µg) and f) tryptic digests of MCF-7 (4.0 µg). Molecular mass standards (M) are i) myosin (200 kDa), ii) phosphorylase b (97 kDa), iii) BSA (68 kDa), iv) ovalbumin (45 kDa) and carbonic anhydrase (30 kDa). Commassie blue staining. ➤Indicates the position of MFGP-70 and MFGP-53.

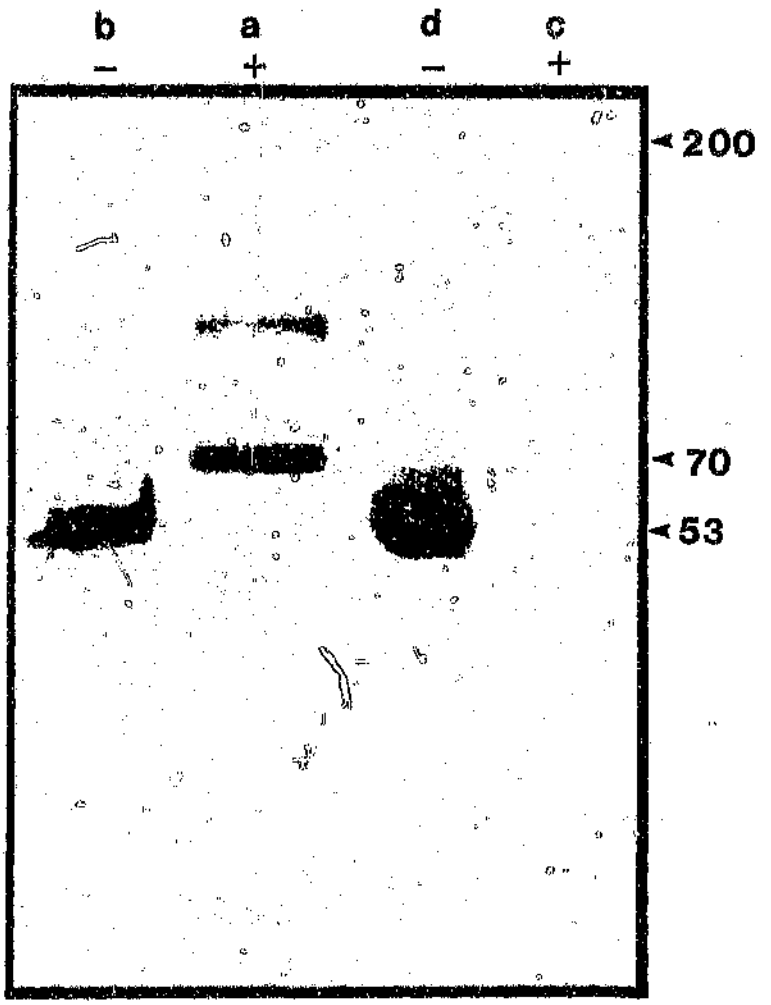


Figure 4.3.1. Immunodetection of human and primate MFGP with anti-MFGP in human milk (2.5 µg) a) with (+) and b) without (-) thiol reduction and lactating primate mammary tissue (2.5 µg) c) with (+) and d) without (-) thiol reduction.➢Indicates the position of MFGP-200, MFGP-70 and MFGP-53.

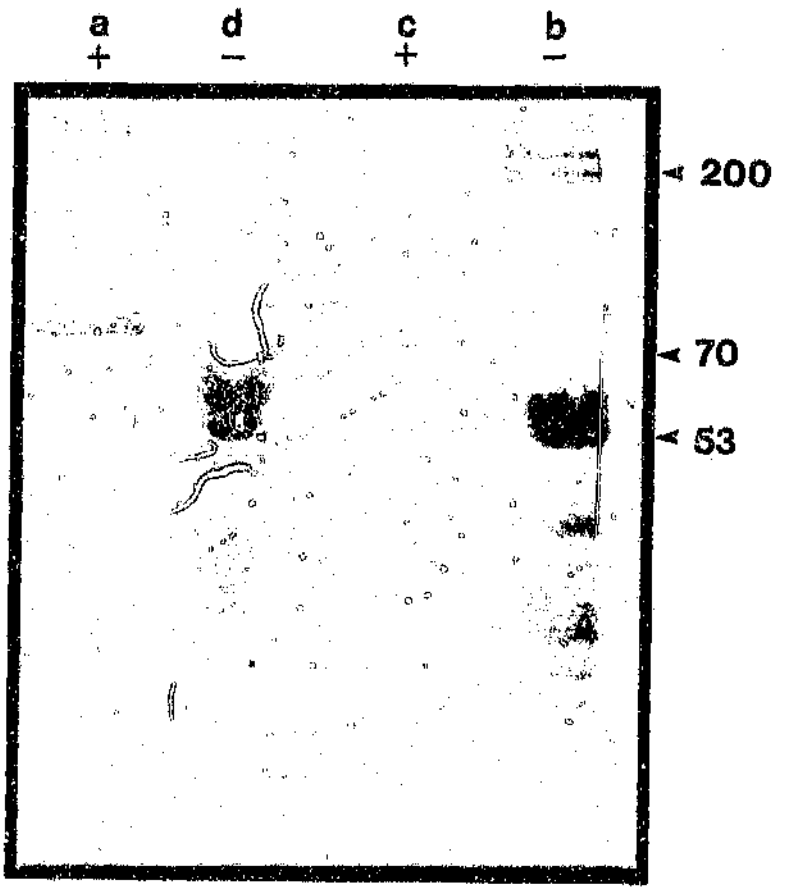


Figure 4.3.2. Immunodetection of tryptic digests of human and primate MFGP with anti-MFGP in human milk (2.5 µg) a) with (+) and b) without (-) thiol reduction and lactating primate mammary tissue (2.5 µg) c) with (+) and d) without (-) thiol reduction. ➤Indicates the position of MFGP-200, MFGP-70 and MFGP-53.

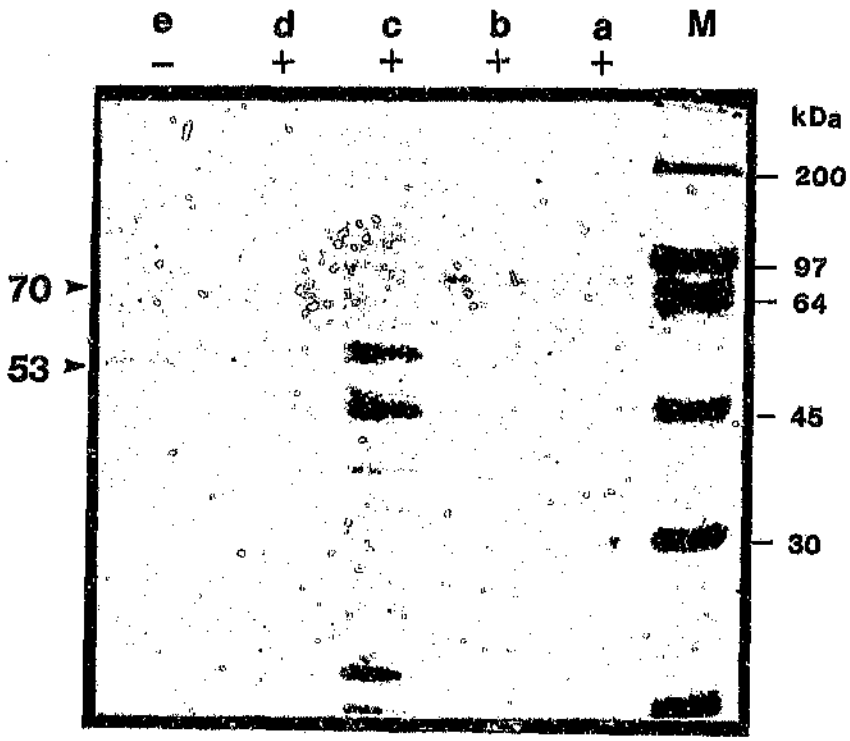


Figure 4.4.1. SDS-PAGE of a) tryptic digests of human (2.5 µg) and b) primate (2.5 µg) MFGP, c) MCF-7 cell lystate (8.7 µg), and d) tryptic digests of MCF-7 cells (4.0 µg), (a-d) with (+) thiol group reduction and e) tryptic digests of MCF-7 (4.0 µg) cells without (-) thiol group reduction. Molecular mass standards are i) myosin (200 kDa), ii) phosphorylase b (97 kDa), iii) BSA (68 kDa), iv) ovalbumin (45 kDa) and carbonic anhydrase (30 kDa). Commassie blue staining. ➤Indicates the position of MFGP-70 and MFGP-53.

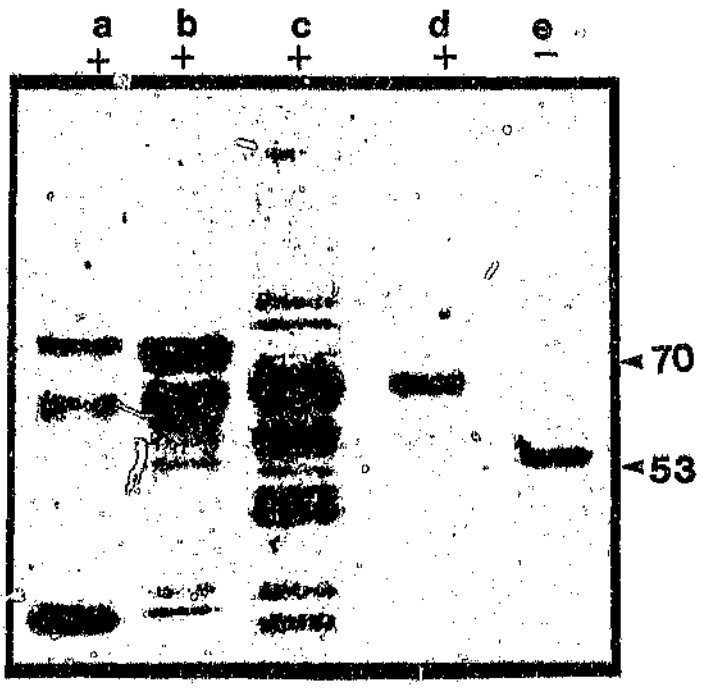


Figure 4.4.2. SDS-PAGE of tryptic digests of a) human milk (2.5 µg) and b) tryptic digests of primate milk (2.5 µg), c) PMEC lystate (10.0 µg), and d) tryptic digests of PMEC (6 µg), a-d) with (+) thiol group reduction and e) tryptic digests of PMEC (6.0 µg) without (-) thiol group reduction. Commassie blue staining. ➤Indicates the position of MFGP-70 and MFGP-53.



Figure 4.5.1 Immunodetection of MFGP isolated from a) human milk (5 µg), b) lactating primate mammary tissue (5 µg), c) MCF-7 (4 µg), d) PMEC lysate (6 µg), trypsin digests of e) MCF-7 cell (4 µg) and f) PMEC lysates (6 µg), a)-f) with thiol reduction (+). ➤Indicates the position of MFGP-200 and MFGP-70.

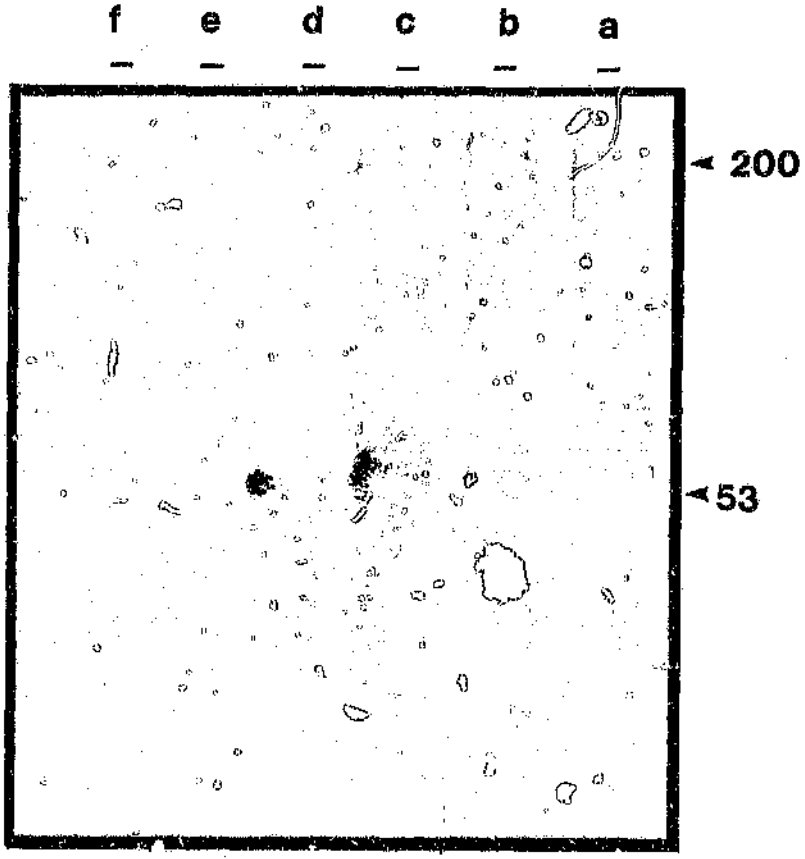


Figure 4.5.2 Immunodetection of MFGP isolated from a) human milk (5 µg), b) lactating primate mammary tissue (5 µg), tryptic digestion of c) human milk (2.5 µg), d) lactating primate mammary tissue (2.5 µg), e) MCF-7 (4 µg) and f) PMEC lysate (5 µg), a)-f) without (-) thiol reduction. ➤Indicates the position of MFGP-200 and MFGP-53.

4.5 DISCUSSION

The MFGP fraction was isolated from human and lactating primate mammary tissue. The primate MFGP fraction consisted of two major proteins with a M_R of 66 kDa and 70 kDa. It has been reported that repeated extraction of bovine MFG membrane with solutions containing high concentrations of salts leads to the enrichment of the butyrophilin at the expense of more peripheral components of the membrane (64,69). The primate MFGP fraction was extracted from lacerated primate mammary tissue suspended in tissue culture medium with a high salt content which led to the enrichment of the 70 kDa and 66 kDa components. Immunoblotting with polyclonal antibodies raised against human MFGP showed cross reactivity with the primate MFGP-70 and not the 66 kDa protein. Heid *et al* (150) identified butyrophilin as a 67 kDa transmembrane protein, found in the apical membrane of lactating mammary epithelial cells which was unrelated to MFGP-70 identified by Imam *et al* (69). Sequence analysis (147) of bovine butyrophilin reveals a close sequence homology to the ret finger protein which is expressed by a number of tumour cell lines, mouse testis and embryonic tissue which like the mammary gland undergoes periods of rapid cell division. Mather and Jack (142) have postulated that the human MFGP fraction may contain a butyrophylin-like protein, based on criteria such a M_R, isoelectric point and peptide composition.

Duwe and Ceriani (141) described the presence of a 63 kDa protein associated with MFGP-70 in the human MFGP fraction and both were released from a large mucin (>400 kDa) under reducing conditions. SDS-PAGE under non-reducing and immunodetection revealed that the MFGP-70 of the human and primate MFGP fraction was covalently bound to a larger molecule, but not the 66 kDa primate protein. A detailed analysis is required to verify whether this 66 kDa component is primate butyrophilin.

The presence of a linkage protein bound by disulphide bonds to a larger molecule is a common characteristic of a family of mucin proteins from diverse species and types

of tissue (143-146). Ultrastructurally these mucins consist of high molecular mass subunits joined by disulphide bonds to a smaller linkage protein. The epitopes of the small linkage proteins are "cryptic" and are probably shielded within the folded protein domains by large glycosylated moieties. This could account for the poor immunocytochemical staining of normal and cancerous tissue observed by Peterson *et al* (151) when using a monoclonal antibody specific for MFGP-70. Proteolytic digestion, forms fragments composed of oligosaccarides that are tightly packed on a central protein core (145). Thiol reduction leads to an enrichment of this linkage protein. Under nonreducing conditions the 70 kDa protein disappears while a 53 kDa component and less than 53 kDa components are detected. Therefore MFGP-70 is possibly a linkage protein bound by disuphide bonds to a larger molecule found in the MFGP fraction of human and primate. Expression of a 200 kDa protein is only detected by immunoblotting in MFGP fractions derived from human milk.

Immunocytochemical analysis of normal and tumour derived HMEC with antibodies raised against human MFGP-70 is specific for the apical membrane of these cells (66,67,68). No positive staining has been observed by immunoblotting and this has been ascribed to an altered antigenic determinant following electrophoresis. However, possible alteration in the antigenic determinant during preparation of cellular lysates is unlikely, as the same effect would be observed for the MFGP fraction isolated from human and primate MFGP fraction. The absence of MFGP-70 in trypsin digests of MCF-7 cells and PMEC implies that homologous epitopes are present in other mammary epithelial cell proteins. A cDNA specific for the 70 kDa protein of the human MFGP fraction, recognizes a 1.8 kDa RNA which is expressed in MCF-7 lines (160).

Electrophoresis of tryptic digests of a MCF-7 monolayer and PMEC following thiol reduction revealed the presence of a 65 kDa and a 62 kDa protein respectively, linked to a larger protein and which does not stain with anti-MFGP. The possible relationship between this protein and MFGP-70 must still be determined.

Without thiol reduction a 53 kDa protein, MFGP-53, possibly consisting either of two

smaller proteins linked by disulphide bonds or a single protein with an antigenic determinant dependent on an intact disulphide bond and which stains positively with anti-MFGP is detected in MCF-7 and PMEC. A family of related proteins, PAS-6, PAS-7 from bovine (153,154), MFG-E8 from mouse (155), GP-55 from guinea pig (156) and a 46 kDa component of human MFGP fraction has been identified (157). PAS-6 and PAS-7, with M_R of 53 kDa and 58 kDa and MFG-E8 isoforms with M_R of 55 kDa and 66 kDa are the result of glycosylation of a 51.5 kDa polypeptide. Mather *et al* (156) observed a high degree of amino acid sequence homology between MFG-E8 and GP-55. The human 46 kDa protein appears to represent a truncated version of the 66 kDa isoform of MFG-E8. This family of proteins have a sequence homology with human coagulation factors, VIII and V regions involved in phospholipid binding while MFG-E8 also has epidermal growth factor like domains (156). Aoki *et al* (153) identified bovine PAS 6 (58 kDa) and PAS 7 (53 kDa) as proteins resistant to trypsin digestion. The sequence of the related human 46 kDa protein reveals the absence of trypsin digestion sites and possible sites for thiol linkage (157). A cDNA probe, detects a 2.2 kb RNA in a number of carcinoma cell lines including the MCF-7 cell line (159). The expression of a 53 kDa MFGP by MCF-7 and PMEC related to bovine PAS-6 and PAS-7, mouse MFG-E8, and guinea pig GP-55 needs to be verified using DNA probes.

Another important group of mucin proteins are the sialomucins, which are not composed of homo- or heteromeres linked by disulphide bonds (160-162). Antibodies against MAM-6 recognizes two glycoproteins with a M_R >400 kDa. HMFG2 recognizes an antigenic determinant of MAM-6 which in MCF-7 cells recognizes a group of proteins with a M_R of 250-80 kDa. HMGF2 is highly species specific and does bind high molecular mass components of the primate milk fat globule proteins. Sialic acid residues contribute greatly to the species and tissue specificity (163,164) and the complexity of MAM-6 antigenicity could be attributed to differential glycosylation (74,162). The core protein of many mucins proteins is conserved between different tissues and species. The SM-3 antibody recognizes the core protein of HMFG-400, it only reacts positively with tumour cells and immortalized cell lines where adherent glycosylation exposes this epitope.

Therefore only with the development of species specific antibodies can the expression of sialomucins in PMEC be determined. Alternatively, as glycosylation is a posttranscriptional modification which is highly species, differentiation and tissue dependent use of DNA coding for a domain of the core protein can be used to determine mRNA expression.

MFGP present lactating primate mammary tissue are structurally and immunologically related to the human MFGPs. Furthermore, a MFGP of 53 kDa expressed by PMF *vitro*, is immunologically and structurally related to a similar protein in the huma primate MFGP fraction and the MCF-7 cell line.

CHAPTER 5:

THE EFFECT OF DIMETHYLBENZ(A)ANTHRACENE ON PRIMATE MAMMARY EPITHELIAL CELL SURVIVAL.

5.1 INTRODUCTION

The 7,12-dimethylbenz(a)anthracene (DMBA) rat model for mammary carcinogenesis has furthered the understanding of the mechanisms involved in tumour initiation, hormonal and dietary modulation and promotion (10,22,26,115,165,166). DMBA is metabolized to the 3,4 diol-1,2-oxide that interacts with DNA (164,165) and induces mammary tumorigenesis in Sprague-Dawley rats. However, normal HMEC are highly resistant to cellular transformation with chemical carcinogens (99,100,102,103,104). Exposure of HMEC to a number of carcinogens have lead to the identification of partially transformed phenotypes (99). Characteristic of these cells is increased survival, colony formation efficiency, alterations in karyotype and amplification of the ras proto-oncogene (99).

The cytotoxic effect of DMBA was studied by measuring mitochondrial succinate dehydrogenase activity, crystal violet uptake and the direct measurement of cellular DNA. The MTT/succinate dehydrogenase inhibition assay (167,168) correlates with colony formation down to survival levels of 10% for a number of cytotoxic agents and radiation (168). The number of cells in culture following exposure to DMBA was determined by measuring crystal violet uptake (131) and total cell DNA (170).

A sensitive method routinely used for the microdetermination of DNA estimates the increase in fluorescence, induced by Hoechst 33258 when the dye complexes with DNA (171-174). Hoechst 33258 (bisbenzamide: [2-(4-hydroxyphenyl)-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole])(Hoe) binds specifically to the adenine-thymidine

base pairs of DNA (174-176) and because of its high quantum efficiency and specificity for DNA, it can be used for the determination of the DNA content in most biological tissues (175,177). However, it was observed that the background fluorescence is enhanced in the presence of 0.05% sodium dodecyl sulphate (SDS), resulting in non-linear calibration curves (169,178, 179). This phenomenon has restricted the use of Hoe for the quantification of DNA in cell cultures. Therefore to quantify DNA in cell cultures the masking effect of SDS must be reduced to be able to combine the effectiveness of SDS in cell dissolution with the specificity and sensitivity of Hoe for DNA analysis.

A simple assay to determine cellular DNA content (170) was developed and this method was compared with the crystal violet assay (131) to determine the effect of DMBA on PMEC number *in vitro* while cell viability was determined by measuring succinate dehydrogenase activity (167,168).

5.2 MATERIALS

SDS, Triton X100 (TX100), cetyltrimethyl ammonium bromide (CTAB) was purchased from Merck (Darmstadt, Germany). Cholic acid, sodium salt and Hoechst 33258 were obtained from the Sigma Chemical Company (St. Louis, USA). Butanol and all other reagents were of analytical grade and were from Merck (Darmstadt, Germany). Calf thymus DNA (high molecular mass) was obtained from Boehringer Mannheim (Mannheim, Germany). MCF-7 cells, medium and other tissue culture products were purchased from Highveld Biologicals (Sandton, South Africa). 7,12-Dimethylbenz(a)-anthracene and dimethylsulphonyloxide (DMSO) were purchased from the Sigma Chemical Company (St. Louis, USA). Crystal violet, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)(MTT) was purchased from Merck (Darmstadt, Germany).

5.3 METHODS

Exposure of PMEC to DMBA for 3, 24 and 48 hours.

PMEC were plated at a concentration of 8.5x 10^3 cells/cm^2 in a 96 well microtiter plate. After incubation for 24 hours at 37^{0}C in 5% CO_2, the cells were exposed to the following concentrations of DMBA, 0-16 µM for 3 hours and 0-1.95 µM for 24 and 48 hours. A stock solution of 0.4 mg/ml DMBA in DMSO was prepared and stored in the dark at 4°C. Prior to each experiment an appropriate dilution of DMBA was prepared with a final DMSO concentration for all assay points of 0.15%. After an incubation period of i) 3 hours, ii) 24 and iii) 48 hours, the DMBA containing medium was removed, the cells were washed with medium and replaced with DMEM:F12 (RC) supplemented as described previously. The cells were maintained in this medium with regular media changes for a further 6 days.

Crystal Violet assay.

Cell number was determined by the crystal violet assay as described by Kueng *et al* (131).

Succinate dehydrogenase inhibition assay/MTT assay.

The medium was replaced with 250 µl supplemented DMEM:F12(RC) containing 50 µl of a 1mg/ml MTT stock solution (168). The cells were incubated in the presence of MTT at 37^{0}C in a humidified atmosphere in the presence of air/CO_2. After a 4 hour incubation period, the medium was carefully removed, the adherent cells were washed with PBS and the insoluble formazan product was solubilized in 100 µl acid isopropanol (0.04 M HCl in isopropanol). Absorbance is measured at 590 nm using a Titertek Multiscan plate reader. All assays were done in quadruple.

Effect of SDS on Hoe fluorescence.

A 0-8 mM (0-0.23%) SDS concentration series was prepared in 10 mM Tris/HCl buffer, pH 7, with or without 3.11 mM cholate or in 10 mM Tris/HCl, 0.5 M NaCl, pH 7 with or without 3.11 mM cholate. A 0-9.2 mM cholate concentration series in 10 mM Tris/HCl, pH 7, containing 0.5 M NaCl was also prepared.

Effect of n-butanol on SDS-Hoe fluorescence.

To 1.15 mM samples of SDS in 10 mM Tris/HCl 1 M NaCl, pH 7, increasing amounts of n-butanol were added.

The effect of SDS, Cetyltrimethylammonium bromide and Triton X100 on Hoe fluorescence.

Sodium dodecyl sulphate at a concentration of 4.6 mM (0.13%) was prepared in the following buffers: 10 mM Tris/HCl buffer, pH 7, with or without 3.11 mM cholate and 10 mM Tris/HCl, 0.5 M NaCl, pH 7, with or without 3.11 mM cholate. CTAB at a concentration of 3.66 mM (0.13%) was prepared in 10 mM Tris/HCl buffer pH 7 with or without 3.11 mM cholate and 10 mM Tris/HCl, 0.5 M KBr, pH 7, with or without 3.11 mM cholate. TX-100 at a concentration of 0.13% was prepared in the same buffers as for SDS.

The effect of pH on SDS-Hoe interactions.

SDS at a concentration of 4.6 mM was prepared in a 10 mM Tris/HCl containing 1 M NaCl at pH 7, 8, 9 and 10.

DNA Standard curves.

Calf thymus DNA, 100ug/ml, (high molecular mass)(Boehringer Mannheim, Germany) was prepared in 10 mM Tris/HCl, pH 7, and the final concentration was determined at 260 nm with a molecular coefficient of 6800 $M^{-1}cm$ (179). Serially diluted DNA standards of 0-200 ng/ml were prepared from this stock solution. The DNA samples were prepared in 10 mM Tris/HCl, 0.5 M NaCl, pH 7, with or without 4.6 mM SDS. An identical DNA concentration series was prepared in 10 mM Tris/HCl, pH 7, 0.5 NaCl, 3.11 mM cholate, pH 7, without or with 2.3, 4.6 or 6.9 mM SDS. The same DNA series was prepared in 10 mM Tris/HCl, pH 9, containing 1 M NaCl with or without 4.6 mM SDS.

Fluorometric Determination.

From a stock solution (2.34×10^{-4} M) of Hoechst 33258 a fresh working solution of 2.34×10^{-6} M Hoe was prepared daily. The final concentration of the working solution was determined spectrophotometrically at 388 nm using a molar extinction coefficient of 42 200 $M^{-1}cm^{-1}$ (174). The final volume of all samples was 2 ml, to which 1 ml of Hoe working solution prepared in 10 mM Tris/HCl at the appropriate pH and salt concentration was added. All detergent, DNA, and cell concentrations are calculated in terms of the final assay volume. Each sample was mixed well and the fluorescence was measured in duplicate with the excitation wavelength at 350 nm and emission wavelength at 460 nm in a Hitachi F-2000 fluorescence spectrophotometer.

Determination of cellular DNA.

MCF-7 cells were maintained in EMEM containing 10% FCS at 37°C. The cells were trypsinized and washed in PBS. A standard series of $1x10^4$ - $5x10^5$ cells in 50 µl PBS was prepared, and a 200 µl aliquot of a 96 mM (2%) SDS solution in 10 mM Tris/HCl, pH 7, containing either 0.5 or 2 M NaCl was added to each sample. The samples were

mixed well. A duplicate group of cells was exposed to 500 ug/ml proteinase K prepared in the appropriate buffers. All samples were incubated at 65^{0}C for 1 hour. To each sample 200 µl of a 2% cholate solution prepared in 10 mM Tris/HCl, pH 7, containing 0.5M NaCl was added. Samples are sonicated on a low energy setting and each series was made up to 2 ml in 10 mM Tris/HCl, pH 7, containing 0.5 M NaCl. All samples were processed further as previously described.

Brief protocol for the quantification of DNA in cell culture.

Plate cells in a 24 well multidish at a cell density of 5 x10^3 - 1 x 10^4 cells/cm^2.
To determine the DNA content of the cells in each well,
aspirate the medium and wash the monolayer twice with 10 mM Tris/HCl, pH 7.
Blot dry by inverting the multidish on a piece of tissue paper.
Add 200 µl of a warm (46°C) 2% SDS solution prepared in 10 mM Tris/HCl, pH 7 to each well.
Mix by gentle tilting until the monolayer forms a viscous mixture.
Add 200 µl of a 10 mM Tris/HCl, 4 M NaCl, pH 7, solution as well as 50 µl of a 2 mg/ml proteinase K solution prepared in 10 mM Tris/HCl, pH 7.
Incubate at 65^{0}C for at least 1 hour.
While still warm add 200 µl of a 10 mM Tris/HCl, 3.11 mM cholate, 0.5 M NaCl, pH 7, buffer.
Mix well.
Samples can be stored at 4^{0}C.
Transfer to test tubes.
Add 350 µl of a 10 mM Tris/HCl, pH 7, buffer containing 0.5 M NaCl.
Sonicate on a low energy setting.
Add 1 ml Hoe working solution (2.34 x 10^{-6} M Hoe in 10 mM Tris/HCl, 0.5 M NaCl, pH 7).
Measure fluorescence at EX 350 nm and EM 460 nm.
Prepare a standard curve from a standard series of cells as previously described.

Hoechst-DNA assay.

A standard curve for the determination of the number of PMEC attached following exposure to DMBA was prepared as follows. Monolayers derived from 2 different cultures were trypsinized, the cells were washed with PBS and a series of 0.5-10 x 10^4 cells in 100 µl, 10 mM Tris/0.5 M NaCl, pH 7.0 was prepared. These PMEC as well as adherent PMEC which were exposed to DMBA were lysed with 100 µl, 2% SDS and processed further as described above.

5.4 RESULTS

The effect of DMBA on PMEC succinate dehydrogenase activity.

PMEC was exposed to 0-9.75 µM DMBA for 3 hours and 0-1.95 µM DMBA for 24 and 48 hours and succinate dehydrogenase activity was measured 6 days later. A sigmoidal decrease in succinate dehydrogenase activity was observed (Fig. 5.1). A procedure was developed for the measurement of cell DNA and this procedure was compared with the standard methods of cell enumeration. The number of cells attached following DMBA exposure was determined by measuring crystal violet uptake and cell DNA content.

The interaction between Hoechst and SDS micelle.

In the absence of DNA and at a constant concentration of Hoe a sigmoidal increase in fluorescence is observed with increasing concentrations of SDS (Fig. 5.2). This increase in fluorescence occurs in the region of the critical micellar concentration (CMC); (8.2 mM without and 0.51 mM in the presence of 0.5 M NaCl) (179,180) of SDS. Furthermore, the addition of butanol, an agent which disrupts SDS micelle structure, causes a rapid decline in fluorescence (Fig.5.3).

Increasing concentrations of SDS in the presence of 0.5 M NaCl at a constant Hoe concentration results in an increase in fluorescence at lower SDS concentrations with a 38% decrease in total fluorescence at higher SDS concentrations (Fig. 5.2) when compared to fluorescence measured in the absence of NaCl.

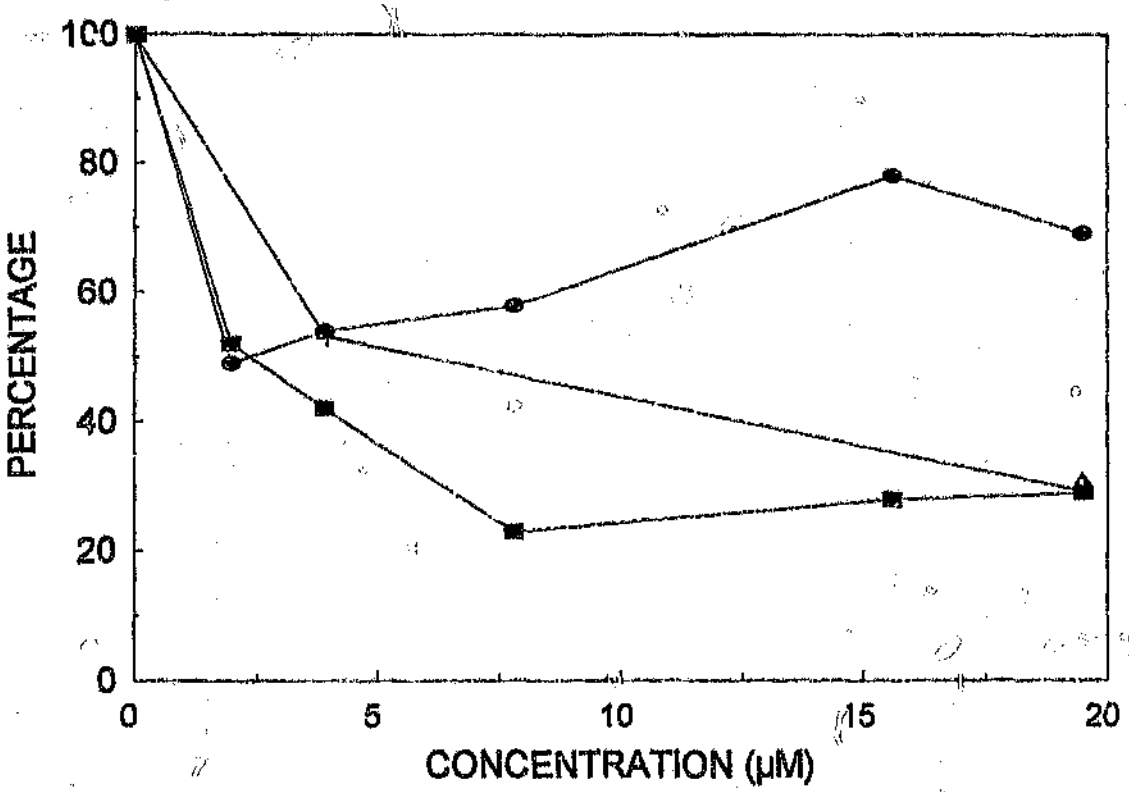


Figure 5.1. The effect of 3 (–▲–), 24 (–●–) and 48 (–■–) hours DMBA exposure on PMEC viability as measured by the succinate dehydrogenase activity.

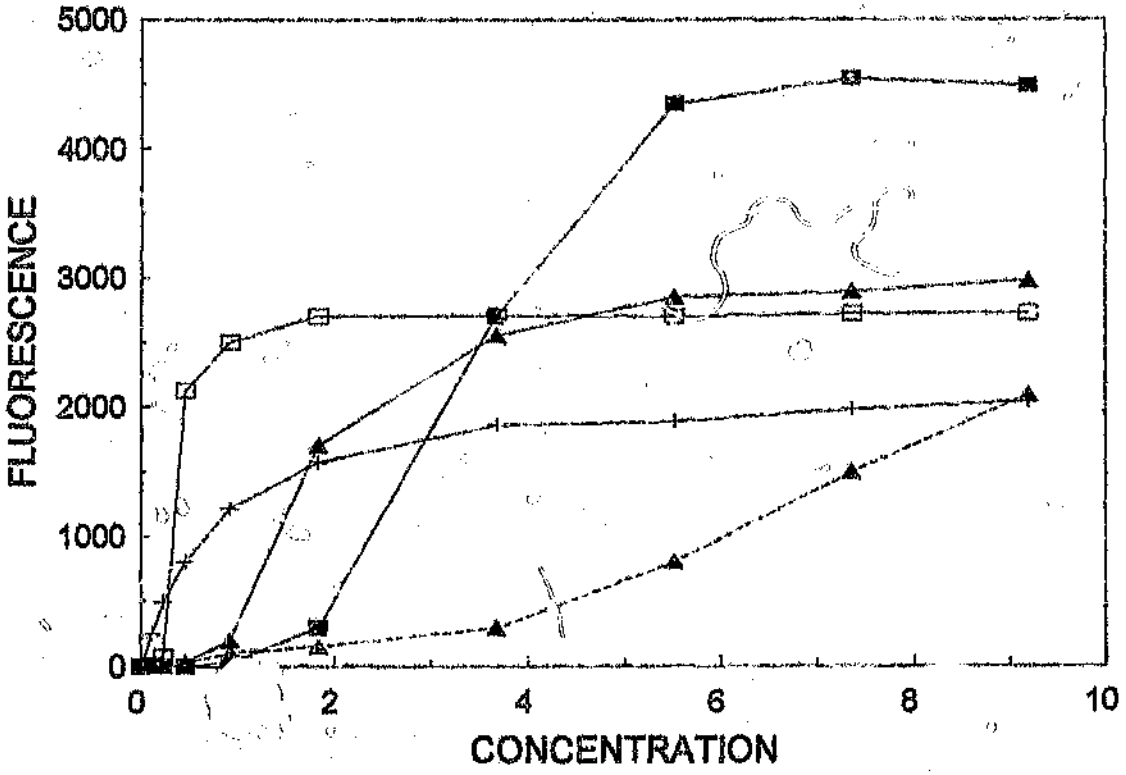


Figure 5.2. Non-specific fluorescence enhancement with increasing concentrations of a) cholate with 0.5 M NaCl (–▲– –) and b) SDS (i) without (–■—), with ii) 0.5 M NaCl (–□—), iii) 3.11 mM cholate (–▲—) and iv) 0.5 M NaCl and 3.11 mM cholate (–I—).

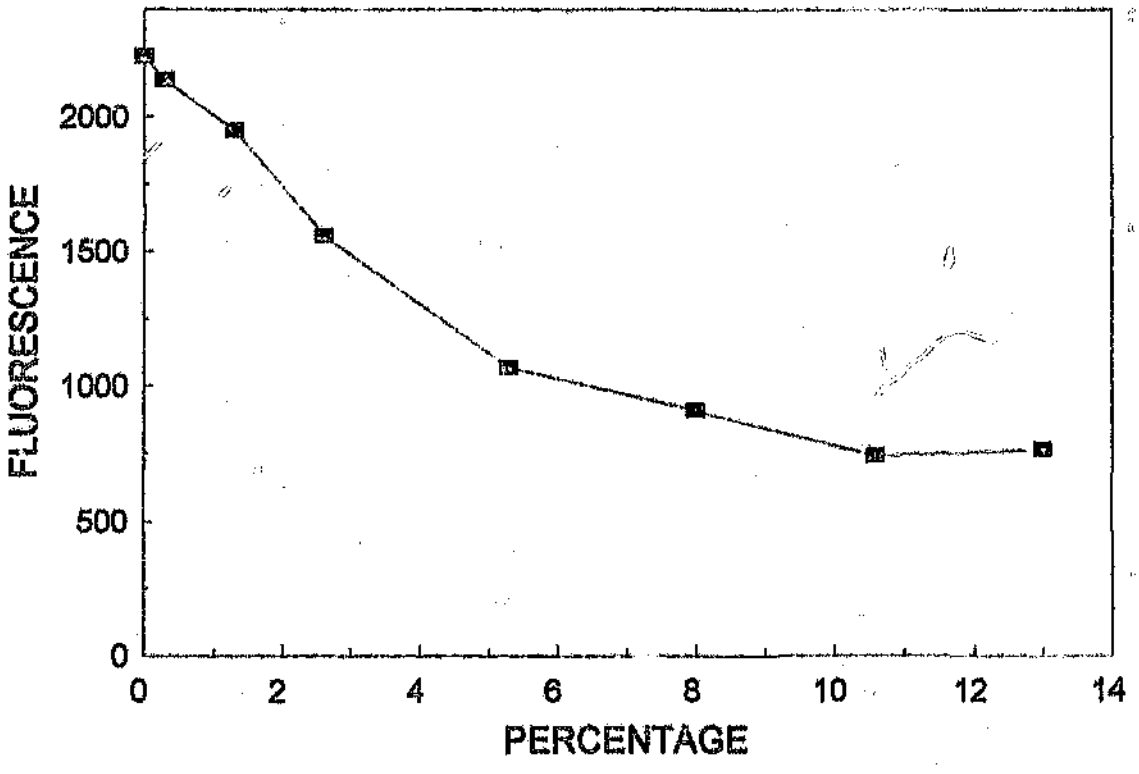


Figure 5.3. Effect of increasing n-butanol concentration on SDS-Hoe induced non-specific fluorescence at SDS concentration of 1.15 mM.

The non-specific absorbtion that is observed as a result of the interaction between Hoe and the SDS micelle, is not limited to SDS but the same effect is observed for other detergents such as the cationic detergent, CTAB and the nonionic detergent TX-100 at concentrations above their respective CMC's (Fig. 5.4).

The increase in fluorescence above the CMC of CTAB is further enhanced following an increase in the counterion concentration to 0.5 M, whereas for SDS in the presence of 0.5 M NaCl a decrease in fluorescence was observed (Figs. 5.2 and 5.4).

A mixture of cholate and CTAB results in the formation of mixed micelles (181) which also exhibits increased fluorescence in the presence of Hoe. An increase in the counterion, Br^- concentration results in a further enhancement in fluorescence which is greater than that observed for the pure CTAB micelle in the presence of 0.5 M KBr.

The addition of cholate to a SDS solution above its CMC results in a 30% decrease in fluorescence which is diminished by a further 30% with an increase in the counterion concentration (Figs. 5.2 and 5.4). At a constant Hoe concentration, an increase in fluorescence is also observed with increasing concentrations of cholate, however no decrease in fluorescence was observed in presence of NaCl (Fig 5.2).

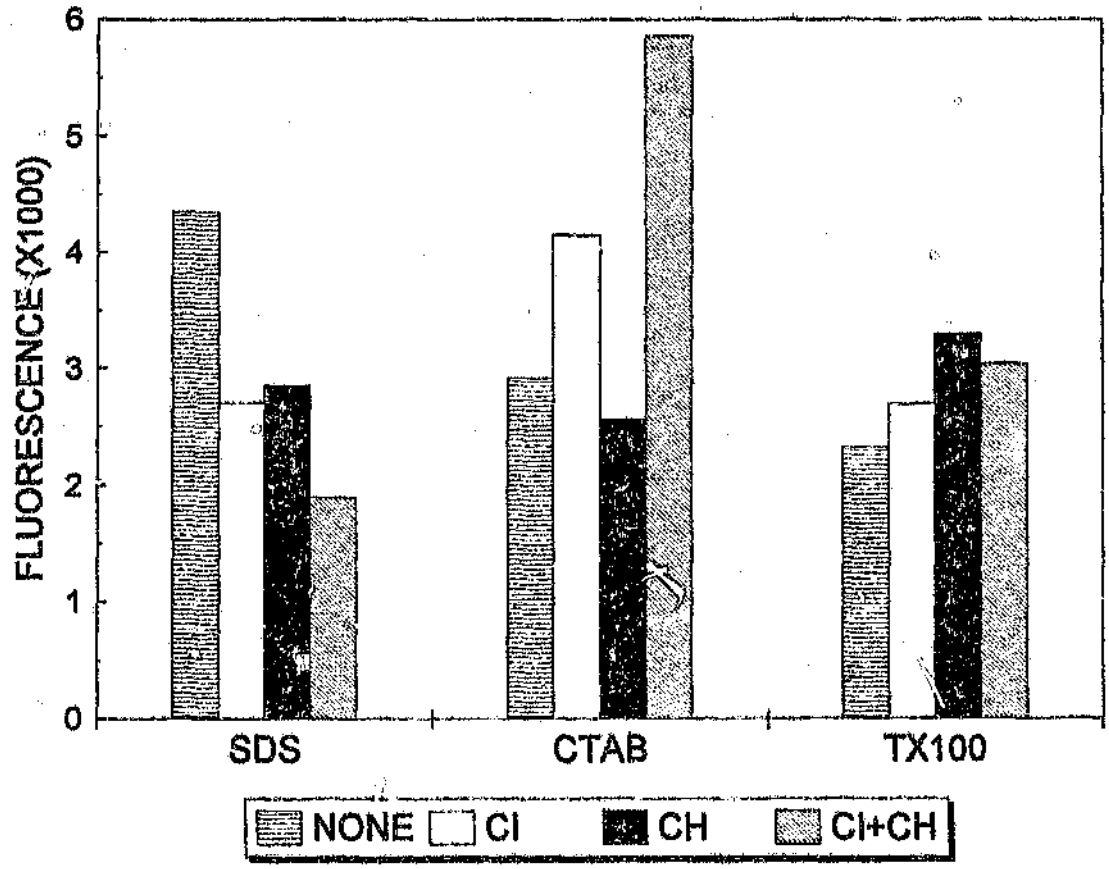


Figure 5.4. The effect of a) none, b) the counterion (Cl), 0.5 M NaCl for SDS and TX100, and 0.5 M KBr for CTAD c) 3.11 mM sodium cholate (CH) and d) 0.5 M Cl and 3.11 mM CH on fluorescence of 4.6 mM SDS, 3.66 mM CTAB and 0.13% TX100.

DNA standard curves.

With Hoe as fluorochrome a linear increase in fluorescence was observed with increasing DNA concentration (Fig. 5.5). In the presence of 2.3 mM SDS it is not possible to determine less than 200 ng DNA and it is only above this DNA concentration that a linear correlation between fluorescence and DNA concentration is obtained. The inclusion of cholate had no effect on the gradient of the DNA standard curve. In the presence of 2.3, 4.6 and 6.9 mM SDS and 3.11 mM cholate there is a decrease in the gradient of the DNA standard curve. However, linearity is maintained and it is possible to accurately measure as little as 15 ng/ml DNA in the presence of 0.06, 0.13 and 0.2% (2.3, 4.6 and 6.9 mM) SDS.

The interaction between the SDS micelle and Hoe is pH dependent. By increasing the pH of the buffer from 7 to 9 there is a 96% decrease in Hoe-SDS micelle fluorescence while at pH 9 there is only a 40% decrease in Hoe-DNA fluorescence (Fig. 5.6). At pH 9 with Hoe as fluorochrome a linear increase in fluorescence is observed with increasing DNA concentration (Fig. 5.7). At the same Hoe concentration in the presence of 4.6 mM SDS there is a decrease in the gradient of the standard curve. Linearity is maintained and it is still possible to accurately measure less than 15 ng/ml DNA in the presence of 0.13% (4.6 mM) SDS.

Assaying DNA in the presence of high concentrations of SDS facilitates the direct quantification of cellular DNA following cell dissolution with SDS (Fig. 5.8). Increasing the NaCl concentration from 0.5 to 2 M results in a 50% enhancement of fluorescence which can be further enhanced by 10%, following a 60 minute incubation of the cellular lysate with proteinase K. With this assay it is possible to measure as little as 5×10^3 cells/ml (Fig 5.9.) .

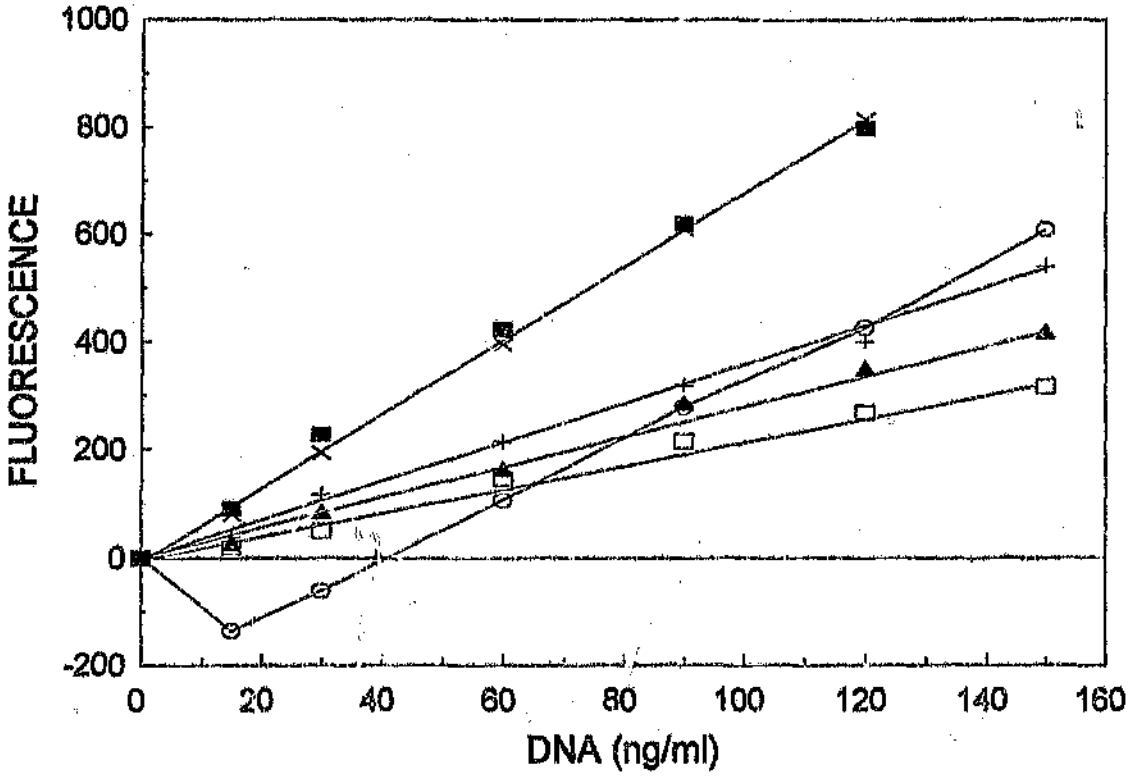


Figure 5.5. DNA standard curve, a) without (–■—), with b) 2.3 mM SDS (–o—) and c) 3.11 mM cholate (–x—), in the presence of i) 2.3 mM SDS (–+—), ii) 4.6 mM SDS (–▲—) and iii) 6.9 mM SDS (–□—).

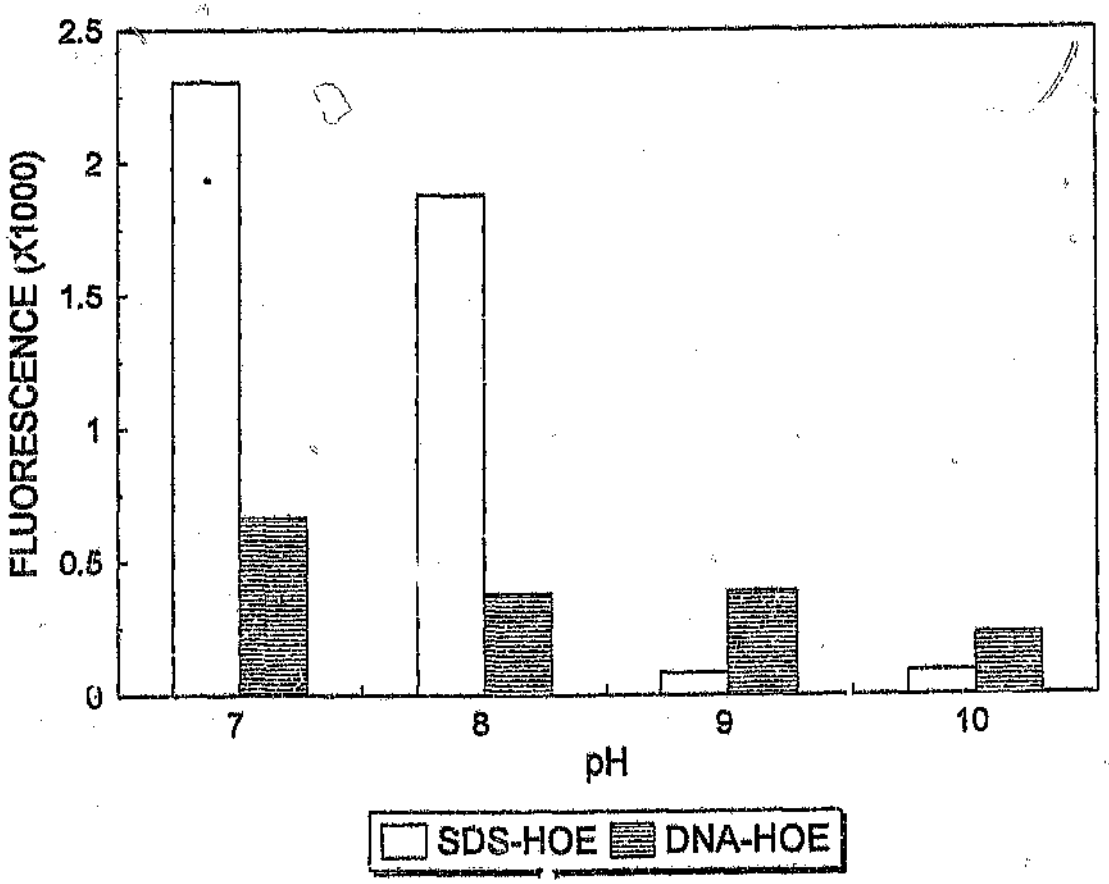


Figure 5.6. The effect of increasing pH on the interaction between Hoe-SDS and Hoe-DNA

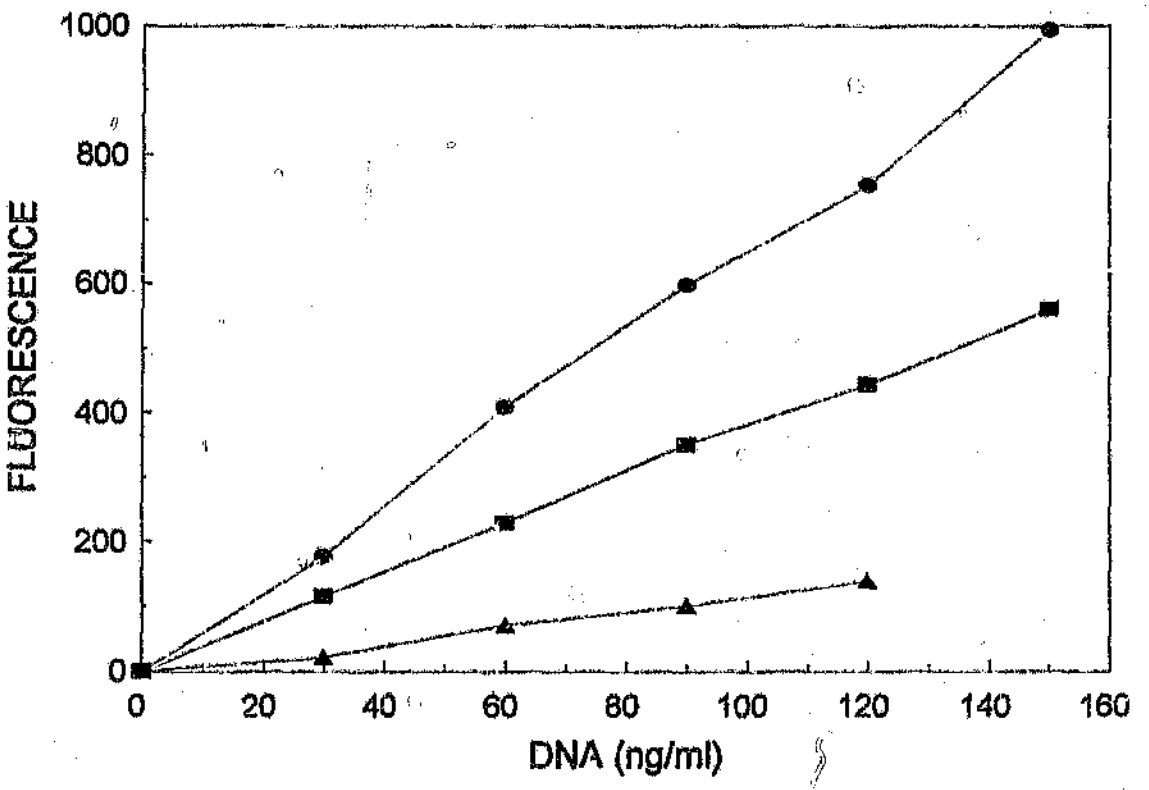


Figure 5.7. DNA standard curve a) pH 7 (–●—), b) pH 9 (–■—) and c) pH 9 in the presence of 4.6 mM SDS (–▲—).

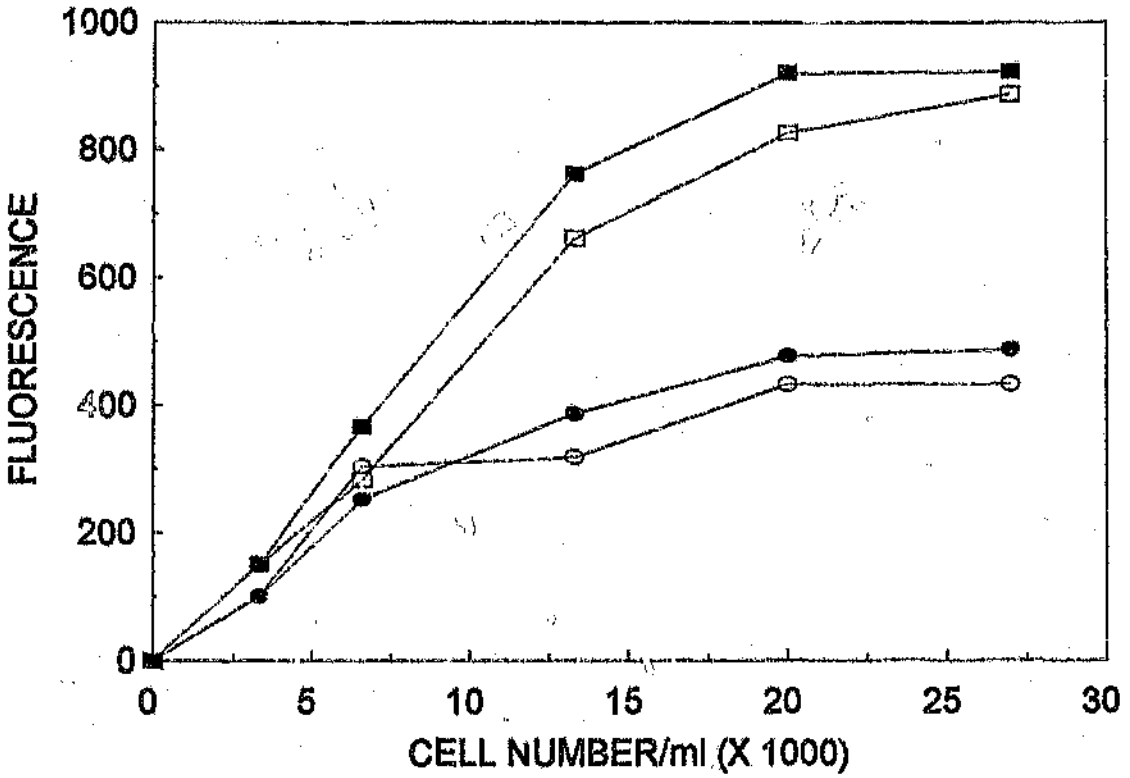


Figure 5.8. Measurement of cellular DNA in the presence of 4.6 mM SDS and 3.11 mM cholate in the presence of a) 0.5 M NaCl (–○—) and b) 500 ug/ml Proteinase K (–●—) c) 2 M NaCl (–□—) and d) 500 ug/ml Proteinase K (–■—).

The effect of DMBA on cell survival measured by the crystal violet, the succinate dehydrogenase inhibition and Hoe-DNA assay.

The effect of DMBA on cell survival was determined by measuring the decrease in succinate dehydrogenase activity, the uptake of crystal violet and cellular DNA. These parameters were measured, 6 days after 3, 24 and 48 hours exposure to DMBA. Two controls (i) without DMBA and DMSO and (ii) without DMBA and with DMSO were included. The presence of DMSO without DMBA increased cell growth measured by both methods by 15% (SEM 8%, for 5 different cultures). Exposure of PMEC to increasing concentrations of DMBA resulted a sigmoidal decrease in succinate dehydrogenase activity.

Exposure of PMEC to 0-16 μM DMBA for 3 hours at 0.05 μM (Fig. 5.10) resulted in a rapid decrease in succinate dehydrogenase activity of 70% and number of cells of 30% and 2% determined by crystal violet uptake and the Hoe-DNA assay respectively. Differences in the number of cells *in vitro* was observed for DMBA concentrations from 0-7 μM, while at DMBA concentrations greater than 7 μM both methods measured a 30% decrease in cell number.

Exposure of PMEC to 0-1.95 μM DMBA for 24 hours (Fig. 5.11) resulted in a 50% decrease in succinate dehydrogenase activity, an average of 30% decrease in the percentage of cells attached from 0-0.78 μM DMBA. At DMBA concentrations greater than 0.78 μM, no differences were measured by all three methods. Exposure of PMEC to 0-1.95 μM DMBA for 48 hours (Fig. 5.12) resulted in a sigmoidal decrease to 75% at 0.78 μM DMBA in succinate dehydrogenase activity and number of cells measured by crystal violet uptake. A less rapid decrease in the number of cells was observed when measuring cellular DNA. At DMBA concentrations greater than 1.56 μM DMBA no differences were observed for all three methods.

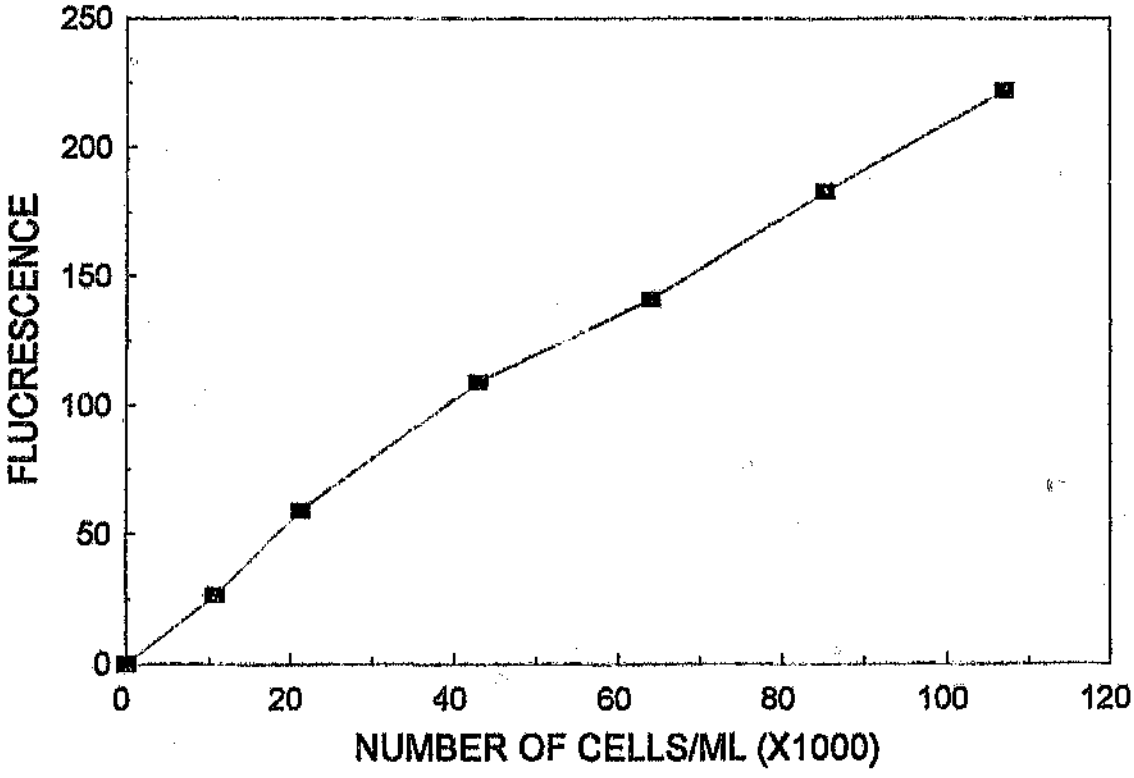


Figure 5.9. Standard curve for the determination of the number of PMEC in tissue culture. Monolayers derived from 2 different cultures of PMEC were trypsinized, washed and a concentration series of 0.5-10 $x10^4$ cells in 100 µl was prepared and the DNA content of each sample was determined with the Hoe-DNA assay.

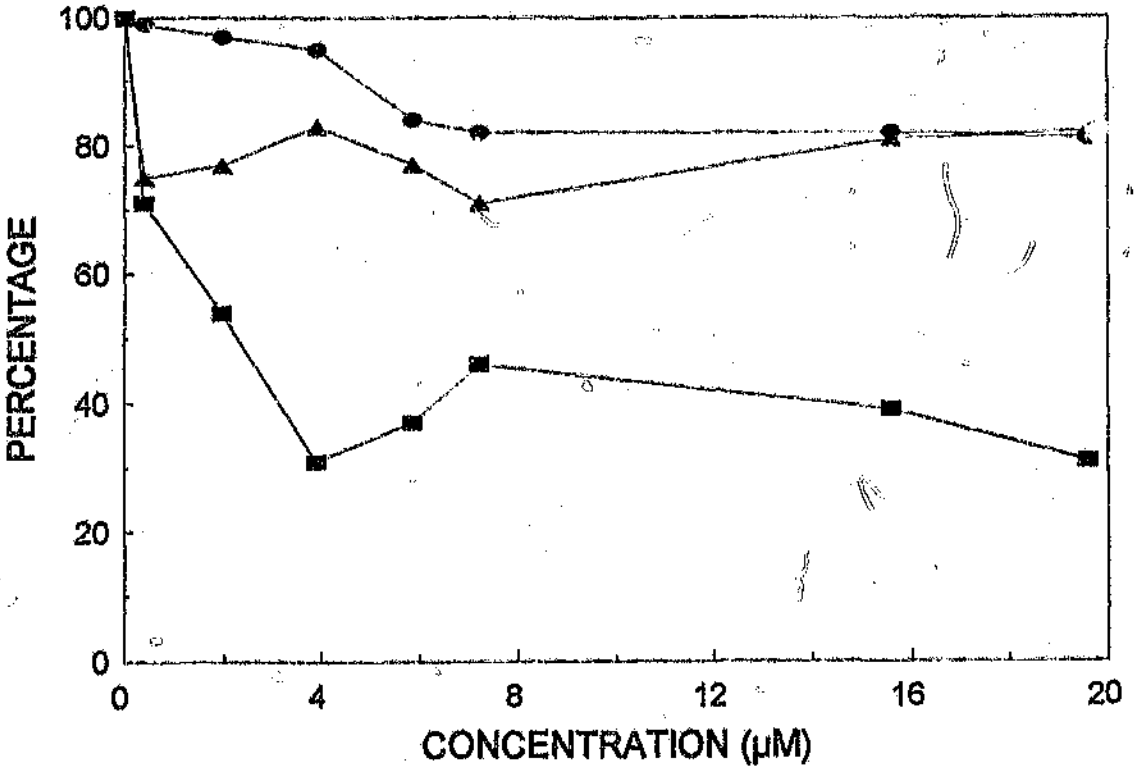


Figure 5.10. The effect of 3 hours exposure to increasing amounts of DMBA on cell survival as measured by the crystal violet (–■—)(n=4), the succinate dehydrogenase inhibition assay (–▲—)(n=4) and Hoe-DNA assay (–●—)(n=2).

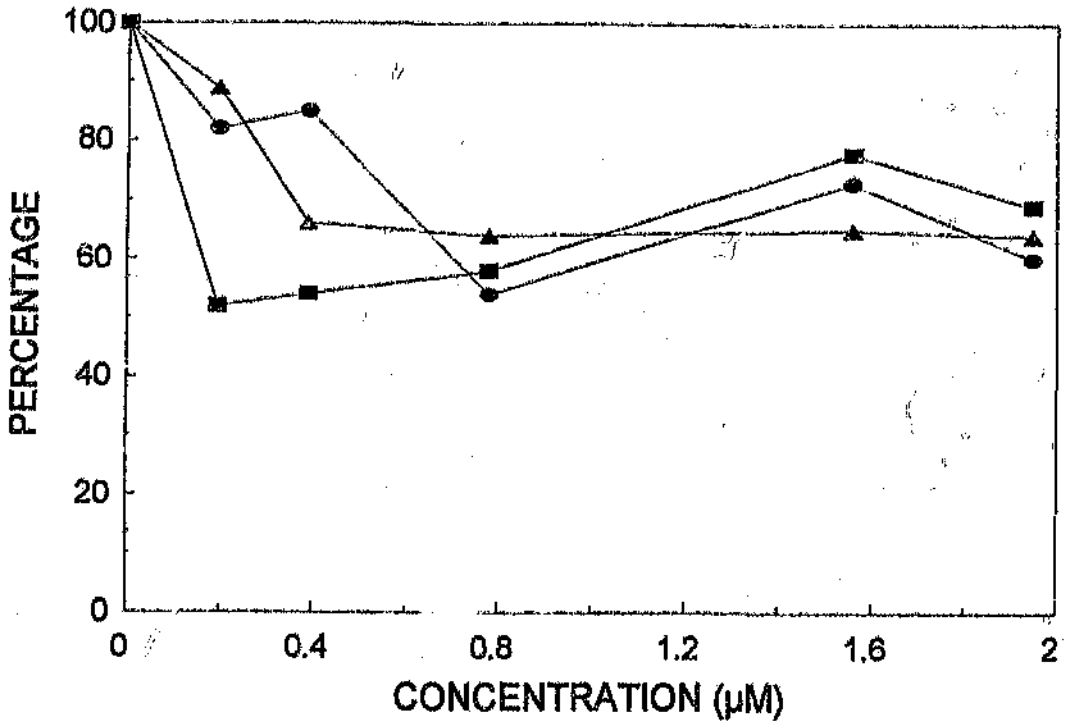


Figure 5.11. The effect of 24 hours exposure to increasing amounts of DMBA on cell survival as measured by the crystal violet (–■—)(n=4), the succinate dehydrogenase inhibition assay (–▲—)(n=3) and the Hoe-DNA assay (–●—)(n=2).

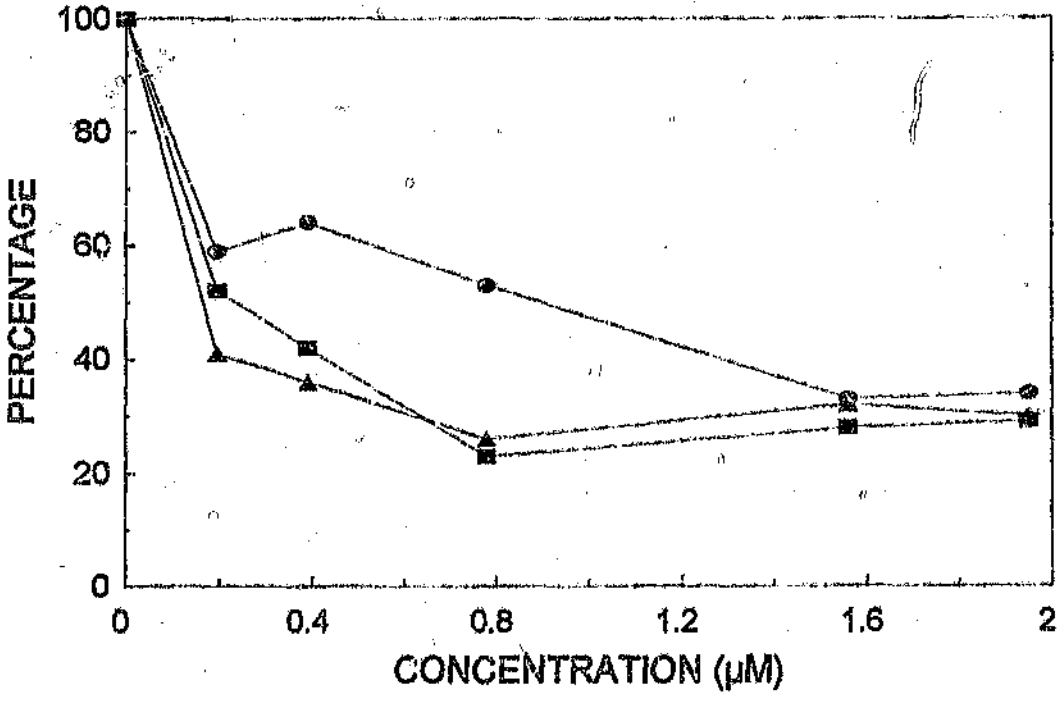


Figure 5.12. The effect of 48 hours exposure to increasing amounts of DMBA on cell survival as measured by the crystal violet (–■—)(n=2), the succinate dehydrogenase inhibition assay (–▲—)(n=3) and the Hoe-DNA assay (n=2)(–●—).

5.5 DISCUSSION

Colony formation is frequently used to determine cell survival following exposure to cytotoxic agents. This procedure is dependent on the sustained proliferation of single cells (166,168). Use of this method to determine cell survival is limited as PMEC harvested from each culture are few, high seeding concentrations are required and PMEC cultured in reduced calcium medium form diffuse colonies that are difficult to score. Furthermore, the results may require 2-3 weeks to obtain by which time many epithelial cell cultures may have undergone terminal differentiation.

Most methods used to determine cell number involve the measurement of total cellular protein or the activity of a specific enzyme. An important criterion when studying the effects of carcinogens, hormones, growth factors, and metabolic inhibitors on cell growth in culture is that the parameter used to determine cell number remains essentially unaltered. Cell DNA content remains essentially constant under most experimental conditions and is therefore directly proportional to the number of cells in culture.

Succinate dehydrogenase is part of the inner mitochondrial membrane is a component of Complex II of the electron transport chain and is highly sensitive to changes in cell homeostasis (167,168). The MTT assay measures succinate dehydrogenase activity with a tetrazolium dye (168), that serves as a hydrogen acceptor and is reduced to strongly absorbing formazan products which absorb at 565 nm. The development of a microassay overcomes limitations associated with few cells and a large number of samples. Furthermore, this method correlates with colony formation down to survival levels of 10% for a number of cytotoxic drugs and radiation (167,168).

A growth period of 6 days following exposure of PMEC to DMBA, effectively measures the resultant effect of DMBA uptake, adduct formation and removal, DNA repair and subsequent growth and/or cell death. C3H mouse embryo fibroblasts exposed to 0.1 μM DMBA for 24 hours, showed increased viability and transformation frequency after

6 days in culture (180).

Russo *et al* (126) exposed 3 different HMEC cell types, in tissue culture medium with normal and reduced calcium concentrations to DMBA for 24 hours. After 7 days in culture the toxic effect of low carcinogen concentrations were more pronounced in cells grown in low calcium medium, however at DMBA concentrations of above 1.95 µM the survival in both mediums were the same. Medium containing 1.05 mM calcium was more selective for measuring the long-term effect of the carcinogen on cell growth. An exposure time of 24 hours to DMBA results in a 50% decrease in cell survival comparable to HMEC *in vitro*.

Microassays developed for the determination of the total number of cells in culture measures the uptake of dye (131), total protein (168) or specific enzyme activity (168,181). However when studying the effect of carcinogens, hormones, growth factors and metabolic inhibitors on cell growth the parameter that is measured must stay unaltered. Cell DNA content remains essentially constant under most experimental conditions and is proportional to the number of cells in culture.

A sensitive method routinely used for the microdetermination of DNA estimates the increase in fluorescence, induced by Hoechst 33258 when the dye complexes with DNA (171-174). However, it was observed that the background fluorescence is enhanced in the presence of 0.05% sodium dodecyl sulphate (SDS), resulting in nonlinear calibration curves (175-177) This phenomenon has restricted the use of Hoe for the quantification of DNA in cell cultures. Therefore, the masking effect of SDS must be reduced to combine the effectiveness of SDS in cell dissolution with the specificity of Hoe for DNA analysis.

Micelles are aggregates of detergent molecules in which the hydrophobic moieties of the detergent molecules form the core of the micelle. Immediately surrounding the core is the Stern layer which not only contains the polar detergent head groups but also the counterions (181-184). James *et al* (185) proposed that cationic dye molecules such

as acridine orange undergo two types of interaction with the SDS micelle: an electrostatic interaction between the surface charge of the SDS micelle and the dye molecule as well as hydrophobic binding to the interior of the SDS micelle. Moulik *et al* (185) suggested that the electrostatic forces were the principal source of interaction and that hydrophobic interaction was secondary and only occurred in the presence of the first. The extent to which a dye molecule penetrates the hydrophobic interior of the SDS micelle depends on the type of polar head groups, the charge on the dye, and the micelle surface charge.

The Hoe molecule is a crescent-shaped cationic molecule with flat aromatic rings containing hydrogen-bond-forming NH groups along its concave edge (174,175). Normally the Hoe binds in the minor groove of DNA and displaces the spine of hydration that is present in the free DNA. With the formation of H-bonds there is a dramatic increase in fluorescence. Hoe therefore exhibits a high specificity for DNA and because of its high quantum efficiency it is ideal for the quantification of low concentrations of DNA. However, routine use of this assay in tissue culture has been limited due to nonspecific fluorescence observed in the presence of SDS (169,178,179).

Nonspecific fluorescence occurs above the CMC of SDS, and an increase in NaCl concentration results in an enhancement in fluorescence at lower SDS concentrations while the addition of n-butanol reduces fluorescence, therefore implying that fluorescence is caused by Hoe binding to the SDS micelle.

Binding of Hoe to the SDS micelle as well as the CTAB and TX-100 micelle implies that the interaction between SDS and Hoe is not purely an ionic interaction but that there is also a hydrophobic component involved.

Therefore, to measure DNA in the presence of SDS, the interaction between Hoe and the SDS micelle must be reduced. This could be achieved by altering the charge on the surface of the micelle, by increasing the counterion concentration, or by inducing the

formation of mixed micelles. Alternatively it may be possible to reduce the charge on the Hoe molecule by altering the pH of the buffer. However, it is essential that Hoe must still bind to DNA quantitatively so that it is still possible to determine nanogram amounts of DNA.

An increase in counterion concentration leads to a decrease in the CMC, associated with an increase in aggregation number and a decrease in the surface potential of the micelle. An increase in the NaCl concentration results in a reduction in fluorescence at concentrations of SDS above its CMC. James *et al* (184) proposed that a decrease in the surface potential of the SDS micelle is associated with increased penetration of cationic dyes into the SDS micelle. We found that the decrease in fluorescence, associated with an increase in counterion concentration, was not caused by a shift in the emission maximum, but rather by decreased hydrogen bonding as the fluorochrome shifts into the more hydrophobic environment of the inner core.

This is supported by the observation that in the case of the cationic detergent CTAB, an increase in the KBr concentration resulted in an increase in fluorescence at detergent concentrations above the CMC. A decrease in the surface potential results in the surface of the CTAB micelle becoming less positive, leading to an increase in the electrostatic interactions between the CTAB micelle and the Hoe molecule. Furthermore, a mixture of cholate and CTAB results in the formation of mixed micelles with the production of aggregates which are small, approximately spherical, and only slightly charged (185,187). Lower fluorescence is observed for the mixed CTAB-cholate micelle than for the pure CTAB micelle. However, an increase in the counterion concentration results in an enhancement of fluorescence greater than that observed for the pure CTAB micelle in the presence of 1 M KBr.

Although cholate and SDS are not known to form mixed micelles, we have observed that in the presence of cholate there is a decrease in fluorescence which is further enhanced following an increase in the counterion concentration. The mechanism involved is unknown. It can be speculated that since cholate micelles also bind Hoe,

the ..ate micelle competes with SDS for Hoe binding or alternatively that cholate promotes the absorbtion of the Hoe molecule into the SDS micelle.

James *et al* (185) observed that an increase in the pH of the environment of a cationic dye favours absorbtion. It can be speculated that, with an increase in the pH of the buffer system used the Hoe molecule becomes more neutral and shifts to a more hydrophobic environment within the micelle. Therefore, a decrease in H-bonding is associated with a decrease in fluorescence. At pH 9 the Hoe molecule is still able to quantitatively bind to DNA.

By increasing the counterion concentration and adding cholate or increasing the pH of the buffer used it is possible to accurately determine DNA in the presence of SDS. In the absence of cholate and high levels of NaCl, at a SDS concentration of 2.3 mM (0.13%), it is only possible to accurately determine DNA concentrations above 66.6 ng/ml. The gradients of the DNA standard curves in the presence of SDS and cholate differ from standard curves determined in the absence of SDS. Cholate alone does not cause a decrease in the gradient of the DNA curve. The results obtained strongly suggest that Hoe still binds to the SDS micelle but factors which promote absorbtion into the inner core, result in a significant reduction in the masking effect of SDS, allowing the accurate determination of DNA in the presence of SDS.

We have found that the nonspecific fluorescence observed as a result of Hoe binding to the SDS micelle can be reduced by adding butanol, by increasing the ionic strength or the pH of the buffer used, or by adding cholate.

Although it is possible to reduce the masking effects of SDS by using a buffer system at pH 9, the addition of NaCl at concentrations required to reduce histone-DNA interactions (188) results in the precipitation of SDS. Therefore, we favour the use of a combination of cholate and a buffer system with an increased ionic strength to reduce the masking effect of SDS. The addition of cholate not only has the advantage of reducing the level of nonspecific fluorescence, associated with Hoe binding the SDS-

micelle, but it also increases the solubility of SDS in high salt solutions. Furthermore, buffers containing NaCl not only reduces nonspecific fluorescence but also NaCl concentrations above 2M promote the dissociation of histone proteins from DNA, resulting in a 50% enhancement in fluorescence, as reported by Labarca and Paigen (172). The addition of proteinase K results in increased stability of fluorescence and a further 10% enhancement of fluorescence.

A sigmoidal decrease in succinate dehydrogenase activity was observed with increasing concentrations DMBA following exposure for 3, 24 and 48 hours. At 1.95 µM DMBA for 3 hours, viability of PMEC decreases to 40% and the number of cells attached to 99 or 75 percent, measured by crystal violet uptake or DNA-Hoe assay. At 1.95 µM DMBA for 24 and 48 hours viability and percentage attachment of PMEC decreases to 60 and 30 percent respectively.

Criteria developed by Kung *et al* (189) to study the cytotoxic effects of cell cycle phase specific agents can be applied to PMEC exposed to DMBA. The criteria for the end points of "cell death" are the loss of membrane integrity without detachment, cell lysis or cell detachment and the classical clonogenic survival assay. Loss of membrane integrity may account for the differences observed between number of cells measured either by crystal violet uptake or Hoe-DNA assay at low DMBA concentrations. Increased membrane permeability results in increased protein leakage and an underestimation of cell numbers determined by crystal violet uptake (170). At higher concentrations of DMBA cell lysis with cell detachment is the dominant effect and accordingly no differences are observed in both assays used to determine cell numbers *in vitro*. A correlation between succinate dehydrogenase activity and crystal violet is only observed when PMEC are exposed to DMBA for 24 and 48 hours.

Indications are that cellular transformation of PMEC in vitro with DMBA is a rare event which is further corroborated by the inability to induce tumours in the primate, *Cercopithecus aethiops pygerthrus* (190) with this carcinogen. Factors such as cell density (180), medium conditioning (180) and calcium content (126, 191,192) of

medium, may modulate a cell's ability to undergo cellular transformation. Other elements, including mammary gland differentiation (126), expression of genes such as bcl-2, p53, c-myc (189-193) and cyp1b1 (180) may predetermine the eventual result of exposure to a carcinogen.

CONCLUDING DISCUSSION

The development of rodent model systems to study the process of breast carcinogenesis has increased our knowledge of mammary gland development, differentiation and mammary carcinogenesis. *In vitro* studies with normal HMEC, has revealed that human mammary carcinogenesis is a complex multistage process and extrapolation between both species is difficult. Mammary epithelial cells from the non-human primate, *Cercopithecus pygerythrus* were established in cell culture to determine whether studies within this cell population could address some of the differences observed between rodent and human mammary cancer models.

Normal mammary epithelial cells established in cell culture should reflect the heterogeneity of the normal population and must express biological markers associated with normal mammary tissue. Furthermore, tissue culture conditions which extend the longevity of normal mammary epithelial cells *in vitro* would permit more detailed studies within a single cell population.

Selective collagenase and hyaluronidase digestion results in the isolation of organoids which are clusters of epithelial and myoepithelial cells. Organoids have retained much of the lobular structure of the tissue of origin. As for rat and human mammary tissue four lobule types (10,22,23,25), Lob 1-4 were identified in primate mammary tissue. Using a classification described for human organoids, cultures derived from primate mammary tissue were grouped according to the degree of lobular development. Less differentiated cultures contained organoids with Lob 1 development, moderately differentiated, a mixture of Lob 1 and 2, and Lob 2 and well differentiated, Lob 3. Cultures derived from tissue with Lob 1 and Lob 1 and 2 development attached more rapidly than those derived from mammary tissue with Lob 2 and Lob 3 development. Subsequent organoid growth reflected the lobular development of the mammary gland of origin. Organoid growth in cultures derived from less differentiated mammary tissue, grew more rapidly than those derived from moderately and well differentiated mammary tissue. Russo *et al* (126) observed that human mammary organoids reflect in culture

certain properties of the intact tissue, where less differentiated lobular structures with a greater proliferative rate attached and grew more efficiently than the more differentiated and less proliferating lobules. Although primate organoid attachment and growth is more rapid than observed by Russo *et al* (126) for human organoids, it does reflect mammary gland differentiation.

Growth in a tissue culture medium was limited and a reduction in the calcium content increased the lifespan of PMEC *in vitro*. Calcium *per se* does not promote cell growth but it does uncouple terminal differentiation from cell proliferation thereby keeping PMEC responsive to the mitogenic effects of growth factors. The mechanisms of this process must still be determined. Furthermore, a reduction in calcium has a dramatic and reversible effect on cell morphology. *In vitro* PMEC grow as typically cuboidal cells with a high degree of intercellular contact, desmosomes and few microvilli. In contrast a decrease in the calcium content of the tissue culture medium causes PMEC to become rounded with little cell-cell contact, the number of microvilli on the surface increased and few desmosomes were observed. This effect is also observed in other epithelial cell types (34,35,61,78-81). No contact inhibition occurs and at confluence new cells were shed into the tissue culture medium. These cells could be collected and plated to give rise to subsequent cultures of PMEC. The doubling times of cultures derived from less and moderately differentiated mammary tissue are highly variable. The population doubling times determined for HMEC (126) derived from less and moderately differentiated mammary tissue and grown in the same medium as PMEC also varied considerably. These differences in cell growth for cultures derived from mammary tissue with the same degree of lobular development could possibly be ascribed to the genetical heterogeneity of the population.

The expression of specific cytokeratins are not only unique to epithelial cells of the mammary gland but can also distinguish between the luminal, myoepithelial and possibly a combined phenotype. Myoepithelial cells express keratins K5, K14, K15 and K17, luminal epithelial cells express keratins K7, K8, K18 and K19 (54,56,62), while an intermediate "stem" cell types expresses both types of keratins (60,66).

Cultures derived from human milk express K7, K8, K18 and K19 associated with the luminal cell phenotype. HMEC derived from reduction mammaplasty organoids, express K7 and K14, but not not K19 while in later passage an increase is observed in K18 (54). The HMEC line, MCF-10 and MCF-12 and subsequent sublines developed from fibrocystic mammaplasty tissue expressed predominantly K7, K8, K15, K16 (77). The presence of K14 and K19 was variable and expression of K19 was lost in later passage. Dairkee *et al* (62) quantified keratin expression in HMEC *in vitro* and observed that K6 followed by K5 and K14 were the predominant keratins expressed by HMEC. K7, K8 and K18 were only minor components.

K8 is the predominant cytokeratin followed by K6/K11 (indistinguishable due to similarities in M_R) and K7 in primate mammary tissue. PMEC expresses the same cytokeratins as primate mammary tissue, with increased expression of K7 and K6/K11 with respect to K8 is observed. The mitogenic effect of growth factors on HMEC results in increased expression of K6 (58,62). K11 is also a luminal keratin found in mouse mammary epithelial cells (10). The use of a monospecific antibody would distinguish between K6 and K11. K5 as well as K8 are minor components of PMEC *in vitro*. Although the expression of K14 still needs to be determined in PMEC *in vitro*, increased expression of K7 and decreased expression of K5 may verify that PMEC *in vitro* express cytokeratins associated with the luminal phenotype. The expression of a combined phenotype by PMEC can only be established by immunohistochemical procedures.

The MFGP fraction isolated from milk contains membrane proteins representative of the epithelial cell of the mammary gland. Human anti-MFGP is specific for all components of the human MFGP fraction but only the 70 kDa (MFGP-70) protein present in the primate MFGP fraction. A linkage protein bound by disulphide bonds to a larger molecule is a common characteristic of a family of mucin proteins (143-146). Ultrastructurally these mucins consist of high molecular mass subunits joined by disulphide bonds to a smaller linkage protein. The epitopes of the small linkage

proteins are "cryptic" and are probably shielded within the folded protein domains by large glycosylated moieties. Proteolytic digestion, forms fragments composed of oligosaccarides that are tightly packed on a central protein core (145). Subsequent thiol reduction leads to an enrichment of this linkage protein. In both the human and primate MFGP fraction MFGP-70 was resistant to trypsin digestion and was covalently bound by disulphide bonds to a larger protein. Electrophoresis, without reduction of disulphide bonds revealed the presence of a 53 kDa protein in both fractions that stained positively with anti-MFGP.

Peterson *et al* (151) ascribed that the poor immunocytochemical staining of normal and cancerous tissue observed with an antibody specific for MFGP-70, to the cryptic nature of MFGP-70. Anti-MFGP did not identify MFGP-70 in lysates of MCF-7 and PMEC, or trypsin digests of MCF-7 and PMEC cell lysates following reduction of disulphide bonds. A 65 kDa and a 62 kDa protein, thiol-linked to a large protein, were detected in cell lysates of MCF-7 cells and PMEC respectively. Both proteins did not stain with anti-MFGP. Under non-reducing conditions an immunopositive protein of 53 kDa was detected in cell lysates of MCF-7 cells and PMEC. This protein may consist of two smaller proteins which are thiol linked or be a single protein with an antigenic determinant dependent on an intact disulphide bond. Whether this protein is related to other MFGP with similar M_R such as bovine PAS-6, PAS-7, mouse MFG-E8 and guinea pig GP-55 (153-157) must still be determined.

Antibody HMFG-2 specific for human sialomucins (74,77,160-162) does not stain MFGP derived from lactating primate mammary tissue or PMEC. The development of species specific antibodies would greatly assist in the detection of primate associated sialomucins. Furthermore, the core protein of human mucins is highly conserved and mucin molecules from different sources differ in the degree and type of glycosylation. As glycosylation is a posttranslational modification use of human cDNA encoding for a domain of the core protein can be used to determine mRNA expression in PMEC (159).

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Dimethylbenz(a)anthracene (DMBA) has been identified as a potent carcinogen that induces mammary tumours in rodents (10,22,26,115,166). Exposure of HMEC *in vitro* to DMBA results in the expression of a partially transformed phenotype with no tumour formation in SCID mice (102-104). The effect of DMBA on the PMEC survival was determined by measuring carcinogen induced succinate dehydrogenase inhibition. The number of PMEC in culture was determined by measuring crystal violet uptake and cellular DNA. A sensitive assay was developed for the direct measurement of cellular DNA in cell lysates using fluorochrome Hoechst 33258 (170). The routine use of Hoe for the determination of the DNA content of cell lysates has been limited due to nonspecific fluorescence as a result of Hoe binding to the SDS micelle. This effect could be reduced by increasing the concentration of the counterion, the addition of cholate or increasing the pH of the buffer. An assay was developed combining the effectiveness of SDS in cell dissolution with the sensitivity of Hoe to determine cellular DNA (170).

PMEC were exposed to 0-16, 0-1.95, and 0-1.95 μM DMBA for 3, 24 and 48 hours respectively. A 6 day growth period following DMBA exposure effectively measures the resultant effect of DMBA uptake, adduct formation, removal, DNA repair and subsequent growth and/or cell death. A sigmoidal decrease in succcinate dehydrogenase activity was observed for 3, 24 and 48 hours exposure to DMBA. Following 3 hours exposure to DMBA a 60% decrease in cell activity is associated with a significantly lower decrease in cell numbers when measured by crystal violet uptake (131) and cellular DNA (170). At longer exposure times of 24 and 48 hours, differences between all three methods are observed at low DMBA concentrations which become insignificant at higher DMBA concentrations. A decrease to 60% and 30% succinate dehydrogenase activity and cell number is observed following 24 and 48 hours exposure to 1.95 μM DMBA. These observations can be explained according to the criteria of Kung *et al* (189) used to describe the end points of cell death. These are loss of membrane integrity without dettachment, cell lysis associated with cell dettachment and the classical clonogenic survival assays or inhibition of succcinate dehydrogenase activity. Loss of cell integrity without dettachment accounts for differences observed

beween the crystal violet uptake and DNA measurement at low DMBA concentrations. This can also account for the differences in the percentage cells measured by both methods and succinate dehydrogenase activity following 3 hours exposure to DMBA. It is only at longer exposure times and higher concentrations of DMBA that decreased cellular viability is assocciated with cell lysis and cell dettachment.

Russo *et al* (126) exposed 3 different HMEC cell types, in tissue culture medium with normal and reduced calcium concentrations, to DMBA for 24 hours. After 7 days in culture the toxic effect of low carcinogen concentrations were more pronounced in cells grown in low calcium medium, however at DMBA concentrations above 1.95 µM the survival in both mediums were the same. HMEC exposed to 1.95 µM DMBA for 24 hours resulted in a 50% decrease in cell survival comparable to PMEC *in vitro*. Furthermore exposure of HMEC derived from less differentiated mammary tissue to DMBA only resulted in partial transformation associated with increased survival, colony formation efficiency alterations in karyotype and activation of the *ras* proto-oncogene. These cells did not form tumours in nude mice. Indications are that cellular transformation of PMEC *in vitro* with DMBA is also a rare event which is further corroborated by the inability to induce tumours in the primate, *Cercopithecus aethiops pygerthrus* with this carcinogen (190).

The growth responsiveness of primate organoids and primate mammary epithelial cells *in vitro*, reflects mammary gland differentiation. Cytokeratins expressed *in vitro* by PMEC are characteristic of the mammary tissue of origin. PMEC *in vitro*, express a MFGP, MFGP-53 that is present in the human and primate MFGP fraction as well as the MCF-7 cell line. In conclusion, the PMEC *in vitro* has growth properties, expresses keratins and MFGP antigens associated with the primate mammary gland, and which are analogous to the HMEC *in vitro*. The carcinogen DMBA induces a dose and time related decrease in PMEC cell surival measured using the succinate dehydrogenase inhibition assay. Routine measurement of cell numbers by measuring crystal violet uptake underestimates cell numbers *in vitro* (170). Subsequently a new method, for cell

enumeration by measuring cellular DNA was developed. In conclusion, many attributes of the PMEC *in vitro*, are similar to the HMEC *in vitro*. This identifies the PMEC *in vitro* as an cell culture system that can contribute significantly to understanding some of the mechanisms involved in mammary carcinogenesis.

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