# REGULATION OF PHENOTYPIC EXPRESSIONS IN LUNG CANCER MODELS IN VITRO AND IN VIVO

*By*

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## ABBREVIATIONS

| | |
|---|---|
| ABC | Avidin-biotin complex |
| AP | Alkaline phosphatase |
| APUD | Amine precursor uptake and decarboxylation |
| BLI | Bombesin-like immunoreactivity |
| BSS | Balanced salt solution |
| CEA | Carcinoembryonic antigen |
| CKBB | Creatine kinase BB isoenzyme |
| DAB | Diaminobenzidine |
| dbcAMP | N6-2'-O-dibutyryl adenosine 3':5'-cyclic monophosphate |
| DCG | Dense core granule |
| DDC | Dopa decarboxylase |
| Dex | Dexamethasone |
| DMEM | Dulbecco's modification of Eagles minimum essential medium |
| DMF | Dimethylformamide |
| DMSO | Dimethyl sulphoxide |
| DOPA | Dihydroxyphenylalanine |
| ECM | Extracellular matrix |
| EDTA | Ethylene diamine tetra-acetic acid |
| EGF | Epidermal growth factor |
| ELISA | Enzyme linked immunoadsorbant assay |
| F10 | Ham's F10 medium |
| FBS | Foetal bovine serum |
| FIF | Formaldehyde Induced Fluorescence |
| GAGS | Glycosaminoglycans |
| HBSS | Hanks, balanced salt solution |
| HCG | Human chorionic gonadotrophin |
| HEPES | N-2-Hydroxyethylpiperazine-N'-2-ethane sulfonic acid |
| HMBA | N,N'-Hexamethylene bisacetamide |
| HRP | Horse radish peroxidase |
| i.p | Intraperitoneal |
| i.v | Intravenous |

| | |
|---|---|
| IP | Immunoperoxidase |
| LDH | Lactate dehydrogenase |
| M. Wt. | Molecular weight |
| MES | 2-[N-morpholino]ethane sulphonic acid |
| MOG | Medical Oncology Glasgow |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| Na-But | N-Butyrate, sodium salt |
| NBF | Neutral buffered formalin |
| NE | Neuroendocrine |
| NEB | Neuroepithelial bodies |
| NMF | N-methylformamide |
| NSCLC | Non-small cell lung cancer |
| NSE | Neuron specific enolase |
| p.s.i | per square inch |
| PA | Plasminogen activator |
| PAP | Peroxidase anti-peroxidase |
| PBS | Phosphate buffered saline |
| PDGF | Platelet derived growth factor |
| PHF | Pre-cultured heart fragment |
| PU | Plough units |
| RIA | Radioimmunoassay |
| RPMI 1640 | Roswell Park Memorial Institute medium 1640 |
| RSV | Rous sarcoma virus |
| SCLC | Small cell lung cancer |
| SDS | Sodium dodecyl sulphate |
| SEM | Scanning electronmicroscopy |
| TBS | Tris-buffered saline |
| TCA | Trichloroacetic acid |
| TEM | Transmission electronmicroscopy |
| TGF | Transforming growth factor |
| TNF | Tumour necrosis factor |
| TPA | Tumour promoting agent |
| TRIS | Tris-(hydroxymethyl)-aminomethane |
| W/V | Weight/volume |

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## DEDICATION

**To my mother**

## DECLARATION

I declare that the work presented in this thesis has been carried out solely by me, except where indicated in the text and below.

The studies with the *in vitro* invasion assay were performed in collaboration with Professor L.I. de Ridder, Laboratory of Histology, State University of Ghent, Belgium. Southern blotting was performed by Dr Jas Lang of the Beatson Institute.

The oncogene studies with mink lung cells (Chapter five) were presented at the 30th Annual General Meeting of the British Association for Cancer Research in Glasgow (May, 1989), at the 37th Annual Meeting of European Tissue Culture Society in Graz, Austria (August, 1989), at the Gordon Research Conference on Cancer in Newport, Rhode Island, USA (August, 1989), and at the Meeting of the Pathological Society of Great Britain and Ireland in London (January, 1990).

The work performed on the studies of human SCLC models (Chapter three) has been accepted for presentation and publication in the Third International Conference of Anticancer Research in Marathon, Greece (October, 1990).

## SUMMARY

The objectives were to investigate the relationship between lung cancers and to determine the effects of micro-environmental and genetic manipulation on regulation of phenotypic expression.

A small cell lung carcinoma (SCLC) cell line, NCI-H69 was selected as a model for the study of heterogeneity. Two adherent sublines (H69V and H69VZ) have been isolated, and characterized, for species and lineage confirmation, and for expression of neuroendocrine markers. They have also been characterized for their growth, morphological properties, and invasiveness *in vitro*, radiation- and chemo-sensitivity. The cell lines have also been investigated for tumorigenicity, invasion and metastases formation *in vivo*. The cell lines were also investigated for c-myc expression by immunostaining.

Significant morphological differences have been found in culture, between the parental and the derivative lines. While the parental line grew as densely packed free floating cellular aggregates, the sublines grew as a monolayer of epithelioid cells. Both the derivative lines either expressed very low levels or did not express neuroendocrine cell markers including L-dopa-decarboxylase, creatine kinase-BB isoenzyme, bombesin-like immunoreactivity, neuron specific enolase, and neurosecretory type granules, compared to the parental line. All the lines stained positively for epithelial markers. Karyotypic analysis of the derivative lines showed features of SCLC. Differences have also been observed in their growth characteristics, both *in vitro* and *in vivo*. The growth rates of both the derivative lines are faster than the parental line, with doubling times closer to non-small cell lung carcinoma (NSCLC) cell lines. *In vitro* invasion studies of the cell lines showed early, single cell invasion in the parental cells, while solitary invasion was absent in both the derivative lines. Both the parental and the derivative lines were tumorigenic in nude mice. The parental line was both invasive locally, and formed multiple metastatic lesions in visceral organs, while local invasion was not found in either of the derivative lines, and they were non-metastatic. Xenograft histology of all the lines revealed an anaplastic, pleomorphic and rather large cell type tumours. However, the derivative lines showed focal spindle cell morphology, and spaces filled with an amorphous material, which was alcianophilic, and some of the cells contained glycogen. These features were not found in the parental line. Therefore,

it seems likely that H69 cell line contains cells of both small and non-small cell like phenotypes, suggesting a common origin for these two cell types. Further studies showed that H69 clones were able to give rise to a heterogeneous cell population similar to the parental line. These data suggest a common origin for both derivative and parental lines.

Having established that phenotypic changes can occur spontaneously in H69 cells, they were also investigated further to establish whether similar phenotypic changes could be induced in these cells by micro-environmental manipulation. The cells were exposed to different chemical inducers, and investigated for various parameters as above. The specific aim was to see whether malignant cells still respond to changes in microenvironment, and if they do, whether it was possible to alter the malignant behaviour of cells by micro-environmental manipulation.

Cells treated with hexamethylene bisacitamide (HMBA), dibutyryl cyclic adenosine monophosphate (dbcAMP), and sodium butyrate (Na-But) showed cytostatic effects on cell growth in culture, changes in culture morphology, inhibition of invasion *in vitro*, decrease in final tumour volume *in vivo*, changes in tumour histology, and reduction in expression of some neuroendocrine markers e.g L-dopa decarboxylase, suggesting that the H69 cell phenotype can be regulated be micro-environmental alterations.

A NSCLC cell line (WIL) derived from an adenocarcinoma of lung was selected for the study of phenotypic expression in this group of lung cancer, caused by varying the micro-environmental influences. Growth of cells in conventional monolayer, with and without stromal interactions, was compared with the growth in a histotypic tissue culture model and xenografts *in vivo*. The malignant cells grown in optimum conditions (histotypic culture) also were exposed to various chemical agents, and effects on morphology both at light and electronmicroscopic level, and on the production of mucin-like glycoproteins, and immunohistochemical markers were analyzed. Cells grown on collagen in histotypic culture showed increased production of mucin-like glycoproteins and formation of surface microvilli, and intracellular mucin-like inclusions. Effects in histotypic culture were similar to *in vivo* growth of cells as xenografts in nude mice.

For the study of the effects of oncogene transfection on lung epithelial cells, the MvlLu cell line derived from mink lung, was transfected with human c-myc, and normal and activated Ha-ras oncogenes individually. The parental and the three

derivative lines were characterized following transfection. Though the MvlLu line was already immortalized, it is contact inhibited, has a very low cloning efficiency in suspension and low tumorigenic potential, and shows a normal response to serum and growth factors. Several parameters were selected to evaluate the effects of oncogene transfection on the induction of the malignant phenotype. These included a high saturation density, a high growth fraction at saturation density, serum and anchorage independent growth, response to cytotoxic drugs, plasminogen activator activity, invasiveness *in vitro*, tumorigenicity, invasiveness, and metastasis *in vivo*. were also characterized morphologically, immunohistochemically, and for their growth characteristics both *in vitro* and *in vivo*. Morphology of transfected cells was altered, with a more spindle shaped morphology compared to the epithelioid morphology of the parental cell line. Transfection with each oncogene had increased the growth rate of the transfectants *in vitro*, increased their plating efficiency in monolayer, and in suspension, and reduced their serum dependence. Growth *in vivo* as xenografts was also increased. While a very few tumours were formed from the parental line and after a prolonged lag period, the transfected lines produced tumours with 100% efficiency, and a short lag period. There was no significant increase in PA activity as a result of transfection, and the invasion of the lines *in vitro* in organotypic culture was broadly similar. There was no significant effect on the response of cells to cytotoxic drugs following transfection. A gradual rise in the expression of the malignancy associated phenotype was observed where c-myc exhibited slight differences from the parental line, while transfection with Ha-ras produced more pronounced differences from the parental line, and in general the effects of activated Ha-ras were more aggressive and extreme, with the highest growth rates, plating efficiencies, clonogenicity in soft agar, and shortest lag periods and doubling times *in vivo*. This showed that enhanced expression of the malignant phenotype in a continuous cell line of epithelial origin could result from a single activated gene.

# CHAPTER ONE

## GENERAL INTRODUCTION

**This Chapter provides an overview of the literature on the subject of human lung cancer, with emphasis on the changing phenotypes, and also on the molecular aspects of the disease with special reference to oncogene involvement.**

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## 1.1 HUMAN LUNG CANCER

This is the commonest primary neoplasm, and is one of the major causes of death throughout the world. Over 90% of lung cancer patients will die of the disease. In the beginning of the century it was quite infrequent, but it has since become the commonest fatal cancer in men, and second leading cause of cancer deaths in women age 35-74 years; the incidence in women is rising mainly due to increased smoking. The male to female ratio is 3:1. The incidence of lung cancer is increasing, and it is the second major cause of death in the United Kingdom after cardiovascular disease [HMSO, 1985].

Scotland has the highest incidence of lung cancer in the world. The overall 5 years survival rate remains between 8-12%. At present only 20% of patients with lung cancer have evidence of localized disease at the time of diagnosis. Like other cancers the incidence increases with age, but has been reported in young men in their thirties. The commonest known causative agent is smoking, while other causes are airborne carcinogens, industrial pollutants, atmospheric radiation, occupational factors, genetic susceptibility, diet, viruses, and various chemical carcinogens. Early spread via both lymphatics and blood is common; direct spread to thoracic and abdominal organs is also common in late stages.

Most classifications, including the latest one proposed by the World Health Organization [WHO, 1982], divide lung cancers into four major histological types: small cell carcinoma, squamous cell carcinoma, adenocarcinoma, and large cell carcinoma, plus other subtypes and relatively uncommon forms. However, for various biological [Gazdar et al., 1983; Gazdar, 1984; 1986], pathological [Matthews *et al.*, 1973], clinical and therapeutic [Ihde, 1984] reasons, lung cancers are often divided into two major subdivisions: small cell lung carcinoma (SCLC), accounting for about 25% of all lung cancers, and the remaining 75%, collectively referred to as non-small cell lung carcinomas (NSCLC) including; squamous cell carcinoma 40%, adenocarcinoma 20%, and large cell carcinoma 15%). The clinician has been concerned mainly with this classification, because of different treatment modalities, and response to therapy. Recent advances in the concepts of lung cancer biology, diagnosis, and staging and therapy, suggest an emerging clinical role for sub-classification of NSCLC.

SCLC has characteristic histological features. The tumour appears highly cellular with scanty stroma. Histologically, three types of SCLC have been recognised. (1) Oat cell carcinoma composed of uniform small cells, generally larger than lymphocytes, with dense round or oval nuclei, diffuse chromatin and

inconspicuous nucleoli, and very sparse cytoplasm. (2) Intermediate cell type, composed of small cells with nuclear characteristics similar to the oat cell, but with more abundant cytoplasm. (3) Combined oat cell carcinomas with a definite component of oat cell carcinoma combined with squamous and/or adenocarcinoma.

SCLC has also been classified as a unique clinicopathological entity for several clinical and therapeutic reasons, such as a high tumour cell growth fraction, early potential for metastatic spread, and sensitivity to both radiotherapy and chemotherapy, in comparison to NSCLC. In patients with limited disease, an overall response rate of 80% following combination chemotherapy has been attained, with a complete remission rate approaching 50%. In spite of this dramatic response to induction chemotherapy, the median survival is only about 15 months, and 2 years survival about 10-20% largely due to subsequent relapse with tumour resistant to chemotherapy.

While the basic classification of lung cancer has not changed significantly over the course of last several years, the histological criteria have undergone a considerable evolution, mainly by input from biological studies, in both SCLC and NSCLC. New advances in technology such as in molecular biology and immunohistochemistry have aided the subtyping of some tumours, and have also helped in delineating the accurate typing of lung tumours. The application of clinical protocols directed to specific subtypes of lung cancer will extend these studies further.

### 1.1.1 COMPONENTS OF NORMAL AIRWAYS EPITHELIUM

The normal tracheobronchial epithelium is composed of basal cells, intermediate cells, ciliated cells, and mucous cells [Breeze and Wheeldon, 1977]. There are also other rare cells such as Kultschitzky-like cells (K-cells, small granule cells, neuroendocrine cells), and brush cells. The basal, intermediate, and mucous cells are capable of division [McDowell *et al.*, 1978]. Two types of epithelial cells line the bronchiole. The ciliated and the Clara cells (non-ciliated). Mucous cells develop following injurious stimuli in the latter. There are no basal cells. The Clara cells act as the reserve cell, and differentiate into ciliated and mucous cell [Evans *et al.*, 1978]. The alveoli and the alveolar ducts are lined by type I and II pneumocytes, the latter is the precursor of type I.

In the human adult respiratory tract mucosa the neoroepithelial (NE) cells are found to have special topographic organization. Two well identified components of the system are : 1) the solitary NE-cells, 2) and neuroepithelial

bodies (NEB). Both components have been found extending from trachea to alveolar ducts, being most numerous at bronchiolar level [Lauweryns & Peuskens, 1972; Cutz et al., 1974; Hage, 1974; Stahlman et al., 1985]. The solitary NE-cells are in the majority, located close to the basement membrane, with long dendrite like cytoplasmic processes containing neurosecretory granules. They are scattered throughout the entire length of the tracheobronchial mucosa, and constitute approximately 1-2% of bronchial epithelial cells. The structures now referred to as neuroepithelial bodies (NEB) were described as innervated clusters (knotchen) by Feyrter [1938; 1954] and Froehlich [1949]. They consist of 4-12 cells, which are non-ciliated with clear to slightly eosinophilic cytoplasm. In correctly oriented sections these cells extend from the bronchial basal lamina to its lumen. NEB have been described only in the intrapulmonary bronchial mucosa in human lungs, and have been observed in bronchial, bronchiolar, and alveolar lining epithelium especially at bronchial bifurcations [Cutz et al., 1974; 1978; 1975; Lauweryns et al., 1972]. Both the NE-cells and the NE-bodies show characteristic neurosecretory granules [Cutz & Conen, 1972; Hage, 1973].

The origin of the NE-cell system of lung is controversial. According to the latest hypothesis by Pearse & Takor [1979], the diffuse neuroendocrine system (DNES) is divided into three segments of related, but, distinct embryogenesis, including neural crest derivatives, derivatives of neural tube and ridge, and the derivatives of "neuroendocrine programmed" cells of embryonal ectoblast or epiblast (definitive endoderm precursors). It is possible that cells derived from all three embryonic layers may share a common neuroendocrine programme that enables them to synthesize and variably utilize biogenic amines and peptides common to the nervous and endocrine systems through closely linked systems or a similar responsiveness to common stimuli [Gould et al., 1983]. Pearse [1977] has also suggested a central and peripheral division of DNES, where lung and gastroenteropancreatic axis have been included in the latter category, with a possible origin from embryonal ectoblast.

The functional relationship of NE-cells and NEB is not clear. NE-cells are thought to have an endocrine or paracrine (regulatory) function, whereas NEB are considered analogous to peripheral chemoreceptors.

### 1.1.2 ORIGIN OF LUNG CANCER

The origin of lung cancers, especially their relationship to normal cell types, is mostly controversial, because of the lack of readily accessible material for the detailed study of sequential development of neoplasia. It is generally

accepted now however, that all have a common stem cell origin.

### 1.1.2.1 Small Cell Lung Carcinoma

SCLC and bronchial carcinoid are thought to arise from or are related to bronchial NE-cell [Cutz *et al.*, 1981; Gazdar *et al.*, 1981]. The endocrine behaviour of SCLC [Pearse, 1969; Greco and Oldham, 1979] and the production of biologically active polypeptide hormones such as adrenocorticotropic hormone (ACTH), anti-diuretic hormone (ADH), calcitonin, the expression of NE related enzymes (e.g., dopa decarboxylase) and the presence of dense core granules (DCG) are in support of this theory. A neural origin was suggested by findings of Tischler *et al.* [1977] who have shown that certain functions present in neural cells such as the ability to generate short duration electrical action potentials, could be demonstrated in a number of normal and neoplastic NE-cells, including cultured SCLC. Bell and Seetharam [1979] have shown that certain antigens of neural origin were expressed by SCLC as opposed to other types of human lung cancers.

However, over the past decade an emerging body of data from both clinical and laboratory studies have challenged the validity of the theory for a separate cell of origin of SCLC. An increasing number of biochemical studies have revealed that various polypeptide hormones are indeed found in each of the histological types of lung cancer, and are not restricted to SCLC as it was thought before. Bayline *et al.* [1980], have observed dopa decarboxylase (DDC) activity from surgical and autopsy material of all types of lung cancers. Gazdar *et al.* [1988] have found DDC in 12% of NSCLC cell lines such as adenocarcinoma and large cell carcinoma although the DDC activity was much lower in NSCLC compared to SCLC specimens. Similarly, other hormones and peptides such as bombesin/gastrin releasing peptide, once thought to be the key NE-cell marker, have also been found in about 17% of NSCLC from surgical and autopsy material [Yamaguchi *et al.*, 1983]. It has been well known to pathologists for many years, that the same patient may present with disease that shows SCLC histology in combination with the morphologic characteristics of other lung cancers [Yesner, 1978]. In a series of clinical studies Abeloff *et al.* [1979] have shown that patients diagnosed as SCLC on biopsy material showed that 12% had NSCLC, 15% had mixed small cell-large cell histology at autopsy. These changes in SCLC phenotype suggested a link between different lung cancer types. Bonikos and Bensch [1977] have proposed that if the tendency for this tumour to express NE-cell like properties is interpreted as evidence that the tumour originates from NE-cells, which are scattered in sparse number, then the

incidence of SCLC might be predicted to be much lower.

These data have led the investigators to revive the older, unitarian theory of histogenesis. Several new studies, and some of the previous data indicate a relationship among the major forms of lung cancer including SCLC, and suggested a common embryological origin.

#### 1.1.2.2 Non-Small Cell Lung Carcinomas

NSCLC represents a group of tumours with distinct but overlapping histological features, clinical course, and biological behaviour. Their response to therapy, other than surgical excision, is limited and they cannot be cured once they have disseminated outside the thoracic cavity.

Squamous cell carcinoma is assumed to take origin from the foci of atypical squamous metaplasia in respiratory epithelium, maybe from basal cells. Metaplastic squamous cells can develop from mucous cells [McDowell *et al.*, 1978]. However, it is not clear whether they arise directly from multipotential basal cells or from more differentiated cells. Adenocarcinomas are thought to arise from basal and/or mucous cells. Dermer [1981] has demonstrated from the cellular distribution of glycoproteins after *in vitro* labelling that adenocarcinomas arise from a cell type committed to differentiate into ciliated epithelium. Bronchiolo-alveolar carcinomas arise from bronchiolar or alveolar epithelium, from type II pneumocytes or their precursors, or from Clara cells or the mucous cells that have developed from Clara cells. Typically, these tumours form acinar structures, surface microvilli, mucin production, and other epithelial features on ultramicroscopy.

Large cell carcinomas are the tumours of basal and/or mucous cell origin that have developed beyond the basal cells stage but lack sufficient glandular or squamous differentiation to be classified into these categories. Horie and Ohta [1981] have shown that large cell carcinomas have ultrastructural features of both squamous and adenocarcinoma. They may also even express features of endocrine tumours [McDowell *et al.*, 1981], and it has been suggested that this group may represent a potential stem cell.

### 1.1.3 THE INTERRELATIONSHIP OF LUNG CANCERS

Overwhelming evidence exists for a common origin of all the mucosal cells of bronchial epithelium, and the tumours derived from them [Yesner, 1978; Gazdar *et al.*, 1981]. The main evidence comes from the study of embryonic derivation of bronchopulmonary tract [Andrew, 1976; Fountaine & LeDauarin, 1977]. According to this theory a stem cell undergoes progressive differentiation,

through morphologically undifferentiated intermediate stages committed to specific pathways of differentiation. The fully differentiated cells are end stage cells, and therefore, incapable of further division [Gazdar *et al.*, 1981]. A committed precursor cell may differentiate along more than one pathway depending on environmental conditions (e.g carcinogens, viral infections) and may even switch from one pathway to another, probably due to genetic alterations (e.g oncogenic activation, suppression of anti-oncogene). Pulmonary NE-cells with amine precursor uptake and decarboxylation (APUD) cell properties may be one type of differentiation. It is possible that APUD-cell properties develop in this cell with the onset of endocrine differentiation or else these properties develop in all intermediate cells, and are then selectively retained only in endocrine cells [Baylin and Mendelson, 1981].

Transforming events occur in undifferentiated cells, since fully differentiated cells are not capable of replication. The differentiation status of the transformant may depend on the level of differentiation at which transformation occurs. According to this concept SCLC are regarded as partly differentiated along the endocrine pathway. Large cell carcinoma may represent the stem cells or undifferentiated tumour. This explains the presence of tumours having more than one morphological component, and also the APUD-cell markers in NSCLC. These cell types may represent intermediate stages in the spectrum of lung cancer.

The tumour cell population is propagated by a core of undifferentiated stem cells capable of a large number or infinite divisions, some of the progeny differentiate, while others remain relatively undifferentiated, and maintain the stem cell pool [Figure 1.1]. The stem cell fraction in a tumour may appear morphologically different. It has been demonstrated that most SCLC cultures contain a very small percentage of large cells [Gazdar *et al.*, 1981] which may be stem cells. According to this theory, a changing tumour morphology can be explained by progressive failure of the stem cells to undergo differentiation. Depending on the severity of differentiation failure, a pure small cell or mixed small cell-large cell tumour or pure NSCLC may be found in an originally SCLC with a very small stem cell population.

The stem cell theory can also explain the co-existence of SCLC with other histological types of lung cancer such as squamous and adenocarcinoma. If all lung cancers arise from a single stem cell, conversion from one differentiated cell type to another can occur, by maturation down one particular pathway, resulting in either a pure squamous, adeno or SCLC or even a mixed population of SCLC

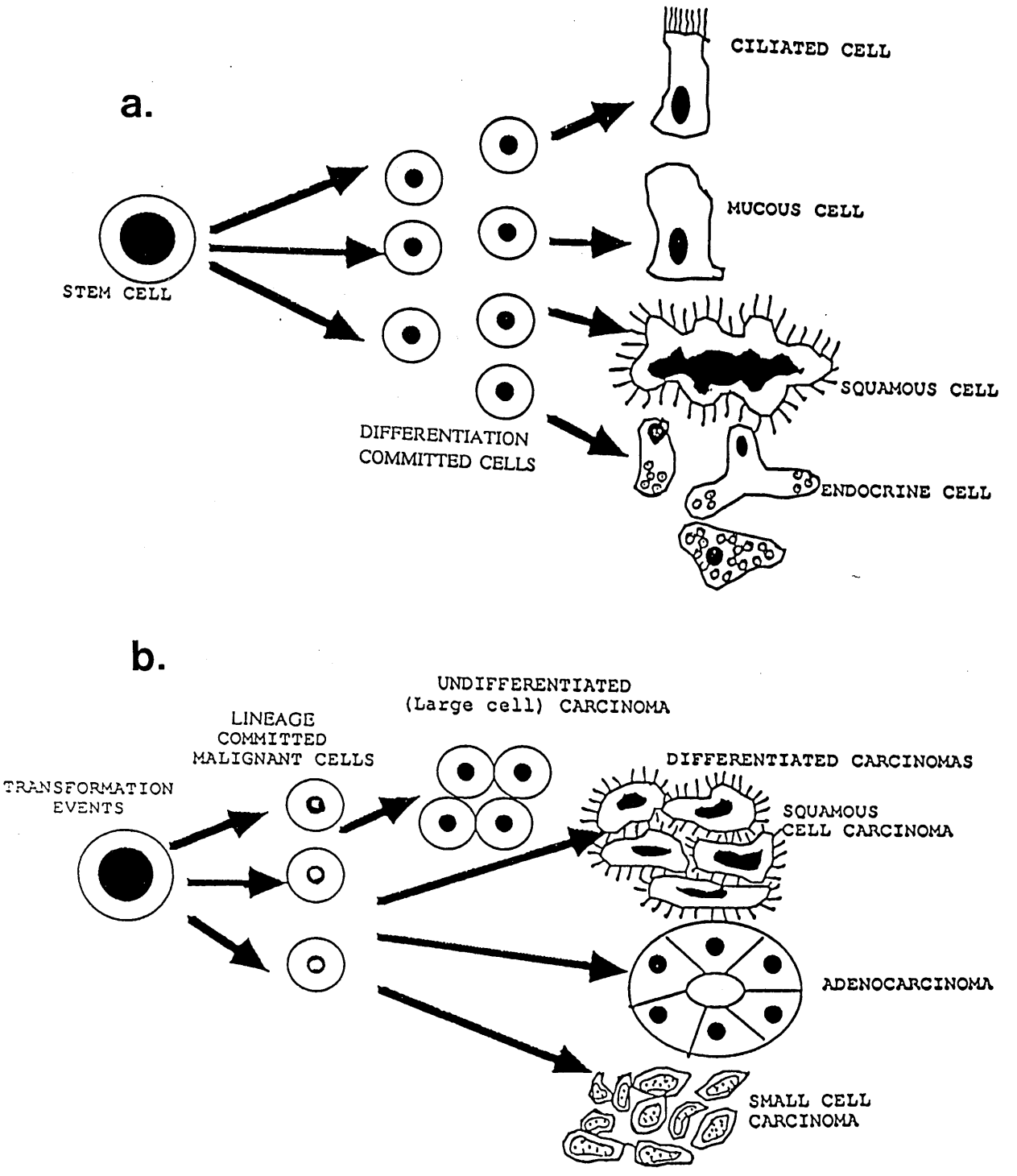


**Figure 1.1:-** **Common stem cell theory of the origin of the cells of the respiratory mucosa (a), and lung cancers (b).**

with any of other lung cancer types. The stem cell concept of lung cancer has also been supported by data derived from clonogenic assays which indicate that only a small percentage of SCLC (1-5%) is capable of giving rise to progressively growing colonies, but that many cells are capable of limited replication [Carney *et al.*, 1980]. In addition, in mixed small cell-large cell tumours, cell kinetic studies indicate greater thymidine uptake by the large cell components, suggesting a greater replicating ability of the latter.

The cells of carcinoid tumours resemble the K-cells of the bronchus. Small cell carcinomas are poorly differentiated with some features of K-cells, but more often resemble bronchial basal cells. It seems most likely that the cell of origin of SCLC is the basal cell [Sidhu, 1979]. It may be the case that carcinoid arise from cells already on the pathway of differentiation toward K-cells. This also explains the epidemiological divergence of SCLC and carcinoid of lung. Therefore, it appears that perhaps only the basal cell is susceptible to the influence that results in an increased incidence of SCLC; cells more differentiated toward K-cell may be less susceptible, explaining why carcinoid have not increased with the rising incidence of SCLC.

### 1.1.4 BIOLOGY OF LUNG CANCER *IN VITRO*

#### 1.1.4.1 Small Cell Lung Cancer Cell Lines

It has been possible now to establish cell lines with over 70% success, due to the use of defined media [Gazdar *et al.*, 1980; 1981; Carney *et al.*, 1981]. Once established, cell lines express characteristic properties [Table 1.1]. They have a relatively prolonged cell population doubling time which decreases with increasing passage number, suggesting a selective process or adaptation to the culture environment. They form colonies in semi-solid media with a low efficiency (1-5%) and form tumours in athymic nude mouse with a characteristic SCLC morphology.

SCLC cells grow in culture either as free floating aggregates or as a partially attached monolayer. In the latter cases, it has been found that as cell density increases, large clumps of cells detach from the surface of the monolayer and float to grow in suspension [Pettengill *et al.*, 1980]. Depending upon the morphological appearances of the cultures, SCLC cell lines have been classified into four types by Carney *et al.* [1985]. Type 1 cells lines grow as spherical aggregates of floating cells with frequent central necrosis in large sized aggregates. Type 2 cell lines grow as floating cellular aggregates with irregular outlines, packed tightly as in type 1, but without central necrosis. Type 3 cell

**Table 1.1:- Biological properties of lung cancer cell lines.**

| | SCLC | | NSCLC |
|---|---|---|---|
| Characteristic | Classic | Variant | |
| Morphology | FFA | FFA/monolayer | epithelioid |
| Substrate adhesion | absent | absent | present |
| Tumorigenicity | + | + | + |
| Ploidy | aneuploid | aneuploid | aneuploid |
| CFE | + | ++ | +++ |
| Histology | SCLC | SCLC | NSCLC |
| Doubling time | long | short | short |
| DCG | + | _ | _ |
| DDC | + | _ | _ |
| BLI/GRP | ++ | _ | _ |
| BLI receptors | + | _ | _ |
| NSE | ++ | + | _ |
| CKBB | ++ | ++ | - |
| Neurotensin | ++ | _ | _ |
| Peptide hormones | ++ | +/_ | _ |
| EGF receptors | _ | _ | + |
| Chromosome 3p del | + | + | _ |
| IF | cytokeratin | cytokeratin | cytokeratin |
| RS | sensitive | resistant | resistant |
| CS | sensitive | resistant | resistant |
| c-myc | + | ++ | _ |
| N-myc | +/_ | +/_ | _ |
| L-myc | +/_ | _ | _ |

FFA, free floating aggregates; CSF, colony forming efficiency; DCG, dense core granules; DDC, dopa decarboxylase; NSE, neuron specific enolase; BLI/GRP, bombesin like immunoreactivity/gastrin releasing peptide; CKBB, creatine kinase isoenzyme-BB; IF, intermediate filaments; RS, radiation sensitivity; CS, chemosensitivity.

lines grow as a combination of loosely adherent cells and floating small irregular aggregates. Type 4 cell lines grow attached to the substrate like monolayer cultures.

Depending on their morphological, and biochemical properties SCLC cell lines have been further classified into two major groups, classic and variant [Carney *et al.*, 1985; Gazdar *et al.*, 1985]. Classic SCLC cell lines exhibit type 1 or 2 morphology in culture, show intermediate cell type histology in xenografts, express elevated levels of dopa-decarboxylase (DDC), bombesin-like immunoreactivity (BLI), neuron specific enolase (NSE), and creatine kinase BB isoenzyme (CKBB). The variant SCLC cell lines lack DDC, and BLI but continue to express NSE, and CKBB, and exhibit type 3 or 4 morphology. The variant SCLC cell lines have been further divided into 1) biochemical variant lines which have typical SCLC morphology, intermediate cell type histology in xenografts, and characteristic SCLC growth properties, but with variable biochemical profiles, and 2) morphological variant SCLC cell lines which have variant morphology, biochemical profiles, altered growth characteristics, exhibit a histological appearance in xenografts of undifferentiated large cell carcinoma, and show amplification of c-myc oncogene. A transitional class of SCLC cell line has also been proposed characterized by mixed classic-variant characters, and there have been speculations as to whether this class represents an intermediate state between pure classic and pure variant groups. This model would represent a transition from SCLC to NSCLC phenotype.

Many clinical specimens and established cell lines of SCLC form tumours when inoculated subcutaneously into nude mice, and tumours can be transplanted with 100% take, and established as continuous cell lines in culture [Sorenson *et al.*, 1981; Gazdar *et al.*, 1981]. Some lung tumour cells have shown differentiation when grown in the mouse [Giovanella *et al.*, 1974]. In general the more poorly differentiated tumours were those that grew best in the nude mice. In general, xenografts retain the morphological characteristics of the parental tumours at light and electron microscopic level, but some features may be lost due to selection as an increased rate of proliferation with serial passage of tumour in animals. Tumours have also been found to express biological markers, which are found in the serum of the animal bearing the tumour. Studies have shown a good correlation between the response of xenografts to cytotoxic drugs and the patient's tumour [Shorthouse *et al.*, 1980].

### 1.1.4.2 Non-Small Cell Lung Cancer Cell Lines

NSCLC cell lines can be readily established from approximately 40% of all specimens obtained [Brower *et al.*, 1986], and can be more readily established from metastatic lesions compared to primary lesions. Once established as permanent cell lines, they can be characterised for different parameters [Table 1.1]. These cells retain the morphology of the original tumour type. They form tumours in athymic nude mice, and colonies in soft agarose, with relatively more frequency compared to SCLC cell lines.

### 1.1.4.3 Molecular Genetics of Lung Cancer

Many cancers have now been associated with specific chromosomal abnormalities e.g retinoblastoma [Cavenee *et al.*, 1983], Wilms tumour [Fearon *et al.*, 1984], leukaemia [Nowell and Hungeford, 1980; Deklein *et al.*, 1982], and Burkitt's lymphoma [Taub *et al.*, 1982]. Molecular analysis of these lesions has resolved different potential mechanisms by which these specific chromosomal abnormalities may play a role in the development of the transformed phenotype.

Various genetic changes have been described in lung cancer cells. These include point mutations, chromosomal rearrangements, deletions, and gene amplifications. The target genes include dominantly acting cellular oncogenes, onco-suppressor genes, and genes for growth factors and their receptors, especially autocrine growth factors. These studies, therefore, suggest that lung cancer can be regarded as a genetic disease, and the products of these altered genes and their biochemical pathways involved are the potential targets for new therapeutic strategies.

Various genetic abnormalities in SCLC have been reported by Whang-Peng *et al.*, [1982]. A modal chromosome number ranging from 40 to 90 has been observed in SCLC cell lines. Many cell lines have been found to be hypodiploid, hyperdiploid, or tetraploid. Other chromosomal abnormalities include the absence of chromosomes 13 and 15, aberrations of chromosomes 1,2,9,10, 14, and X. Another characteristic abnormality is the deletion in the short arm of the third chromosome 3p14-23. This abnormality is found in over 90% of SCLC, and about 25% of NSCLC [Brauch *et al.*, 1987]. It has been proposed that such a deletion may uncover an otherwise recessive mutation residing on the cytogenetically normal chromosome 3, since such mechanisms have been suggested for retinoblastoma (13q24) [Benedict *et al.*, 1983], and Wilms tumour (11p) [Orkin *et al.*, 1984], and the genes inactivated by these chromosomal changes are proposed to be anti-oncogenes. Abnormal expression of various proto-oncogenes have been found in lung cancer cells [see Table 1.2].

Amplification of myc oncogenes has been found in SCLC. It has been shown that SCLC morphological variant cell lines express high levels of c-myc oncogene. Amplified c-myc mRNA has been found in the variant compared to classic cell lines [Little *et al.*, 1983; Gazdar *et al.*, 1985; Nau *et al.*, 1985; 1986]. Other members of the myc family of genes have also been found in association with SCLC.

**1.1.4.4 Growth Factors and Lung Cancer**

Lung cancers, like many other tumours, synthesize and secrete a number of growth regulatory factors. Several growth factors have been identified, which are synthesized and secreted by lung cancer cell lines [Sporn & Todaro, 1980]. Moody *et al.* [1981] have shown that SCLC cell expresses high levels of intracellular bombesin. Others have shown that bombesin and its related peptide GRP are potent mitogens for normal lung epithelial cells [Willey *et al.*, 1984], and in SCLC [Carney *et al.*, 1987]. Cuttitta *et al.* [1985] have shown that the growth of SCLC tumours both *in vitro* and *in vivo* is inhibited by a monoclonal antibody 2AII which binds to the C-terminal portion of bombesin, and inhibits binding of bombesin to its receptor. Macauley *et al.* [1987] have shown other growth factors such as insulin-like growth factor 1 (IGF-1) to be secreted by SCLC cells and to be a potent mitogen for these cells. This may explain the relative ease with which SCLC may be grown in low serum or serum free medium.

Studies of NSCLC cell lines have also shown that these tumours secrete and respond to growth factors [Betsholtz *et al.*,1987]. Gamou *et al.* [1987] and Siegfried [1987] have shown an increased expression of epidermal growth factor (EGF) receptors in NSCLC in contrast to SCLC tumours, and in some cases modulation of the receptors or alteration of binding of EGF to the receptor by monoclonal antibody can inhibit both *in vitro* and *in vivo* growth of tumour cells.

**1.1.4.5 Expression of Marker Proteins in Lung Cancer**

The ability of many pulmonary tumours to produce and secrete hormones and other substances has been used as a tumour marker. Many of these markers are measured in blood, or other body fluids, and still others may only be present within the tumour tissue. Tumour may be associated with humoral markers that are not released by the tumour tissue per se, but result from a secondary effect of the malignancy on some other tissues. The tumour markers or products include hormones, hormone precursors, placental and fetal proteins, and enzymes [Minna *et al.*, 1982]. Various clinical applications of these markers have been suggested such as early detection, diagnosis of undifferentiated SCLC tumours

[Baylin *et al.*, 1980; Marangos *et al* ., 1982], clinical staging of lung cancers [Minna *et al.*, 1982; Gazdar *et al.*, 1981; Carney *et al.*, 1982], and monitoring response to therapy [Minna *et al.*, 1982; Carney *et al.*, 1982].
However there is no definite marker for screening for the presence of lung cancer [Minna *et al.*, 1982].

A variety of markers are produced by all lung cancers, but the concentrations have usually been found to be significantly higher in patients with SCLC than in patients with NSCLC. These include ACTH, which is mainly produced by SCLC, but also by NSCLC tumours, lipotropin [Odell *et al.*, 1979], calcitonin [Silva *et al.*, 1976; 1979], human chorionic gonadotropic (HCG) [Tashjian & Weintraub, 1973], carcinoembryonic antigen (CEA) [Sculier *et al.*, 1985].

Elevated expression of neuroendocrine markers has been reported in NSCLC. Berger *et al.* [1981] have shown elevated expression of L-dopa decarboxylase, and NSE in up to 20% of NSCLC tumours, and Ariyoshi *et al.* [1986] have found elevated serum levels of NSE in up to 15% of patients with NSCLC. These NSCLC tumours with neuroendocrine properties have been shown to be more sensitive to cytotoxic therapy like SCLC [Mulshine *et al.*, 1987]. In a prospective trial preliminary data suggest that endocrine NSCLC are more chemosensitive than non-endocrine NSCLC tumours. Therefore, these data suggest that elevation of the expression of endocrine properties in primary lung cancer may be of prognostic value and may aid in selection of appropriate therapy in such patients.

**1.1.4.6 Chemosensitivity of Lung Cancer**

*In vitro* tests of chemosensitivity and radiosensitivity have been carried out both on fresh specimens and cell lines of lung cancer. Carney *et al.* [1985] have shown a good correlation between *in vitro* chemosensitivity testing and predicted *in vivo* resistance in the majority of cases, and predicted *in vivo* sensitivity in up to 60% of cases. However, due to the low cloning of fresh specimens of lung cancer, the results of tests for specific cytotoxic agents are limited to a small number in fresh specimens. Therefore, the use of cell lines for chemosensitivity testing is another alternative, and has many advantages, when compared to fresh specimens. Using established cell lines of both SCLC and NSCLC it has been shown that many retain a similar pattern of *in vitro* chemosensitivity to that of fresh biopsy specimens [Carney *et al.*, 1983]. Although the results and the pattern of chemosensitivity may vary in different culture conditions, the overall data suggest that such cell lines can be used to predict *in vivo* responses [Carney *et al.*,

1985], and to evaluate drug resistance mechanisms [Shoemaker *et al.*, 1983]. They may also be used for screening new drugs in clinical trials [Carney *et al.*, 1985], and for the investigation of correlations between marker expression and other aspects of the phenotype, such as drug resistance and sensitivity [Johnson *et al.*, 1987; Twentyman *et al.*, 1986].

## 1.2 CARCINOGENESIS

Carcinogenesis is a multistage process. The evidence comes from epidemiological studies in man [Armitage and Doll, 1957], biochemical [Farber, 1984] and histopathological [Foulds, 1954] analysis of tumours *in vitro* and *in vivo*, the studies of experimental cancer induction in animals using chemicals, irradiation and viruses [Spandidos, 1986], and *in vitro* cell transformation assays [Spandidos and Siminovitch, 1978]. The process of carcinogenesis can be divided into three distinct stages: initiation, promotion and progression.

The first stage of tumour formation is initiated by a carcinogenic agent that can be a chemical carcinogen, oncogenic virus, radiation, replication error, or other unknown factors. This step is an absolute requirement which results in irreversible mutational events in the cellular genome. Initiation is a very rapid process which may result following a single application of a carcinogen. It has been shown that some chemical carcinogens induce activation of the ras gene by point mutation. Balmain *et al.* [1984] have demonstrated activation of ras gene in chemically induced benign skin papillomas in mouse. Wiseman *et al.* [1986] have shown mutational activation of the c-Ha-ras proto-oncogene in chemically induced mouse hepatomas. It is possible that a point mutation in the ras gene confers a selective growth advantage on these cells, which in time undergo phenotypic diversification, with subsequent malignant transformation. Point mutations in the ras oncogene have also been demonstrated in lymphomas following X-ray exposure [Guerrero *et al.*, 1984]. *In vitro* transformation assays have shown that over-expression of a single mutated ras oncogene was sufficient to transform primary rodent cells [Spandidos and Wilkie, 1984] and early passaged rodent cells [Wyllie *et al.*, 1987]. The c-myc gene has been found as an initiator of carcinogenesis, in transgenic mice [Stewart *et al.*, 1984].

The application of carcinogen does not lead to the immediate formation of a tumour, but rather there is a series of changes following tumour initiation caused by carcinogen. The initiated cells may persist for a considerable time, until they are acted upon by promoting agents. Tumour promotion may be

facilitated by carcinogen or by other substances called tumour promoters, which do not themselves produce tumours. They also stimulate tumour formation in the initiated cells, and interfere with the differentiation process. The exact role of tumour promoters remains unknown [Parker and Ullrich, 1986].

The third and clinically most significant stage is the progression of the benign tumour to malignant tumour. This stage is characterized by both phenotypic and genotypic changes in the tumour cells. The tumour cells acquire malignant phenotypes such as invasion and metastasis. Tumour progression is also associated with various genetic alterations such as aneuploidy, double minute chromosomes, homogeneously staining regions in chromosomes, chromosomal rearrangements, deletions, mutations, amplifications of specific genes, and increased DNA content of cells.

It is established that cancer is a result of defective regulation of cell growth and differentiation. Regulatory processes which control the events during a normal cell division are deranged in cancer cells [Campisi *et al.*, 1986]. The study of cell cycle events shows that a major regulatory control occurs in G1-phase just prior to the entry of cells into S-phase (restriction point) [Pardee, *et al.*, 1978]. Data strongly suggest that a normal cell needs to make a critical amount of an unstable protein (e.g p68) in order to pass the restriction point [Campisi & Pardee, 1984]. It was found that various transformed cells behave as if this protein is stabilized or made in excess suggesting that greater availability of this or similar proteins may permit the escape of cells from growth control, and this may be under oncogenic control. Synthesis of these proteins requires the presence of growth factors, and is blocked by protein synthesis inhibitors [Campisi *et al* , 1984; Zetterberg & Larsson, 1985]. It has been shown that ras oncogene(s) is activated during 6 hours pre DNA synthesis period [Campisi & Pardee, 1984], and similar results have been shown in other cases [Mulcahay *et al.*, 1985], suggesting a possible oncogenic involvement in this process.

## 1.3 INVASION AND METASTASIS

Alteration of growth properties and uncontrolled proliferation is a primary but not a critical step as such during tumour progression, since neoplastic growth is seen in benign tumours. Proliferation only produces benign tumours, invasion and metastasis are the characteristics of the malignant phenotype. As a rule a benign tumour grows but neither invades nor metastasizes. A benign tumour may remain benign or may become malignant [Foulds 1969, Balmain 1985].

Although tumour growth and proliferation are associated with invasion there is ample evidence that invasion is not dependent on cell proliferation *in vitro* [Mareel *et al.*, 1982, Thorgeirsson *et al.*, 1984] and *in vivo* [Disttelmans *et al.*, 1985]. Drugs such as 5-Fluorouracil (5-FU) that completely inhibited cell proliferation [Bracke *et al.*, 1984, Storme *et al.*, 1985] did not block invasion.

A three step hypothesis has been proposed for the sequences of events during tumour cell invasion: 1) adhesion, 2) matrix hydrolysis, 3) and tumour cell motility or locomotion. A continuous invasion of the matrix takes place by cyclic repetition of these three steps, and all the processes are taking place at the same time.

Certain molecules mediate communications between metastatic cells and their milieu. This is essential for tumour cell detachment from the primary tumour mass, its transportation through matrices, and re-attachment at metastatic sites. These matrix and cell surface molecules can be divided into tumour and stromal components. The tumour cell surface contains receptor molecules, which are thought to be essential for homotypic and heterotypic adhesions. Tumour cell adhesion with matrix may be mediated by specific glycoproteins such as laminin, fibronectin, vitronectin, integrins, and tenacin on the cell surface [Alberts *et al.*, 1989]. Homotypic adhesion is necessary for tumour cells to break in lumps or aggregates and to survive the long journey in the blood or lymphatic channels, and also to help their arrest in capillary wall. Heterotypic adhesion is essential for tumour cell-stromal interactions at different levels of invasion and at metastatic site. Homotypic adhesion is reduced, but heterotypic adhesion may be increased in metastatic tumour cells.

Malignant cells have been shown to express various types of proteolytic enzymes which facilitate invasion and dissemination of tumour cells by matrix degradation. These include various collagenases [Thorgeirsson *et al.*, 1985], cathepsins [Sloane *et al.*, 1981], and plasminogen activators [Liotta *et al.*, 1981]. It has been proposed that when a normal cell or benign tumour cell attaches to the extra-cellular matrix (ECM) it may respond by shifting into a resting or differentiated state, but not a malignant cell [Liotta & Schiffmann, 1988]. Many of these hydrolytic enzymes are cell surface associated, and may require contacts with matrix for lysis to occur. Many of the proteases are secreted in proenzyme form and therefore require activation. The localization of degradative enzymes at the cell surface suggests a spatial organization which may be essential for activation and directional movements.

Cell movements and locomotion have a fundamental role in cancer

spread. Two types of molecular mechanisms have been described for the regulation of locomotive behaviour of cancer cells. Chemokinetic movements are thought to be under influence of autocrine motility factors (AMF) secreted by tumour cells, while directional movements are due to chemotactic factors.

Invasive cells also possess the ability to deform and squeeze through narrow spaces like blood cells, and deformability may facilitate their entry into intercellular spaces and between endothelial cells on the blood and lymphatic vessels. This property may be due to altered cytoskeleton. Alterations in actin polymerization and vinculin phosphorylation and interaction with transmembrane proteins and other similar mechanisms may be involved.

There are also alterations in cell-cell interactions, both at primary tumour site and at metastatic site. Loss of spatial control results from abnormal cell interactions and altered recognition, leading to a loss of orientation, which may be due to loss of specific sites for cell or matrix recognition.

The growth of vasculature in tumour is another important factor in tumour growth and progression. Tumour angiogenesis factors (TAFs) are produced by the tumour, and stimulate migration and proliferation of endothelial cells in the tumour mass.

Although, invasion is an essential step for metastasis [Hart, 1984], all invasive tumours are not necessarily metastatic. Primary brain tumours are highly invasive, but they do not metastasize, likewise the basal cell carcinoma of skin [Dvoretzy *et al*, 1978].

The formation of metastatic tumour is a multi-step process. The first steps are as in invasion; motility and detachment from primary tumour site, invasion of basement membrane and subsequent infiltration of vascular channels and dissemination. Once in the circulation, adhesion to vascular endothelium is essential, and this may be facilitated by mechanical entrapment or injury to the endothelial lining. Tumour cells then penetrate the endothelium, due to their deformability facilitated by proteolytic enzyme secretion, or by specific surface receptor mechanisms. The extravasated cells then penetrate the secondary tissue, followed by tumour growth at the secondary site. Not all of the tumour cells that reach the new organ site are capable of forming tumour. The "seed" has to be able to respond to the "soil", according to "seed and soil" hypothesis, proposed by Paget [1889] that cancer cells, like plant seeds, may travel in different directions, but can only grow if they fall on soil favourable for their growth. Organ specific metastasis has been well documented both in clinical and experimental studies. Both the adhesion and invasion may be facilitated at different fronts by specific

affinity factors. A growth factor has been isolated from lung which stimulates melanoma cells which readily form lung metastases. It is possible that metastatic cells may become responsive to the growth factors by switching on the gene that codes the receptors for it. They have also shown that an increase in EGF receptors on human gastric carcinoma was associated with progressive disease *in vivo*, and a significant correlation was found between the depth of tumour cells invasion into gastric wall and EGF receptors on the tumour cells. Therefore, the growth of the tumour cells in a specific organ microenvironment could be facilitated by the production of organ specific growth factors generated subsequent to organ damage, which in turn is followed by repair process.

## 1.4 HETEROGENEITY IN MALIGNANT TUMOURS

Tumour cell heterogeneity is a well established phenomenon [Fidler *et al.*, 1978; Fidler and Hart, 1982]. Such heterogeneity includes variations in morphology, antigenicity, karyotype, enzymatic profiles, drug resistance, growth characteristics, biochemical, and immunological properties, invasion and metastatic potential. It is now generally accepted that tumour heterogeneity is a manifestation of tumour progression [Heppner, 1984], by a dynamic combination of variant cell generation and selection of those best fitted to survival.

It is well recognised that both naturally occurring and induced neoplasms generally develop from a single transformed cell [Reddy and Fialkow, 1983]. Tumour progression probably starts when tumour cells diversify in their unique microenvironments, and the variants with altered phenotypes arise in the tumour cell population. The cellular variant generation seems to be a stochastic, highly disordered, and multidirectional process, which involves both genetic and epigenetic changes. To survive and grow in a competitive environment, such a variant must have some selective growth and other advantages over other cells [Nicolson *et al.*, 1984; Nowell, 1986]. Several factors [Table 1.2] such as genetic instability of transformed cells [Bishop, 1987; Nicolson, 1987], microenvironmental influences [Sutherland, 1988] and other interactions [Heppner *et al.*, 1983; Nicolson, 1987] play an important role in variant generation changing a homogeneous cell population to a heterogeneous one. Then gradually with time, evolution of more stable variants may result in the eventual dominance of a particular subpopulation of tumour cells with more advanced malignant characteristics [Nicolson, 1984]. However, changes within tumours do not always

**Table 1.2:- Factors influencing cell phenotype.**

## I. INTRACELLULAR

(A). Nuclear events

1. Chromosomal alterations (e.g breakage, translocation).
2. DNA alterations (e.g mutations, deletions).

(B) Cytoplasmic Events

1. Cytosol alterations (e.g second messengers, metabolites).
2. Cytoskeleton alterations (e.g membrane interactions).
3. Post-transcriptional (e.g mRNA processing, post-translational).

## II. EXTRACELLULAR

(A). Cell-Cell Interactions (e.g cell junctions, cell contacts).

(B). Cell-Matrix Interactions (e.g matrix receptors, cell shape changes).

(C). Cell-Factor Interactions (e.g hormones, growth factors).

(D). Iatrogenic Effects (e.g radiation- and chemo-therapy).

result in progression of tumour cells to more malignant phenotypes and rarely may also result in tumour regression [Nicolson, 1984].

Genotypic variations within tumour cell populations have been demonstrated by molecular and karyotypic analyses. A variety of different genetic alterations have been found in malignant cells such as aneuploidy, translocation, homogeneously staining regions, double minute chromosomes, mutations, deletions, and amplifications. More recent molecular studies have shown that these genetic rearrangements and changes can result in expression or suppression of genetic sequences involved in generation of malignant phenotypes. These sequences are oncogenes [Bishop, 1987], tumour-suppressor genes [Todaro, 1986; Spandidos and Anderson., 1989], and a recessive gene associated with cancer predisposition [Knudson, 1986]. Cancer has myriad causes, but probably all work through a final common path of activation or suppression of these genetic sequences.

Most, if not all, malignancy-related gene products are also associated with normal developmental processes, suggesting that virtually all of the cellular characteristics of malignant neoplasms may also be encoded by normal genes. The quantitative changes in gene expression are also evident from the reappearance of fetal characteristics and gene products in malignant cells. It is possible that the genes that regulate embryonic cellular growth and differentiation may be intimately linked in the genome, and their quantitative variations in expression may explain expression of embryonic characteristics in malignant phenotype. Tumour progression is therefore, characterized mainly by quantitative, not qualitative, changes in gene expression, and the role of qualitative changes, such as genotypic alterations, might be to circumvent normal growth and differentiation controls and accentuate developmental heterogeneity leading to quantitative differences in gene expression among tumour cells. This explains the dynamic phenotypic shift seen in malignant cells population, an event that occurs much too quickly to be explained by genotypic changes in a minor cell subpopulation.

In addition to the genetic factors, the phenotypic stabilities in tumour cells are largely determined also by microenvironment [Nicolson, 1984]. Genotypic instability and selection alone cannot explain the rapid rates of tumour cell phenotypic variation that are observed *in vitro* and *in vivo*, since these are orders of magnitude lower than the rate of variant generation. It is important to recognize that phenotypic variability in cellular population is a normal cell attribute that is essential for tissue adaptability, however in malignant tumour it

may be accentuated beyond that required for normal tissue adaptation. Slight differences in tumour microenvironment may generate events that lead to local differences within the tumour. These include differences in cell-cell and cell-stromal interactions within the tumour, soluble inducers, nutrients, growth factors, hormones, enzymes, oxygen, and other different tumour regulators. These factors may play important roles in destabilizing tumour cells, and determining their susceptibility to genetic and epigenetic alterations.

## 1.5 CELL MICROENVIRONMENT AND PHENOTYPIC REGULATION

### 1.5.1 Mechanism of Differentiation Control

The differentiated state is achieved by interactions between parenchymal and mesenchymal elements in a tissue, both during embryogenesis and in adult life. Different germ layers of the embryo interact to give rise to differentiated tissues. Primitive endoderm differentiates into various visceral organs by interacting with the mesoderm in which it proliferates, e.g, lung buds arise from foregut primordium, and start proliferating in somatic mesenchyme. As developing bronchial ducts ramify, epithelium is induced by mesenchyme to form alveolar morphology, while mesenchyme is induced to form elastic tissues in lung substance [Taderera, 1967], suggesting a bilateral stimulatory effect of interacting tissues in order to give rise to the differentiated phenotype of each.

The phenomenon of tissue interaction is also found in adult tissues for the regulation and maintenance of normal structural and functional integrity. In the beginning it was assumed that the particular phenotypes of the cells in different tissues were established in embryonic life and that they would subsequently "breed true" as observed for instance during transplantation of full thickness skin [Billingham and Silver, 1968]. However, now it is established that both morphological and functional properties of various adult tissues are the outcome of interactions with their micro-environment, and they are of a similar nature as in their embryonic counterparts. Degeneration of taste buds following their nerve resection [Zalewski, 1969], maintenance of epidermal organization in combination with dermis [Billingham and Silver 1968], hair and nail formation [Oliver, 1967], amphibian limb regeneration only in presence of nerve supply [Singer, 1965], are just a few of the examples where tissue interactions have been found essential for the maintenance of a tissue.

It has been proposed that at least *in vitro* there could be four major

parameters for the regulation of differentiation [Freshney, 1985; 1987].

### 1.5.1.1 Cell-Cell Interaction

Cellular interactions can be heterologous or homologous. Heterologous cell interaction is probably responsible for initiation and promotion of differentiation. The exchange of information may occur at the cell surface by paracrine signals [Roberts *et al.*, 1988] or by cell surface molecules or receptors [Simon-Assmann *et al.*, 1988]. Homologous cell interactions occur at high cell density probably involving gap junctional communications, which probably harmonizes the expression of differentiation within one cell population [Pitts *et al.*, 1988]. These interactions have been found both during embryogenesis and in adult life, and are often lost in malignant tumours.

### 1.5.1.2 Cell-Matrix Interaction

Matrix, mainly comprised of glycoproteins and proteoglycans, forms a complex within each tissue around each cell. It has been shown that matrix regulates gene expression [Bissell, 1988]. Degradation of matrix in malignant cells would suggest that either they are less dependent on extra-cellular matrix for growth, or less able to interact with it due to loss of specific receptors or surface molecules, or they produce endogenous matrix components essential for their growth. Collagen is a major component of extra-cellular matrix *in vivo* and it has been used as substrate for cultured cells in different studies dealing with morphology [Chambard *et al.*, 1981; 1982], migration [Greenberg & Hay, 1982], and differentiation [Bissel et al., 1981; Kleinman *et al.*, 1981]. Collagen occurs in four forms: collagen type I, II, III, and IV [Bornstein and Sage, 1980]. Collagen used as a substrate in culture work is in the form of either a thin film of dried collagen, or a hydrated collagen gel usually consisting of type I found in acid extract of rat tail.

### 1.5.1.3 Polarity and Cell Shape

Orientation in space, and the shape of the cell are important determinants of the differentiated phenotype. It has been shown that full maturation of hepatocytes growing on collagen gel required release of the gel from the bottom of the dish [Ben-Ze'ev *et al.*, 1988]. Similarly, thyroid epithelial cells showed polarity and expression of differentiated phenotype, when allowed to grow in a filter well assembly [Chambard *et al.*, 1981]. In malignant cells there is disorganization with loss of inter-cellular and cell-matrix interactions, and polarity. Therefore, re-establishment of matrix and cell relationships with restoration of polarity may restore a state more responsive to other inducers of differentiation in malignant cells.

#### 1.5.1.4 Soluble Factors

Various soluble factors have been described as inducers of differentiation. These include endocrine hormones such as hydrocortisone, dexamethasone [Steffen *et al.*, 1988] paracrine factors such as transforming growth factor-$\beta$ [Goustin *et al.*, 1986], fibroblast pneumocyte factor [Post & Smith, 1984] and, vitamins such as retinoic acid [Davies *et al.*, 1988] and inorganic ions. Different physiological and non-physiological inducers have been found to induce differentiation in different cell types (see below).

### 1.5.2 Specificity of Interactions

It has been found that these interactions are not just the result of some general kind of response, rather they are very specific. The epidermis of mouse embryo skin undergoes male urogenital gland morphogenesis when implanted in embryonic mesenchyme of the urogenital sinus and seminal vesicle, suggesting a tissue specification for interaction [Cunha, 1972]. Mouse bladder epithelium was induced by embryonic mesenchyme of the urogenital sinus and seminal vesicle to differentiate into male urogenital gland phenotype [Cunha et al., 1983], suggesting a specification of these cells to respond to inductive influences of embryonic microenvironment. Tissue specificity was also shown by Neubauer *et al.* [1983], in similar studies where expression of prostatic markers was associated with differentiation of epithelial cells in urogenital sinus mesenchyme. The patterns of differentiation expressed by guinea pig epidermis depend on topographic location of the dermis upon which it is grafted [Billingham and Silver, 1968]. Mullerian epithelium forms the lining epithelium of oviducts, uterus, cervix, and vagina. The differences are attributed to regional variation in the inductive properties of the urogenital stroma [Cunha, 1975; 1976]. The adult mouse salivary gland epithelium can undergo branching development when combined with fetal salivary mesenchyme *in vitro* [Auerbach, 1964], and adult vaginal and uterine epithelium respond to fetal mesenchyme [Cunha, 1976]. It has been shown that the capacity for embryogenic morphogenesis persists in the adult mammary epithelium, when epithelial cells make direct contact with syngeneic embryonic mesenchyme from mammary rudiments, while mammary epithelial cells failed to respond to mesenchyme of lung, pancreatic and nephrogenic origin [Sakakura *et al.*, 1976; 1979], suggesting a specificity for a particular tissue in maintenance of differentiated functions.

### 1.5.3 State of Interactions in Malignant Tissues

It has been observed that many factors contribute in creating a micro-environment deficient in interactions in a malignant tissue. There is loss of inter-cellular communication, and contact inhibition. It may be due to changes in cell surface glycopeptides [Hynes, 1976; Sherbert *et al.*, 1982], or altered sialation of cell surface glycoproteins [Van Beck *et al.*, 1973], increased proteolytic activity of tumour cells [Unkless *et al.*, 1974], or mediated by stroma [Gross *et al.*, 1983]. All these factors collectively lead to an uncontrolled cell that has lost its orientation, and response to normal control mechanisms.

### 1.5.4 Induction of Differentiation in Malignant Cells.

In different studies it has been shown that cell interactions can induce or restore a differentiated phenotype in malignant tumours. Teratocarcinoma cells were induced to differentiate when implanted into blastocyst, but only when placed in contact with trophoblast, suggesting a specificity of interaction [Martin, 1975; Mintz and Illmensee, 1975]. Neuroblastoma cells (C1300) were induced to differentiate by implanting at the stage of neural tube development [Pierce, 1984], suggesting that induction depends on the spatial and temporal inductive environment. When B-16 mouse melanoma cells were implanted into the limb buds of 14 days old mouse embryos [Pierce, 1984], melanogenesis was initiated and differentiation and inhibition of proliferation were observed, suggesting that malignant processes can be reversed, but only when the cells are placed in the proper micro-environment.

Attempts have also been made to re-establish cellular orientation by stromal interaction. Malignant cells have been co-cultured with stromal cells from both embryonic and adult tissue. De Coss *et al.* [1973] have shown induction of differentiation in breast carcinoma, following co-culture with embryonic stroma. Cracker and Vernier [1972] have shown differentiation in congenital nephroma of infancy in organ culture, suggesting that tumour cells can be reorientated by stromal interactions. Such interactions are mediated at least in some systems by diffusible factors. Induction of surfactant production by hydrocortisone treated stroma has been reported both in normal and malignant lung tissues [Post & Smith, 1984; Speirs & Freshney, 1989].

Therefore, it is possible that tumour cells may retain the ability to differentiate, if they are induced in appropriate conditions, either *in vitro* or *in vivo*. Induction of differentiation has been proposed as a potential mode of cancer treatment [Spremulli and Dexter, 1984; Freshney, 1985].

### 1.5.5 Markers of Differentiation and Malignancy

Before studying differentiation or malignancy, it is important to define the marker properties, which will allow these phenotypes to be recognized. Different parameters can be used as markers. They can be functional such as enzymes, e.g DDC, NSE, peptides such as BLI, morphological features, lineage specific proteins e.g cytokeratins for epithelium, and protein products e.g mucin for glandular differentiation. Similarly loss of these differentiation associated properties or expression of malignancy associated properties can also be used as criteria for malignancy such as loss of anchorage dependence, serum independence, tumorigenicity, invasion and metastasis. Expression of at least two such properties can be regarded as a change in phenotype.

### 1.5.6 Chemical Inducers

#### 1.5.6.1 Hexamethylene Bisacetamide

Hexamethylene bisacetamide (HMBA), a compound structurally related to N-methylformamide, and dimethyl sulfoxide, is the most potent inducer of differentiation in this group of compounds. *In vitro* studies have shown that HMBA induces differentiation in a number of murine and human leukaemic and solid tumour cell lines including Friend murine erythroleukaemia [Reuben *et al.*, 1980], human HL-60 promyelocytic leukaemia [Collins *et al.*, 1980], glioblastoma [Reiss *et al.*, 1986], mouse neuroblastoma [Kloog *et al.*, 1983], rat hepatoma [Hughes *et al.*, 1982], and embryonal carcinoma [Speers *et al.*, 1979]. *In vivo* studies have shown that rats treated with HMBA after receiving the carcinogen methylnitrosourea developed fewer mammary tumours with a delay in onset compared with untreated animals [NCI-Clinical Brochure, 1984]. HMBA has also been used in phase-1 clinical trials [Egorin *et al.*, 1987].

The mechanism of action of HMBA as an inducer of differentiation remains unknown. Studies of the structure-activity relationships of various polar-planar compounds have demonstrated that the activity is derived from the Lewis base character of the CH3-CO-NH moiety [Matsuo *et al.*, 1984], and that the optimal number of carbon atoms between the functional polar group is five or six for maximum differentiating activity [Reuben *et al.*, 1978].

A polar planar compound must be flexible to penetrate the cell membrane for the induction of differentiation [Marks and Rifkind, 1978]. Cellular uptake of HMBA seems to be essential for its actions, since HMBA linked to glutathione is inactive [Gabrilove, 1986]. HMBA has also been shown to modulate ion transport [Kennedy and Lever, 1985], and has also been shown to be associated

with the membrane fraction of the cell [Reuben, 1979]. Studies on cellular uptake, metabolism, and intracellular distribution of HMBA have demonstrated that the compound was taken up by the cell, and an intracellular concentration equal to the extracelluar concentration was achieved by 6-8 hours of exposure [Reuben et al., 1980], and uptake continued for up to 20-24 hours, but with no further increase in the intracellular concentration of undegraded compound. Commitment to differentiation is both time and concentration dependent, and it takes at least 10 hours after equilibration, before differentiation is detected. Murine erythroleukaemia cells cultured with HMBA exhibited a prolongation of the G1-phase [Terada *et al.*, 1977], and a slight increase in intracellular cAMP [Gazitt *et al.*, 1978]. However, the significance of these phenomena remain unknown. It has been shown that HMBA must be present during DNA synthesis (S-phase) for induction of erythroid differentiation [Levy *et al.*, 1975].

HMBA also induces certain changes in intra-cellular polyamines, which in turn have been shown to be associated with the differentiation processes of the cell [Meilhoc *et al.*, 1986]. Catabolites of HMBA (N-acetyl -1, 6-diaminohexane and 1-, 6-diaminohexane) may be responsible for direct changes in polyamine synthesis in murine erythroleukaemia cells [Meilhoc *et al.*, 1986] and HL-60 cells [Egorin *et al.*, 1988]. *In vivo* studies have also shown similar metabolites of HMBA [Egorin *et al.*, 1988]. The enzymes responsible for HMBA catabolism, such as deacetylase, are involved in acetylation of histone in nuclear chromatin. There is a possibility that HMBA may inhibit histone deacetylation and thereby keep the histone in a hyperacetylated state which in turn might be responsible for gene transcription necessary for differentiation.

**1.5.6.2 Sodium Butyrate**

Butyric acid, a short chain 4-carbon chain fatty acid, occurs naturally in the body and is formed also by the hydrolysis of ethyl-butyrate. It has been shown to have an anti-leukaemic effect *in vivo*, when given to a patient with acute myeloid leukaemia [Novogrodsky *et al.*, 1983]. It produces a variety of effects on mammalian cells in culture depending on the cell type. It has been shown that addition of millimolar concentrations of the compound to actively proliferating cells generally leads to 1) inhibition of DNA synthesis by arrest of the treated cells in G1 stage of cell cycle [Wintersberger *et al.*, 1983, 2) modulation of gene expression [Andrews and Adamson, 1987], 3) induction of terminal differentiation [Prasad & Sinha, 1976; Philippe *et al.*, 1987], 4) alteration in cell morphology and composition of membranes [Fishman *et al.*, 1974], 5) changes in activities of enzymes, and modulation of protein synthesis [Prasad and Sinha, 1976], 6)

increase in histone acetylation [Candido *et al.*, 1978], 7) increase in chromatin decondensation [Fallon and Cox, 1981], 8) DNA hypomethylation in certain cell types [Christman *et al.*, 1980].

While it is well recognized that sodium butyrate causes growth arrest in treated cells in culture, the exact mechanism(s) by which it exerts these effects is not known. G1 is generally thought to be the major regulatory stage of the mammalian cell cycle [Prescott, 1987]. It is this stage which is modulated by conditions which affect growth [Pledger *et al.*, 1978], and cells cease to proliferate [Pardee *et al.*, 1978]. One approach commonly used to study the regulation of mammalian cell growth involved the investigation of the means by which various anti-proliferative agents bring about growth arrest in G1. Sodium butyrate induced arrest does not result from a generalized mechanism, which non-specifically shuts down the expression of growth associated genes, but rather occurs via a more specific mechanism which leads to the reduction in the expression of certain genes, while inducing the expression of others. This involves inhibition of histone deacetylase, leading to hyperacetylation of chromatin associated histone, causing gene expression [Toscani *et al.*, 1988]. Collectively these data suggest that many of the effects of sodium butyrate occur as a consequence of changes in patterns of gene expression.

**1.5.6.3 Cyclic Adenosine Monophosphate.**

Alteration of synthesis of cyclic adenosine monophosphate (cAMP) induced by external ligands act as membrane-generated signals changing the course of many intra-cellular processes. It has been shown to alter phenotypes in a variety of normal cells [Haddox *et al.*, 1980; Hackett *et al.*, 1987], transformed cells [Tagliaferri *et al.*, 1988], and tumour cell lines [Guiffre *et al.*, 1988]. It has been suggested that the altered morphology of transformed cells may be associated with a decreased level of cAMP. Low concentrations and decreased activity of adenylate cyclase have been found in a variety of transformed cell lines [Willingham, 1976].

Cyclic nucleotide dependent protein kinase activity present in the membranes of normal chick fibroblasts, was lost following transformation of these cells with Rous sarcoma virus [Brantan *et al.*, 1978]. However, the reverse transformation or differentiation effects of cAMP are not universal, since other studies have shown that cells transformed with SV-40 virus expressed cytoplasmic cAMP dependent kinase that was not found in parental 3T3 cells [Gharret *et al.*, 1976]. Similar findings have been reported by Somers *et al.* [1977] using a normal rat kidney cell line transformed with a temperature sensitive mutant of

mouse sarcoma virus. These findings suggest that although expression of transformed phenotypes are associated with loss of protein kinase activity, it is not always negatively correlated with the intra-cellular levels of cAMP. Although, cyclic AMP levels seem to modulate phenotypic expressions, the exact mechanism is unknown.

**1.5.6.4 Dexamethasone**

Dexamethasone (Dex) is a synthetic analogue of naturally occurring glucocorticoids. Dex has been shown to inhibit growth of human NSCLC cells [Jones *et al.*, 1978], human glioma [Freshney *et al.*, 1980], human breast [Osborne *et al.*, 1979], prostate [Smith *et al.*, 1985], and smooth muscle [Syms *et al.*, 1984], and various tumour cell lines. A significant reduction of growth inhibition was observed following addition of growth factors [Smith *et al.*, 1985; Syms *et al.*, 1984], suggesting that dex and growth factors have antagonistic effects, on different cells. Glucocorticoids have also been reported to either potentiate [Linebaugh and Rillema, 1977], or antagonize [Osborne *et al.*, 1979], insulin-stimulated macromolecular synthesis in human breast carcinoma cell lines. Growth inhibitory effects of glucocorticoids have been found to be decreased by EGF or TGF in different cell lines [Smith *et al.*, 1985], although, the mechanism is not clearly understood. Dex potentiates anti-proliferative effects of interferon in human cancers, and has been shown to induce differentiation in human lung and brain malignancies *in vitro* [Freshney, 1985].

Glucocorticoids elicit an array of physiological responses in target tissues and cultured cells, including regulation of cellular growth and modulation of gene expression [Rousseau, 1984]. They alter cells by passive diffusion and act via specific receptors. The first step in glucocorticoid action is reversible, noncovalent, high affinity binding to its intra-cellular receptors and translocation to nucleus. Subsequently the receptor hormone complex undergoes some conformational changes, followed by activation of receptor during which it acquires the capacity to interact with chromatin , where it occupies receptor sites composed of DNA and non-histone nuclear proteins [Rousseau, 1984].

The extensive use of glucocorticoids in various therapeutic regimens is largely due to their anti-inflammatory, immunosuppressive, and anti-allergic properties. Their catabolic effects generally result in an inhibition of cellular growth, which might also induce cellular lysis. It appears that glucocorticoids act directly at the level of regulatory mechanisms for cellular proliferation. Two opposite effects have been shown: firstly an inhibitory effect on various cell types, and secondly a stimulatory effect for a limited number of other cell types.

There is evidence for more than one mechanism of action of glucocorticoids [Keller *et al.*, 1982]. In addition to mechanisms in which receptors are involved, there is also a strong inhibitory effect at high concentration, probably the consequence of several metabolic inhibitions e.g carbohydrates, proteins which result in decrease cellular activity [Hainque *et al.*, 1987].

**1.5.6.5 Retinoic Acid**

Vitamin A and its analogues (retinoids) inhibit proliferation and suppress the transformed phenotype of many tumour cells *in vitro*. Vitamin A is essential for normal *in vivo* development, reproductive capacity, vision, and maintenance of several epithelial tissues [Kim & Wolf, 1974]. Retinoids have also been shown to regulate differentiation in different cell types *in vitro*, including malignant melanoma [Lotan *et al.*, 1978], teratocarcinoma cells [Plet *et al.*, 1987], myeloid leukaemia cells [Olsson *et al.*, 1982] and different other cell types. The effects of retinoids have also been shown in differentiation of normal respiratory epithelium *in vitro* [Jetten & Smits, 1985; Jetten *et al.*, 1986]. It is essential for the differentiation and maintenance of normal respiratory mucociliary epithelium. In vitamin A deficiency mucus secreting epithelia such as that of the respiratory tract develop foci of atrophy and keratinizing squamous epithelium and a decrease in number of goblet cells [Bonanni, 1973]. It has been suggested that specific binding proteins mediate the translocation of retinoid to the nucleus where they interact with the chromatin and modify gene expression [Jetten and Smits, 1985]. Several studies have also shown a relationship between retinoic acid, cyclic AMP, and cyclic AMP protein kinase activity in some cells [Fontana *et al.*, 1986].

**1.5.6.6 Transforming Growth Factor-$\beta$**

Transforming growth factor, type-$\beta$ (TGF-$\beta$) is a peptide factor produced by a variety of cells, which can either stimulate or inhibit cell proliferation, and differentiation. The role of TGF-$\beta$ in neoplastic transformation is unknown. As most cells release TGF-$\beta$ in an inactive form and also have receptors for TGF-$\beta$, it has been suggested that at least part of the regulation of its actions is at the post-receptor level or at the level of precursor activation. TGF-$\beta$ is primarily a growth inhibitor and not a classic growth factor [Moses *et al.*, 1978]. It stimulates growth only in fibroblastic cells, and it has been shown that the stimulatory effect of TGF-$\beta$ is mediated through induction of c-sis and autocrine stimulation by a PDGF-like growth factor [Leof *et al.*, 1986]. Therefore, either autocrine stimulation by endogenous TGF-$\beta$ in fibroblastic cells or the loss of the inhibitory

effects of TGF-$\beta$ in other cells, may lead to an increased proliferative potential and thereby contribute to the induction of neoplastic phenotype.

## 1.6 ONCOGENES AND MALIGNANT TRANSFORMATION

An oncogene is a gene that encodes a protein that contributes to the malignant transformation of the transfected cell [Huebner and Todaro, 1969]. Oncogenes are derived from normal cellular genes, expressed in an altered form.

### 1.6.1 Oncogenes in Human Malignancies

Following the discovery of proto-oncogenes, it has been found that a number of altered or overexpressed proto-oncogenes are involved in the genesis of human tumours. Table [1.3] lists genetic lesions that are found at reasonable frequency (varying from 20 to 100%) in these tumours [Varmus, 1984]. Amplification of the myc gene family has been described in SCLC. L-myc proto-oncogene amplification has been found both in primary as well as in relapsed SCLC [Johnson *et al.*, 1987], and in another study a 20-fold amplification was found in SCLC [Chan *et al.*, 1986]. L-myc has been mapped to chromosome 1 [Nau *et al.*, 1985]. Abnormal expression of myc and ras has also been found in human colorectal tumours.

The ras oncogene expression has been found in bladder cancer [Tabin *et al.*, 1982; Reddy *et al.*, 1982]. Ha-ras oncogene activation, and c-myc amplification [Escot *et al.*, 1986] have been reported in breast cancer.

Three classes of genes have been described which are involved directly in carcinogenesis [Spandidos, 1986]. The *first class* is comprised of oncogenes, originally identified by transduction with retroviruses and subsequently by DNA mediated gene transfer [Bishop, 1987]. Oncogenes act dominantly at the cellular level and can convert normal cells to transformed or malignant cells. This is the best studied group and around forty such genes have been directly identified and isolated [see Tables 1.2 & 1.3]. The first human cellular oncogene was cloned in 1982 from T24 bladder carcinoma cells, and was found to be homologous to the viral Ha-ras oncogene [Tabin *et al.*, 1982; Reddy *et al.*, 1982].

The *second class* of genes is comprised of a diverse group of genes whose expression inhibits the cancer phenotype. The existence of these genes was first noticed from studies of cancer associated with heritable single gene traits [Knudson, 1986]. In some cases deletion or inactivation of both alleles have been found in association with tumour formation [Knudson, 1983]. Various descriptive

**Table 1.3:- Examples of proto-oncogenes and associated human malignancies.**

| Proto-oncogene | Neoplasm | Lesion |
|---|---|---|
| c-myc | Burkitt's lymphoma | Translocation |
| | SCLC | Amplification |
| | Ca of breast | Amplification |
| L-myc | SCLC | Amplification |
| | Neuroblastoma | Amplification |
| c-ras | Diverse | Point mutation |
| c-abl | CML | Translocation |
| c-erbB | Squamous cell Ca | Amplification |
| | Glioblastoma | Amplification |

SCLC: small cell lung carcinoma. CML: chronic myelogenous leukaemia. Ca: carcinoma.

terms have been used in literature such as anti-oncogenes [Knudson, 1983], tumour-suppressor genes [Stanbridge, 1986], ortho-, plato-, or emero-genes [Todaro, 1986], onco-suppressor genes [Spandidos and Anderson, 1989]. There is substantial evidence to suggest that malignant transformation may be inhibited by expression of proto-oncogenes and their deletion may release oncogene expression [Spandidos and Wilkie, 1988]. These genes have been found to inhibit the malignancy-related phenotypes in different studies such as morphological transformation, tumorigenicity and metastasis. They have been associated with the predisposition to dominantly inherited disorders such as retinoblastoma [Lee *et al.*, 1987].

A *third class* of genes is associated with predisposition to cancer in patients with recessively inherited disorders such as ataxia telangiectasia, Bloom's syndrome, and Fanconi's anaemia. The patients with these disorders have defects in DNA repair, which indirectly results in increased incidence of malignancy [Knudson, 1986]. One such gene has been identified and cloned, the human excision repair gene ERCC-1 [Van Duin *et al.*, 1986].

### 1.6.2 Origin of Oncogenes

Viruses have been associated with the development of both human and the animal tumours [Bishop, 1985; Varmus, 1986]. Analysis of the genome of oncogenic viruses has revealed that viruses capable of transformation have lost their replicative gene (pol) and in their place are new nucleic acid sequences termed v-onc which confer malignant potential. The transduction hypothesis states that the viral oncogenes were derived from cellular genes that had been "hijacked" by the virus earlier in evolution. These cellular genes have been called proto-oncogenes. Recombination between retroviruses and the cellular genome can implant cellular genes into the viral genome, and in this new setting the cellular genes may become oncogenic. These observations have implied that normal cellular DNA contains genes which when transplanted or transducted into retrovirus become cancer causing genes. Over 50 of such virus isolates have been found to have this property [Table 1.4], and over 20 have been characterized.

### 1.6.3 Activation of Proto-oncogenes

There are several mechanisms by which a proto-oncogene may be activated to a functional oncogene. A single **point mutation** in a proto-oncogene can give rise to a functional oncogene resulting in an amino acid substitution in the gene product that can alter the biological behaviour of the normal cell. An

**Table 1.4:- Examples of viral oncogenes, their cellular counterparts and associated human malignancies.**

| Viral oncogene | Cellular Oncogene | Human Tumour |
|---|---|---|
| v-ras | c-ras (Ha & Ki) | Bladder carcinoma |
| | | Colorectal adenocarcinoma |
| | | Ovarian adenocarcinoma |
| v-myc | c-myc | Prostate carcinoma |
| | | Breast carcinoma |
| | L-myc | Lung carcinoma |
| | N-myc | Neuroblastoma |
| v-erbB | c-erbB1 | Brain tumour |
| | c-erbB2 | Breast cancer |
| v-erbA | c-erbA | Breast carcinoma |
| v-sis | c-sis | Brain tumour |
| v-abl | c-abl | Chronic myeloid leukaemia |

example is the activation of ras proto-oncogenes by point mutations at codon 12, 13, and 61 [Tabin *et al.*, 1982; Reddy *et al.*, 1982]. The **amplification** of a proto-oncogene may also lead to overexpression of gene products, and malignant transformation [Varmus, 1984; 1986]. The c-myc oncogene amplification has been found in leukaemia [Collins and Groudine, 1982], and in SCLC where it has been associated with aggressive clinical behaviour [Little *et al.*, 1983]. N-myc amplification has been found in metastatic neuroblastoma [Schwab *et al.*, 1983]. The **transduction** of a cellular sequence (proto-oncogene) into the viral genome may lead to its activation [Varmus, 1984]. Proto-oncogene activation may also result from **insertional mutagenesis**, when a retroviral promoter integrates adjacent to the proto-oncogene [Varmus, 1986]. More recently, it has been proposed that inactivation of a constitutive suppressor or **anti-oncogene** may also lead to activation of proto-oncogene [Knudson, 1985; Spandidos and Anderson, 1989]. Various **chromosomal abnormalities** have been related to the activation of proto-oncogene into active oncogene. Translocation and break points have been demonstrated in different human malignancies, Philadelphia chromosome 9; 22 translocation in chronic myelogenous leukaemia, and chromosome 8; 2, 14, 22 translocation in Burkitt's lymphoma possibly involving myc oncogene. Malignant cells have also been found to contain homogeneously staining regions (HSRs) and double minutes chromosomes (DMs), which are thought to be the result of gene amplification.

### 1.6.4 Oncogene Product

In a normal cell the expression of a proto-oncogene product is well controlled and appears to play a role in the growth and development of the organism. The oncogene codes for protein product which can be a growth factor, a growth factor receptor, membrane associated and/or cytoplasmic protein kinases, or GTP binding proteins and nuclear proteins [Figure 1.2]. The sis oncogene has been recognized as being homologous to the gene for the $\beta$-chain of platelet derived growth factor (PDGF) [Waterfield *et al.*, 1983]. A number of transmembrane receptors have protein kinase activity, including epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), insulin-like growth factor 1 receptor and colony stimulating factor-1 (CSF-1). The protein product of v-erb-B is homologous to EGFR and the product of vfms is homologous to the CSF-1 receptor. The protein products of v-erb-B, v-fms, and c-fos are undoubtedly concerned with growth control. A number of different oncogenes have been found to have protein kinase activity

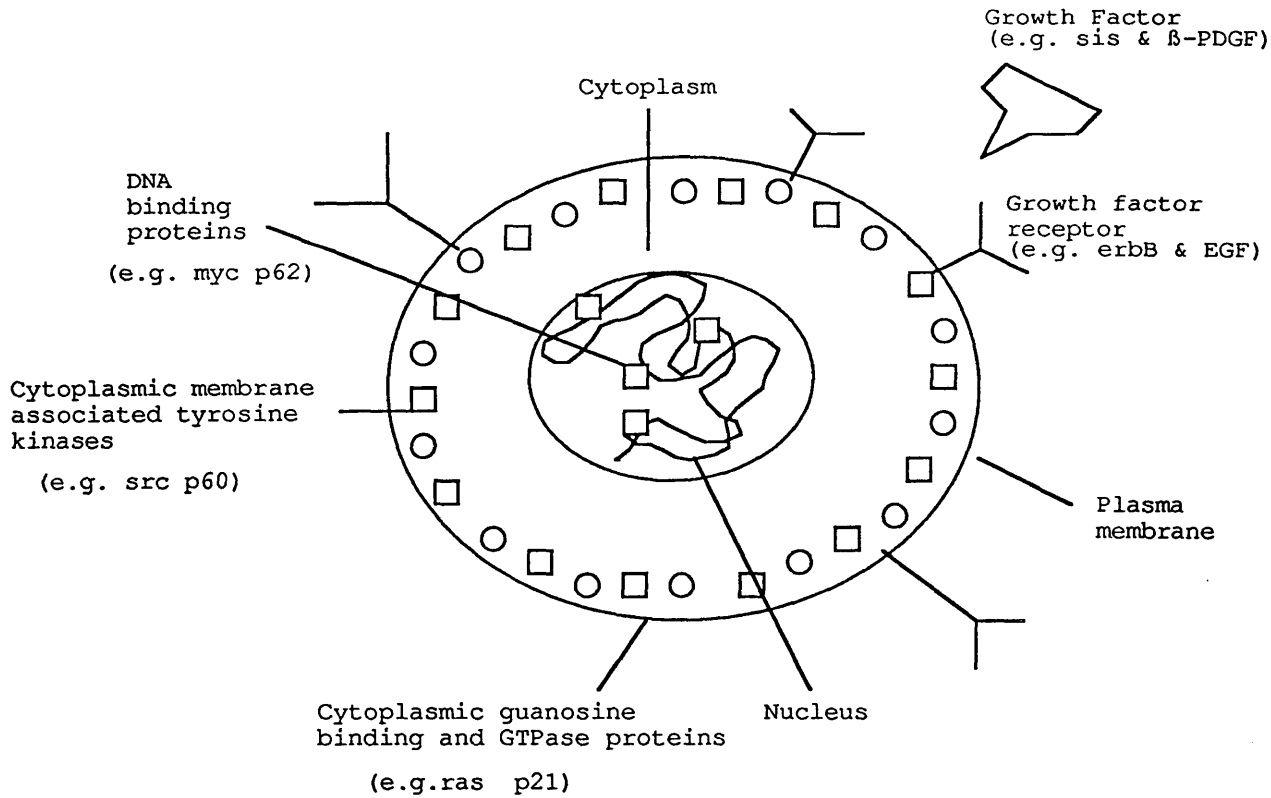


**Figure 1.2-: Sites of action of oncogene products in different areas of the mammalian cell.**

such as abl, fes, fms, erb-B, and ras, ras gene product p21 is a GTP binding protein with GTPase activity. Its submembranous position and the ability to stimulate adenylate cyclase, suggests that it may act as an intermediate between a receptor on the cell surface and adenylate cyclase in the signal transduction pathway. A group of oncogenes including myc, myb, fos, and p53 encode a group of related oncoproteins, which share the properties of nuclear localization, and binding to nuclear matrix or DNA. Several members of this group have been implicated in control of gene transcription [Alitalo *et al.*, 1987] and cell proliferation [Makino et al., 1984], self renewal control [Greenberg & Ziff, 1984] and differentiation control [Pfieffer-Ohlsson *et al.*, 1984; Stewart *et al.*, 1984].

### 1.6.5 THE ras FAMILY OF ONCOGENES

To date three members of the ras gene family have been identified in the mammalian genome. They have been designated Harvey (Ha), Kirsten (Ki) and N-ras. They encode highly related proteins generically known as p21 with molecular weight of 21,000 and 189 amino acid residues [Barbacid, 1987]. The ras gene can become activated and acquire transforming properties by quantitative (amplification) and qualitative (mutation) mechanisms. The latter are more frequent.

Mutations have been identified directly from analysis of tumour DNA with molecular cloning and sequencing of genes, and by this method all mutations occurring in the naturally occurring tumours have been found in the codons for amino acids 12, 13, and 61 where either glycine or glutamine has been replaced by any other amino acid. A second approach was the molecular cloning of normal ras genes, their *in vitro* mutagenesis, and analysis for transforming potentials in NIH 3T3 focus forming assay. Using chemical carcinogens it was found that in addition to the codons found in naturally occurring tumours or tumour cell lines codons 59 or 63 are also potential targets for mutations [Vousden *et al.*, 1986]. Others [Sigal *et al.*, 1986; Walter *et al.*, 1986] have shown that some mutations in codon 116, 119 can also activate ras genes *in vitro*. Various deletion mutations have also been shown to have transforming activity such as deletion of non-coding regions [Chipperfield *et al.*, 1985].

The involvement of ras genes in human cancers is not limited to their activation by point mutations. It is likely that the expression of abnormally high levels of normal ras products may also contribute to malignancy. This could be possible by perturbation of their regulatory sequences [Westaway *et al.*, 1986] or by gene amplification. The overall ras gene amplification in human neoplasia is

estimated to be not higher than 1% [Pulciani *et al.*, 1985]. Enhanced expression or amplification of ras oncogene can be obtained by the insertion of a strong promoter or enhancer in the vicinity of the ras gene [Westaway *et al.*, 1986; Mckay *et al.*, 1986]. Linkage of normal ras gene to retroviral regulatory elements (LTR) results in malignant transformation of NIH 3T3 cells [Chang *et al.*, 1982; Mckay *et al.*, 1986]. Similar results have been shown by amplification of normal genes brought about by integration of multiple copies of a DNA clone of the normal human ras gene [Pulciani *et al.*, 1985] or by deletion of the first (non-coding) exon [Cichutek and Duesberg, 1986]. These cells invariably show 30- to 100-fold higher levels of ras expression than either their normal counterparts or cells transformed by ras oncogenes activated by single point mutations [Chang *et al.*, 1982; Pulciani *et al.*, 1985; Mckay *et al.*, 1986;]. Spandidos and Wilkie [1984] have shown enhanced expression when ras genes were surrounded by both SV40 and Moloney virus LTR enhancers, and this has been used in the present constructs (see below, Chapter five).

**1.6.5.1 Mechanism of Action**

The resemblance of the ras protein to the G-proteins controlling adenylate cyclase has suggested that normal p21 ras proteins are involved in the signal transduction [Gilman, 1984]. According to the currently accepted model [Figure 1.3], the ras proteins exist in equilibrium between an active and an inactive state. Most of ras proteins in a normal cell at rest would exist in their inactive state which is characterized by conformation that allows binding to GDP. They remain in this inactive state until they receive a stimulus resulting in the change of GDP to GTP followed by a conformational change of the ras protein to its active state. Activated ras protein would then interact with the effector molecules. Once the signal transduction has taken place, they would immediately become inactivated by their intrinsic GTPase activity thereby returning the ras protein to the inactive GDP-bound state. According to this model mutations that confer transforming properties to ras genes must reverse the normal equilibrium between the active and the inactive forms. Stabilization of ras proteins in their active state would cause a continuous flow of signal transduction, which would result in malignant transformation. Theoretically, this state can be achieved by: 1) mutations that inhibit the intrinsic GTPase activity of ras proteins. Normally the activated state is terminated by hydrolysis of bound GTP to GDP due to the intrinsic GTPase activity. A loss of GTPase activity would result in a constitutively activated ras protein, most likely due to transforming mutations in codons 12,13 or 61. Sweet *et al.* [1984] have shown that the inhibition of GTPase activity is the preferred

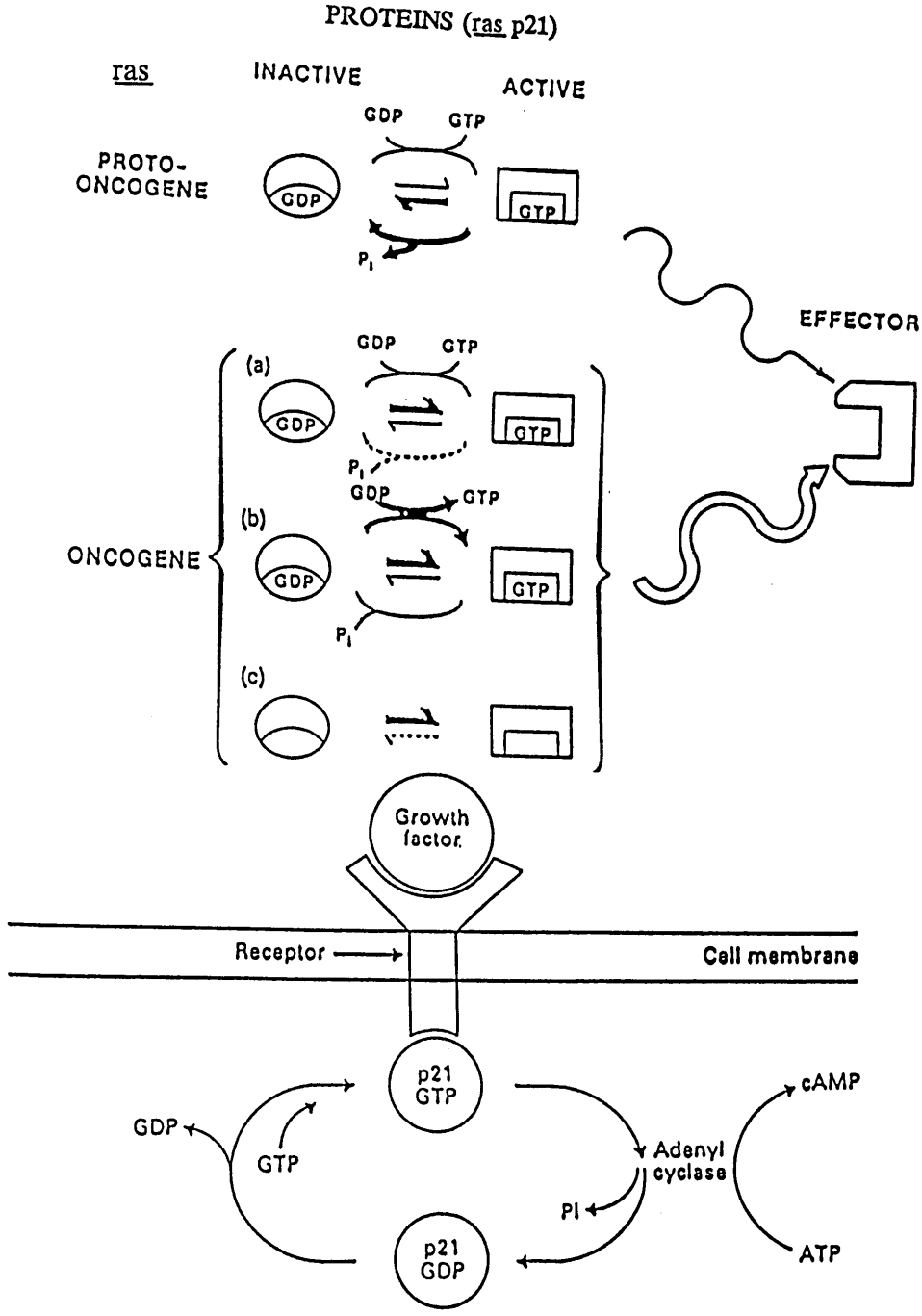


Figure 1.3:- Model for the mechanism of action of normal and transforming ras proteins: signal transmission from growth factor to adenyl cyclase through p21 and growth factor receptor [modified from Barbacid, 1979; Thomas & Waxman, 1988].

mechanism of activation of oncogenic ras proteins as illustrated in Figure [1.3 a], 2) by increasing the exchange rate between GDP and GTP [Figure 1.3 b], 3) by inducing an active conformational change that does not require binding of guanine nucleotide. It has been found that for the mutations in codon 59, 116, and 119 both GDP and GTP still bind to the ras protein, but this binding is less stable [Lacal and Aaronson, 1986]. Since the intracellular concentration of GTP is 10 times higher than that of GDP therefore, GTP will be bound more frequently than GDP and thus the protein will be mostly in its active configuration [Figure 1.3 c].

It is proposed that highly overexpressed levels of normal ras proteins as a result of gene amplification may produce enough molecules in their GTP-bound active state to induce malignant transformation, without affecting the equilibrium between inactive and active forms, which is the characteristic of the normal ras proteins.

**1.6.5.2 Transforming Properties *in vitro***

In addition to their involvement in human cancer and *in vivo* transforming properties, the ras oncogenes have been extensively studied for their transforming properties *in vitro* assays. Cellular ras genes induce morphological and growth transformation in established rodent cell lines in a dominant manner [Barbacid, 1985], suggesting a role in malignant transformation. Shih *et al.* [1979] have shown that ras oncogenes are involved not only in the initiation, but also in the maintenance of the transformed phenotype suggesting a possibility of their continued expression in transformation. Winter and Perucho, [1986] have shown that the level of ras oncogene expression appears to regulate or modulate their transforming potency when used to induce reversible transformation of normal rat fibroblasts. In other studies [Land *et al.*, 1983] cellular ras genes could not transform rat primary embryo cells alone, but required the cooperation of nuclear oncogenes such as c-myc [Schwab *et al.*, 1985], suggesting that a single oncogene may not be sufficient to cause transformation. However, Spandidos and Wilkie [1984] have shown that Ha-ras oncogene can transform rat embryo cells if the surrounding cells are eliminated by selection system, suggesting that the linkage of ras oncogenes to transcriptional enhancers increases the percentage of cells that become transformed under these conditions. Similar results have been shown by Dotto *et al.*, [1985] and Pozzatti *et al.*, [1986], suggesting that normal cells can inhibit transformed phenotypes of ras transfected cells, and this inhibitory effect can be partly overcome by treating the ras containing primary embryo cells with tumour promoters or other selection systems.

Evaluation of the neoplastic properties of ras oncogenes in cells other than cultured fibroblasts has been limited because of the difficulty in transfecting non-fibroblastic cells. However, the use of retroviruses carrying ras oncogenes has circumvented this problem to some extent. Yoakum *et al.* [1985] have shown that the transfection of normal human bronchial epithelial cells by ras oncogenes from DNA clone of Harvey-MSV, yielded clones of transformed cells. Others have been able to transform human epidermal keratinocytes [Rhim *et al.*, 1985] and embryonic kidney cells [Pater and Pater, 1986] with ras containing retroviruses, but with prior acquisition of independent life span. The ras oncogenes have also been found to cause additional neoplastic change to cell lines derived from human tumours [Kasid *et al.*, 1985], such as tumorigenesis, invasion, and metastasis, suggesting a multistep process of carcinogenesis, and involvement of carcinogens (oncogenes) at different stages.

**1.6.5.3 Nature of Normal and Activated ras**

It is important to know whether activated and/or normal ras genes are dominant or recessive. It has been shown that human tumours contain an activated ras allele in addition to a corresponding normal ras allele, and either the normal allele has been lost or the mutated allele has been amplified, and that during tumour progression the ratio between the normal and the activated allele can shift towards the mutated allele [Mckay *et al.*, 1986; Bos *et al.*, 1987]. However, it has been shown by others [Tabin and Weinburg, 1985] that the normal ras allele even at an increased level of expression had no effects on the transforming capacity of the mutant allele. Theillet *et al.*, [1986] and Fearon *et al.* [1985] have shown in several different tumours that one of the Ha-ras alleles was deleted suggesting suppression of normal ras genes might have a role in a tumour development. The loss of a normal allele in cells carrying ras oncogenes has been observed by others also [Santos *et al.*, 1984]. These observations suggest that the deletion of the Ha-ras allele coincides with the locus of another gene located in the proximity of the Ha-ras gene. Possible candidates are the tumour-suppressor genes, which when present can suppress the oncogenic phenotype. Suppressor genes have been located on chromosome 11 in some of the cell fusion studies [Saxon *et al.*, 1986], suggesting that if the ras gene was located close to a tumour-suppressor gene it could be deleted simultaneously, such as in Wilms tumour.

### 1.6.5.4 The ras Expression: Early or Late Event

Expression of ras proto-oncogenes or oncogenes in various stages of carcinogenesis, have suggested their involvement at different stages of disease. The ras expression has been shown in early, intermediate, and late stages of carcinogenesis. Malignant transformation of early passage rat cells with a single oncogene, the T24 Ha-ras1 has been shown by Pozzati *et al.* [1986]. Spandidos and Wilkie [1984] have shown that when linked to transcriptional enhancers, the mutant T24 Ha-ras1 gene induces the complete malignant transformation of early passage rodents including Chinese and Syrian hamsters and rat. These results suggest that the ras gene may be involved in carcinogenesis at early stages of tumour initiation. Yoakum *et al.* [1985] have shown that transfection of human early passage bronchial epithelial cells by a recombinant plasmid carrying the viral Ha-ras oncogene caused immortalization and malignant conversion of these cells. *In vivo* studies have shown similar results. Balmain *et al.* [1984] have shown that chemically induced papillomas of mouse skin contain a transforming cellular Ha-ras gene as evident from elevated transcript levels in these tumours, suggesting an early involvement of ras in carcinogenesis. Elevated ras transcripts have been found in several human tumours such as pre-malignant and malignant tumours of the colorectum [Spandidos and Kerr, 1984], tumours of head and neck region [Spandidos *et al.*, 1985], malignant tumours of human breast [Spandidos and Agnantis, 1984], suggesting that ras activation may be early event in carcinogenesis *in vivo*. Williams *et al.* [1985] have shown expression of ras p21 protein by immunocytochemical methods, using monoclonal antibody to p21 protein in various tumours of colorectum and experimental models. These findings have suggested that ras may be involved in tumour initiation.

There is enough evidence for the involvement of ras genes in the intermediate stages of carcinogenesis such as focus formation anchorage independence, and tumorigenic conversion of immortalized cells, and also in clinical tumour specimens. Malignant transformation of early passage rodent fibroblasts by a single ras gene have been shown by Spandidos and Wilkie [1984], and in colorectal tumours [Spandidos and Kerr, 1984]. Recent evidence suggests that ras oncogene may also be involved in the late stages of carcinogenesis. This has been demonstrated in rodent fibroblasts transfected with ras oncogene [Spandidos and Wilkie, 1984], and in hamster cells [Spandidos and Siminovitch, 1978], and in primary rat embryo cell after ras transfection [Pozzati *et al.*, 1986], in human lymphoma [Vousden and Marshall, 1984], and in human teratoma cells [Tainsky *et al.*, 1984]. Vousden *et al.* [1986] have shown enhancement of

spontaneous metastasis of mouse carcinoma cells transfected with activated Ha-ras oncogene, suggesting an involvement of ras also in the late stages of the malignancy. Albino *et al.* [1984] have shown various metastatic clones of human melanoma expressing ras oncogene. Thorgeirsson *et al.* [1985] have shown that NIH3T3 cells transfected with human tumour DNA containing activating ras oncogene expresses metastatic phenotype in nude mice. These data suggest that ras oncogenes are involved in addition to early stages, in the late stages of the malignant progression, such as invasion and metastasis.

Thus on the one hand activated ras genes have been shown to convert immortalized cells or benign tumours into malignant tumours or malignant tumours into tumours with metastatic capacity, on the other hand activated ras genes have been found in pre-malignant cells or benign tumours. These observations suggest that ras may be involved in tumour initiation and maintenance.

**1.6.5.5 The ras Oncogene and Metastasis**

The ability of ras oncogenes to induce the metastatic phenotype is not fully established. Both *in vitro* and *in vivo* studies have shown that some ras containing tumours are metastatic, but others are not. It has been shown that NIH 3T3 and primary rat embryo cells transfected with ras oncogene formed metastatic foci in lung when injected subcutaneously in nude mice [Pozzatti *et al.*, 1986; Thorgeirrsson *et al.*, 1985], suggesting a possible role of ras oncogene in metastatic progression of the tumour. Bradley *et al.* [1986] have observed metastatic nodules only when ras transfected 3T3 cells were injected intravenously, suggesting that the route of injection may also be important in the metastatic growth, and that all ras expressing cells are not necessarily metastatic. In a comparative study, Muschel *et al.* [1985] used different mouse cell lines transfected with the ras oncogene. NIH 3T3 cells were metastatic but C127 cells were non-metastatic. These studies have suggested that the induction of the metastatic phenotype is not an intrinsic property of ras oncogenes, but may depend upon the cell type and other factors. Studies on human tumours both from primary and metastatic sites have shown a similar incidence of ras oncogene expression. Therefore, it may be that ras is not directly involved in the metastatic process, but provides better growth potential to the cells which indirectly may give them a better chance to form metastases, and this may vary among different cell types and target tissues.

### 1.6.6 THE myc FAMILY OF ONCOGENES

To date a number of related genes have been included in the ever expanding family of myc genes. These are characterized by the presence of conserved myc homology regions, of highly conserved amino acid sequences of c-myc protein, nuclear targeting of gene products, and DNA binding capacity. The myc family consists of three well characterized members, c-myc [Varmus, 1984], N-myc [Schwab *et al.*, 1983], L-myc [Zimmerman *et al.*, 1986], and newly found oncogenes such as R-myc, U-myc, one pseudogene and functional activity of at least one other gene P-myc has also been described. The v-myc genes were first identified and characterized as transforming genes from several different strains of avian retroviruses [Coffin *et al.*, 1981]. Like the discovery of many other proto-oncogenes, an attempt to discover a cellular counterpart of this viral sequence has led to the discovery of the first member of this family, the c-myc gene. The human c-myc gene has three exons with the coding regions located on exon 2 and 3 [Watt *et al.*, 1983]. The remainder of the gene is untranslated, encoding a transcript with large 5, and 3, untranslated regions [Stanton *et al.*,1984].

#### 1.6.6.1 The myc in Growth and Differentiation

The myc gene displays a distinct pattern of expression during normal growth and development. The c-myc expression has been observed in cells progressing from a resting state to a dividing state [Makino *et al.*, 1984]. It is thought that myc is involved in cellular proliferation and differentiation, and that when activated, it can induce cellular proliferation and block differentiation. The level of myc gene expression decreased rapidly in tumour systems induced to differentiate *in vitro* following treatment with agents such as DMSO, sodium butyrate, retinoic acid [Lachman and Skoultchi, [1984], and reappeared later in a cell cycle restricted manner [Lachman *et al.*, 1985]. In another study Grosso and Pitot [1985] have demonstrated that c-myc expression was abruptly repressed when susceptible murine tumour cell lines were induced to differentiate by a variety of inducing agents such as retinoic acid and sodium butyrate. The c-myc has also been shown to inhibit DMSO induced differentiation in mouse erythroleukaemia cells [Dmitrovsky *et al.*, 1986]. It may be involved during differentiation in human cells as c-myc mRNA copy number shows a peak at 4-5 weeks in developing placenta [Pfeiffer-Ohlsson *et al.*, 1984], and a peak during spermatogenesis, with stem cells and mature sperms showing very low levels [Stewart *et al.*, 1984]. These observations suggest that c-myc gene might be associated with the process of cellular proliferation prior to differentiation.

### 1.6.6.2 The c-myc and Malignant Transformation

Deregulated expression of c-myc gene in many different tumours, its enhanced expression during cell division, and a reduced expression in differentiated cells, suggest that the aberrant expression of c-myc can contribute to the development of neoplastic transformation in different tumour types by influencing the rate of cellular proliferation [Varmus, 1984; Bishop, 1985]. Amplification of c-myc has been found in various human cancers and tumour cell lines, including leukaemia [Collins and Groudine. 1982], colonic tumours [Alitalo *et al.*, 1983; Stewart *et al.*, 1986], and both NSCLC [Yokato *et al.*, 1988], SCLC [Wong *et al.*, 1986], and SCLC cell lines in culture [Little *et al.*, 1983; Nau *et al.*, 1985]. Several human epithelial tumours express c-myc p62 protein at a level higher than in the adjacent normal tissue, such as testicular and colonic tumours [Sikora *et al.*, 1987] and breast tumours [Spandidos,1987], suggesting that myc oncogene expression is associated with human malignancy.

Activated c-myc gene has been found to be implicated in immortalization, transformation, and tumour progression depending upon the model used. The c-myc is expressed in virtually all cells in their proliferative phase. It seems most likely that the same biochemical activity and targets promote tumour cell growth, whereas the cell background determines how this activity is going to be manifested. It has been shown that when linked to a transcriptional enhancer the c-myc proto-oncogene induced immortalization of early passage rodent fibroblast cells [Mongueau *et al.* 1984]. The myc transfection can trigger tumorigenic conversion of immortalized cells [Spandidos, 1985]. It has also been shown that c-myc gene converted a non-tumorigenic cell line to tumorigenic [Keath *et al.*, 1984], and induced metastatic properties in tumorigenic cells [Wyllie *et al.*, 1987]. Moguneau *et al.* [1984], and Kelekar & Cole. [1986] have shown that the c-myc transfection of normal fibroblasts could induce immortalization and tumorigenicity in nude mice.

The early studies with the myc oncogene utilized the acute oncogenic avian retrovirus (MC29) containing the cellular myc gene. They have been demonstrated in different lymphomas e.g., feline lymphomas [Mullins *et al.*, 1984].

The study of transgenic mouse strains with abnormal c-myc expression has demonstrated a variety of tumours including testicular, breast, lymphoid and mast cell origin [Leder *et al.*, 1986; Adams *et al.*, 1985], hepatocellular carcinoma [Messing *et al.*, 1985], and pancreatic carcinoma [Hanhan, 1985].

### 1.6.6.3 Regulation of c-myc Expression

The expression of c-myc in many cell types, both normal and malignant, has suggested that it is in a pathway present in most cells. Observations of the quantitative differences in the levels of c-myc mRNA expression in normal and tumour tissue have suggested that the regulatory mechanisms might be operating at both transcriptional and post-transcriptional levels. At the transcriptional level a mitogenic stimulus to quiescent fibroblasts leads to a transient rise in c-myc expression [Kelly *et al.*, 1983], suggesting an association between c-myc expression and entry of cells into cell cycle (Go to G1). Land *et al.* [1983] have shown that the increased transcription rate observed during the initial stage of mitogenic stimulation was not sufficient to account for the 40-fold increase in the c-myc mRNA level, suggesting a post-transcriptional control responsible for the remainder of the increase in mRNA level. In further studies it has been demonstrated that the c-myc mRNA level can be modulated in the absence of any changes in the transcription rate of the gene [Knight *et al.*, 1985]. Teratoma cells induced to differentiate, and lymphoma cells treated with interferon therapy suppressed their c-myc mRNA level at least by 20-fold without any change in the rate of mRNA synthesis [Dani *et al.*, 1985].

These findings therefore, suggest that the primary mode of c-myc mRNA regulation in the transition from quiescent to proliferating cells or vice versa is post-transcriptional. Nevertheless, transcriptional control of c-myc may be important at certain stages of cellular differentiation. Post-transcriptional modulation of c-myc mRNA level may result either from altered mRNA transportation from nucleus to cytoplasm or its stability. It has also been shown that the amount of c-myc protein p62 correlates directly with the mRNA level, and that the protein half life does not change with the cell growth rate [Persson *et al.*, 1985] suggesting that an increased level of myc transcription in tumour cells may be due to the deregulation of the negative feedback inhibition between the myc product and the myc gene.

The c-myc protein, a 439 amino acid sequence, has been isolated and characterized from human cell lines. It is a nuclear phosphoprotein with a molecular weight of 62 kD [Ramsey *et al.*, 1984]. The turnover of both the c-myc protein and its mRNA is rapid with half lives in order of 20 to 30 minutes in exponentially growing cells [Hann *et al.*, 1985]. However, protein species with longer half lives have also been described [Persson *et al.*, 1985]. It has been shown that the synthesis and half-life of the c-myc protein are constant throughout the cell cycle in normal and transformed avian and human cell lines

growing in log phase in culture [Hann *et al.*, 1985].

### 1.6.6.4 Activation of c-myc Oncogene

Unlike the ras oncogenes which are activated as a result of mutations within the protein coding regions, no amino acid changes have been found in the c-myc activation. Different mechanisms are involved in the activation of the c-myc proto-oncogene, such as proviral insertion, chromosomal translocation, and gene amplification. Hayward *et al.* [1981] were first to demonstrate that avian leukosis virus was integrated into the c-myc locus in viral induced B-cell lymphomas. A general model has been described by Varmus [1984] that retroviral insertion activates cellular genes by enhancer insertion which elevates transcription from the normal c-myc promoters. The c-myc gene was found to be located at the chromosomal translocation breakpoints in Burkitt's lymphoma and in murine plasmacytoma [Adams *et al.*, 1985; Erikson *et al.*, 1983]. Amplification of the gene has been found in large cell variant of small cell lung cancer [Little *et al.*, 1983; Nau *et al.*, 1985;1986]. Proto-oncogene amplification in tumour cell line was first described in the c-myc gene locus [Collins and Groudine, 1982; Alitelo *et al.*, 1983]. It has been shown in HL-60 that c-myc gene is not deregulated and can be suppressed by inducers of differentiation [Westin *et al.*, 1982], suggesting that amplification would promote tumour progression by rapid growth and proliferation.

### 1.6.6.5 The myc Expression: Early or Late Event

There is some evidence for the involvement of myc in early, intermediate, and late stages of the carcinogenesis both *in vitro* and *in vivo*. The myc oncogenes have been shown to cause immortalization of early passage fibroblasts in culture [Land *et al.*, 1983; Mougueau *et al.*, 1984]. The myc has also been shown to cause tumorigenic conversion of mammalian fibroblasts [Vennstram *et al.*, 1984; Kelekar and Cole, 1986]. The involvement of myc has also been shown in late stages of human lung carcinoma [Little *et al.*, 1983], and in human neuroblastoma [Schwab *et al.*, 1984], therefore, suggesting that the expression of myc oncogene may be significant in tumour initiation and maintenance.

## 1.7 RATIONALE FOR THE WORK PRESENTED IN THIS THESIS

Heterogeneity and phenotypic changes have been well documented in human lung cancer, but relatively little is known about the nature and the regulatory control mechanisms underlying these phenotypic drifts.

The tumour cell phenotype can be modulated by several different factors [see Table 1.2]. The most important are genetic and microenvironmental. The microenvironment of the tumour is determined by the matrix synthesized by both normal and tumour cells, as well as host stromal components secreted by fibroblasts and other normal cells present in the surroundings. It has been shown that the manipulation of extracelluar matrix and stroma can lead to alterations in gene expression and cell phenotype [Lee *et al.*, 1985]. The differing influences of extra-cellular elements synthesized by parenchyma cells, endothelial cells, mesothelial cell, fibroblasts, and other host cells may be important in modulating state of differentiation in tumours [Nicolson, 1984]. Although malignant cells have altered response to extracelluar matrix and stroma, they can still be partly or differentially responsive to microenvironmental influences [Freshney, 1987]. Therefore, the tumour cell microenvironment is extremely significant in modulating phenotypic expressions of cells, probably by modulation of gene expression.

This thesis describes the development and characterization of models for the study of phenotypic shifts in lung cancer. Three model systems have been utilized in this study including: SCLC, NSCLC, and normal and oncogene transfected (transformed) lung epithelial cells. A SCLC cell line NCI-H69 was selected as a model because of its heterogeneous properties both in term of morphology and drug resistance. The initial studies were performed to investigate whether spontaneous phenotypic changes can take place in this model [Chapter three-A]. It was possible to isolate two sublines of H69 with different morphologies. H69 with its two derivative lines provided a very useful model for the study of heterogeneity, interrelationship, and origin of lung cancers. The studies were designed to ask specifically whether SCLC can change to NSCLC, by comparative studies of the SCLC (parental), the NSCLC, and the derivative lines.

Having established that phenotypic shifts can occur in H69 cells spontaneously, and that H69 cells may represent a stem cell population, studies were extended further [Chapter three-B] to investigate the effects of microenvironmental manipulation (i.e using phenotypic inducers) on tumour cell

phenotypic expressions. These studies investigated how malignant cell can alter its phenotype in response to chemical stimuli.

The effects of extracellular matrix and stromal interactions on malignant phenotype were also investigated in a NSCLC cell line [Chapter four]. These studies examined the phenotypic changes in a cell line derived from an adenocarcinoma of lung. Assuming that tumour cells often acquire a quasi-embryonic phenotype, fetal fibroblasts were selected for stromal interaction studies. Studies were also designed to see if other factors for expression of differentiation associated properties were important for induction of differentiation in these cells, such as polarity, and spatial orientation. The cells were allowed to grow in a histotypic culture, and then investigated for features associated with differentiation and malignancy. The effects of three dimentional culture on tumour cell (WIL) phenotype were compared to conventional monolayer culture on plastic substrate.

Studies were also performed to elucidate the effects of of genotypic manipulation on the phenotypic expression of the cell [Chapter five]. An immortalized lung epithelial cell line MvlLu transfected with human c-myc and normal and activated Ha-ras oncogenes individually, provided a model to confirm the proposed hypothesis, that phenotypic changes are controlled by these specific genes. This model was characterized for a variety of different malignancy and differentiation associated characteristics both *in vitro* and *in vivo*, in order to determine a shift in transfected cell phenotype compared to untransfected parental cells. It has been found that both normal and activated ras and c-myc could exert malignancy associated changes in transfected cells. Therefore, in addition to spontaneous phenotypic changes, both genotypic and microenvironmental factors were investigated for their possible roles in regulation of normal and malignant cell phenotype *in vitro* and *in vivo*.

## 1.8 THESIS LAYOUT

This thesis comprises seven Chapters. Chapter one introduces the background related to the work performed in this thesis. Chapter two outlines the general methodology adapted in these studies, which is applicable to whole thesis. Chapters three-A, three-B, four, and five together form the main body of the practical work and the data derived from it, each Chapter is self-contained with its own introduction, methods and materials, results and discussion. Chapter three concentrates on phenotypic changes in SCLC models. Spontaneous phenotypic changes in a SCLC, H69, are investigated in Chapter three-A, while Chapter three-B deals with the induced changes in H69. Chapter four presents a short account of phenotypic studies in a NSCLC model, with special emphasis on microenvironmental influences. It is also divided into three sections, the first deals with studies in conventional monolayer culture, while the second highlights the effects of three dimensional culture on cell phenotype and last section of this Chapter describes the effects of *in vivo* growth on malignant cell phenotype. Transfection of normal (immortalized) lung epithelial cells with human oncogenes and the effects of transfection on cellular phenotype are detailed in Chapter five, which is again divided into five sections, each concentrating on a specific aspect of phenotypic change. Chapter six gives a brief account of the overall findings of the thesis as a whole, drawing conclusions with possible implications, further experiments, and future prospects.

The contents are provided in the beginning of each chapter. The location of figures, plates and tables is indicated by the preceding page in the text. Methods applicable to more than one chapter are described in General Methods, and methods specific to a particular chapter are described in the appropriate chapters.

# CHAPTER TWO

## GENERAL METHODS

**The main purpose of this chapter is to describe the experimental methods in general which have been used in more than one chapter in this thesis. Methods specific to a particular chapter will be described in the appropriate chapters in the individual "Materials and Methods" section.**

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## 2.1 TISSUE CULTURE

### 2.1.1 Aseptic Techniques

All the experimental work involving tissue culture was conducted under strict aseptic conditions, in microbiological safety cabinets class-II (M.A.T Ltd., UK), with vertical laminar air flow. All the equipment, glassware etc., was sterilized by dry heat at 160 $^0$C for a minimum of one hour. All heat stable solutions were sterilized by autoclaving at 120 $^0$C, and 15 p.s.i. (2 bar), for 20 min (dry materials) or 40 minutes (wet materials).

### 2.1.2 Maintenance of Cell Lines

The cell lines growing as adherent monolayer cultures were maintained in tissue culture grade plastic flasks (NUNC, 25 cm$^2$, 75 cm$^2$, 150 cm$^2$). Cells were grown in 1:1 v/v Hams F10, and Dulbeccos Modified Eagles Medium F10:DMEM (Gibco, Paisley, Scotland) or RPMI1640 medium (Northumbria Biological Ltd; NBL) supplemented with 10% foetal bovine serum FBS (NBL or Gibco) with 8 mM sodium bicarbonate (Gibco), and a 98% air and 2% $CO_2$ gas phase. Cells were fed with fresh medium as indicated by the fall in pH. Confluent or near confluent cultures were passaged weekly, using 0.25% trypsin in 1 mM EDTA, in phosphate buffered saline (PBS, Gibco). It was called PET-solution. PET was removed after 30 sec and then incubated at 37 °C for 5 min and disaggregated by pipetting. The cells were then resuspended in fresh medium, and transferred into a sterile 30 ml plastic universal container (Sterilin, Middlesex). The cell number was determined using an electronic counter (Coulter, Luton, Beds.). Cell viability was confirmed by dye exclusion with 0.1% trypan blue, and also by tetrazolium [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide or (MTT)] dye reduction. Subcultures were set up in sterile flasks with 1x10$^4$ to 1x10$^5$ cells per ml, in 25 ml fresh medium in 75 cm$^2$ flasks. Cell cultures were replaced with fresh stocks from freezer every three months approximately.

SCLC cell lines growing in suspension were maintained in RPMI1640 medium containing 10% FBS. 1 to 3 x10$^6$ cells were seeded in 25 cm$^2$ flasks (Falcon) in 5 ml fresh medium, and passaged by a 1:10 split in late log phase. Cultures were fed before being split by allowing the cells to sediment and replacing three quarters of the spent medium.

### 2.1.3 Cell Freezing, Thawing and Storage

A cell suspension (1-5x$10^6$ cells/ml) in culture medium containing 10% dimethyl sulphoxide (DMSO), analar grade (BDH) was frozen, with an approximate cooling rate of 1 °C per minute, by placing the ampoules in an insulated box, in the -70 °C freezer, and subsequently transferring the ampoules to liquid nitrogen for storage. Tumour pieces were also stored by the same method.

Cells were thawed in water at 35 °C for 5 min, transferred to small flasks (NUNC, 25 $cm^2$), and culture medium added slowly to avoid osmotic shock. The DMSO containing medium was replaced with fresh medium as soon as possible, usually within 6 to 12 hours of plating the cells in the flask in monolayer cultures, when cells had got attached to the substrate. Medium with preservative was removed from the non-adherent cells by centrifuging at 200g for 3 min and resuspending in fresh medium.

### 2.1.4 Mycoplasma Testing

Cell cultures fixed with 25% acetic acid in methanol were screened for mycoplasma contamination by staining with the fluorescent DNA stain Hoechst 33258 (Sigma), at 0.5 $\mu$g/ml for 15 min, at room temperature [Chen, 1977]. The detection of the extra-cellular DNA by fluorescence microscopy was indicative of the presence of mycoplasma in the culture.

### 2.1.5 Estimation of Cell Growth Rate

The growth characteristics of each cell line used in this work were determined in the beginning, to optimize the maintenance conditions. Cells were seeded in 24 well plates at low cell concentrations in their respective growth media. The cells were then allowed to attach and grow for 24 to 72 hours, depending upon the particular cell type. Thereafter, the cells in triplicate wells were counted daily, and the rest fed with fresh medium, until the cell growth reached a plateau, usually 8 to 12 days after seeding, depending on the cell line used. Growth curves were constructed by plotting the cell concentration on a log scale, against time in days on a linear scale. The doubling time was determined at mid log phase and the saturation density at plateau.

### 2.1.6 Morphological Observations

Morphological observations were performed on live cultures by phase contrast microscope, and on Giemsa stained fixed cultures by light microscopy.

## 2.2 INVASION ASSAY *IN VITRO*

Embryonic chick heart fragment-tumour cell confrontation assay has been adapted from Mareel *et al.* [1979; 1987] and Mareel [1983]. A schematic outline of different steps of the assay is given in Figure [2.1].

### 2.2.1 Formation of Monolayer Fragments or Suspension Cellular Aggregates

Approximately 0.2 mm mechanically detached fragments of monolayer culture or cell aggregates in suspension cultures were selected under the stereomicroscope using fine dissection needles, to get a suitable rounded up mass of cells for confrontation with the heart fragment.

### 2.2.2 Preparation of Embryonic Chick Heart Fragments

Fresh heart fragments were dissected from a 9 day old embryonic chick as described by Mareel *et al* (1979). Briefly chick heart ventricles were cut into 0.3-0.4 mm blocks (50 to 100), and cultured in 2 ml MEM (Eagle's minimum essential medium, Flow Labs. Ltd. Irvine) with 5% fetal bovine serum (Gibco, Biocult) on a gyratory shaker at 70 r.p.m., in 50 ml siliconized, tightly stoppered Erlenmeyer flasks at 37 °C for 2 to 3 days. During this period heart fragments formed well circumscribed spheroids consisting of a central core of myoblasts and fibroblasts, and surrounded by a well defined layer of extracellular matrix. These rounded fragments were termed pre-cultured embryonic heart fragments (PHF). For confrontation assays PHF with a mean diameter of 0.5 mm were selected, since previously it has been found that only small size fragments could maintain their morphology for up to 10 days in culture, without central necrosis and deterioration. Routine observation of PHF was carried out under a dissecting phase contrast stereomicroscope. A normal and healthy growing fragment appeared reddish and with evenly rounded edge.

### 2.2.3 Co-Culture of Heart Fragments and Test Cells

PHF were placed on a dish with 0.4% semisolid agar medium. All medium was removed carefully and PHF were placed in a circular array with a central space reserved for a fragment or aggregate of potentially invasive test cells. PHF and test cells were confronted, making sure that no medium was left on top of the agar, otherwise there could be a risk of fragments being moved apart by the movement of any fluid left on the agar surface. About 12 confrontations were made per dish.

Test cells were placed in close contact with PHF and the attachment was

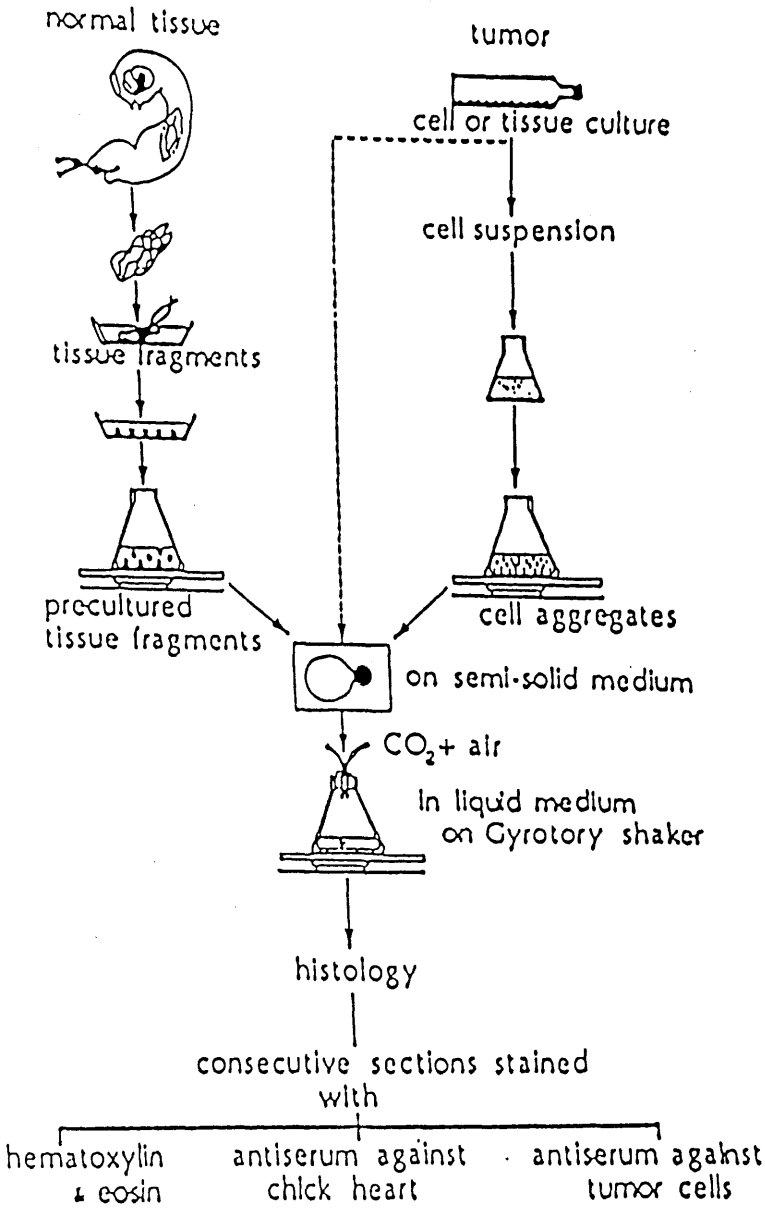


**Figure 2.1:- Schematic representation of the organ culture assay for in vitro invasion.**

In this assay test cells are co-cultured with normal tissue fragments followed by sequential evaluation of invasion using various histological techniques [Mareel *et al.*, 1979; 1987].

assessed during first 24 hours, under the microscope, and later on by histology of the confronted tissue. Each confronting pair was subsequently transferred to a 5 mm Erlenmeyer flask containing 2 ml culture medium, and shaken at 120 rpm on a gyratory shaker, at 37 °C, perfused with 5% $CO_2$ in air.

### 2.2.4 Tissue Processing and Staining

Living co-cultures were examined and photographed daily, at 1, 2, 4, 7, and 10 day, and at least 3 confronting pairs were examined at each time point. They were fixed in Bouin-Hollande solution, embedded in paraffin wax, and 1 and 6 $\mu$m thick complete serial sections were cut, and stained with haematoxylin and eosin (H&E), which was usually sufficient to differentiate tumour cells from chick heart tissue. Confirmation was achieved using an indirect immunoperoxidase technique with a polyclonal rabbit anti-chick primary antibody as described by Mareel *et al.* [1981]. Specific antibodies were also used to confirm the identity of the tumour tissue in the confronting co-cultures. These are described in appropriate experiments.

### 2.2.5 Analysis of Invasion

Invasion activity in this assay was based on the occupation of the normal tissue (PHF) by test cells. Therefore with a three dimensional confrontation assay system the invasive ability had to be deduced from the histological sections of the confronting tissue fixed *in situ*, after various time intervals. Analysis of occupation and degradation or replacement of normal tissue by tumour tissue was mandatory to grade the degree of invasion. Interaction of the confronting tumour cells with the heart fragments was classified according to the grading system described by Bracke *et al.* [1984]. Invasion of the test cells or tissues into the heart fragment in a confronting assay was divided into five grades according to this semi-quantitative grading or scoring system.

**Grade 0** :- Serial sections of complete co-cultures showed no tumour (test) cells. The causes could be 1) test cells non-adherent to PHF, 2) failure to attach for technical reasons, 3) maladaptation of the cells in new culture conditions, and 4) non-invasive cells.

**Grade I** :- The confronting cells were found at the periphery of the outer fibroblast layer of the heart fragment, so that cells surrounded the PHF. Grade I confrontation was interpreted as modelling the natural situation of epithelial

(tumour cells), and stromal (fibroblast layer of PHF) confrontation *in vivo*.

**Grade II:-** Type II co-cultures were usually seen when two normal tissue were brought together so that they either fused at the site of attachment (IIa) or one of the confronting partners surrounded the other (IIb). IIa indicated that the cells were occupying the outer fibroblast layer or had formed a cap at the pole of attachment or had surrounded the heart fragment. Grade IIb indicated that the cardiac muscles had surrounded the nodule of confronting cells with a minimal area of contact (circular in section), [Mareel *et al.*, 1983].

**Grade III:-** Test cells or tissue had replaced less than one half of the heart fragment.

**Grade IV:-** Test cells or tissue had replaced more than one half of the heart fragment.

Grades III & IV fulfilled the criteria for invasiveness [Mareel *et al.*, 1982, 1983]. Invading tumour cells could easily be identified in confronted cultures because of their distinct morphological and antigenic characteristics. The number of cells on surface and with in each fragment was assessed and the percentage of PHF invaded by test cells was determined.

## 2.3 CYTOTOXICITY ASSAYS

Two types of assays were used. Microtitration or MTT assay for quick screening of many samples, and clonogenic assay for more detailed analysis of cell survival. The methods for MTT assay were adapted from Plumb *et al.* [1989], established within the Department of Medical Oncology. The assay is based on observations made by Mosmann [1983], Cole [1986], and Carmichael *et al.* [1987].

### 2.3.1 Monolayer Cloning

Exponentially growing cell cultures were treated with a range of drug concentrations in 25 $cm^2$ flasks (Nunc, Gibco), for a given period of time (1-3 days), the drug being replaced daily. The cultures were then trypsinized and a single cell suspension was obtained. Each cell suspension was diluted to the final concentration in growth medium, to give required cell number per ml by determining the number of cells in the control flasks.

Cells were plated out in 60 mm sterile petri dishes (Nunc) in 5 ml culture

medium, incubated for 2-3 weeks at 37 °C, 98% air, 2% $CO_2$, in a humidified incubator. When discrete colonies were formed the medium was decanted, and the dishes washed with PBS, fixed in graded alcohol and stained with 1% crystal violet.

Colonies were counted both manually and with the help of an automatic colony counter [Artek Counter, Modal 1980]. Plating efficiency (PE) was calculated according to following formula:

$$\text{Plating Efficiency} = \frac{\text{number of colonies formed}}{\text{number of cells seeded}} \text{ X } 100$$

and the surviving fraction was calculated as below:

$$\text{Surviving Fraction} = \frac{\text{mean colony number of tests}}{\text{mean colony number of control}}$$

The surviving fraction was plotted against drug concentration as percentage of control. The $IC_{50}$ and $IC_{90}$ were then determined as the drug concentrations required to inhibit colony formation by 50% or 90% respectively of untreated control.

### 2.3.2 Microtitration Assay

The assay is based on the selective ability of living but not dead cells to reduce a yellow tetrazolium salt [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide or (MTT)] to a purple formazan product, which may be measured spectrophotometrically. The viable cell number per well is directly proportional to the absorbance at 570 nm.

The assay can be divided into four parts: 1) an initial growth phase, 2) period of drug exposure, 3) recovery period, and 4) MTT assay.

Slightly different protocols were employed for adherent and non-adherent cell lines. For adherent cultures a total of 200 μl of the cell suspension at a concentration designed not to reach plateau by the end of the assay were added to all but two outer columns of triplicate 96-well microtitration plates (Linbro). The outer columns received 200 μl of culture medium only, and were used as blank.

Cells were incubated at 37 °C in an atmosphere of 2% $CO_2$ in air, and

allowed to attach and proliferate for 2 to 3 days. Medium was then removed from the exponentially growing cultures and replaced with 200 μl of fresh culture medium containing drug or solvent control, and six to eight drug concentrations were used with six replicates at each.

For drug exposure time longer than 24 hours, fresh drug was added every 24 hours in 200 μl fresh medium. Thereafter, drug containing medium was removed and plates fed daily with 200 μl fresh medium. The recovery period was determined for each cell line, depending on growth rate of the cell line, to allow approximately 2-3 cell population doublings, but not reaching plateau.

On the last day of the assay, cells were fed as usual with 200 μl per well fresh medium, containing HEPES buffer (10 mM) (Gibco), followed by the addition of 50 μl per well MTT (Sigma) solution (see below). Plates were then incubated in the dark (wrapped in aluminium foil) at 37 °C for 4 hours. Medium containing the non-reduced MTT was removed from the wells and the formazan crystals were dissolved in 200 μl DMSO per well. To each well were added 25 μl glycine buffer (BDH). Sorensen's Glycine Buffer: 0.1 M glycine + 0.1 M NaCl, pH 10.5. All formazan present was as a single species with an absorbance maximum at 570 nm single wavelength, using a microtitration ELISA plate reader (Bio-Rad, Model 2550 EIA Reader, Watford, Herts, U.S.A.), interfaced with an Amstrad PC1512 computer.

The optimum MTT concentration for a particular cell line was determined by plating a constant cell number per well and incubating with different concentrations of MTT, and the concentration which gave the maximum absorbance without cytotoxicity selected.

All non-adherent cells were treated as above except as follows: Cells were plated in 96-well plates (round bottomed wells, Nunclon) in 100 μl culture medium at 2x final cell concentration. Drug was added on the same day (100 μl of a 2x final concentration). Plates were centrifuged at 200g for 10 min, to pellet the cells before adding drug and subsequent feeding.

A graph was plotted for absorbance (Mean $\pm$ SEM) on a linear scale against drug concentration on a log scale. The amount of drug required to give a 50% reduction in absorbance at the end of the assay was calculated as $IC_{50}$. As a linear relationship can be demonstrated between absorbance and cell number, it was assumed that a 50% reduction in absorbance implied a 50% reduction in viable cell number, although confirmation of this was not attempted in each assay.

## 2.4 CLONOGENIC ASSAYS

### 2.4.1 Soft Agar Clonogenic Assay

A modification of the double layer soft agar petri-dish clonogenic assay was used.

One ml of cell suspension in 0.3% agar was pipetted on a pre-formed 1% agar under-layer. The final cell concentration varied among different cell lines, but was between $10^3$-$10^4$ cells per dish. Following application of the cell layer to the dishes, they were transferred to refrigerator at 4 °C for 5 min, then to room temperature for 10 min, to allow proper and uniform gel formation. Thereafter plates were incubated at 37 °C, in 98% air, 2% $CO_2$, in a humid incubator. On the following day, the dishes were checked for the presence of a single cell suspension. Plates with cellular aggregates were not included. One ml of growth medium was added on top of the agar in each dish on the next day. Colony formation was examined at least twice a week, using an inverted phase contrast light microscope. Colonies consisting of more than 50 cells could be identified after 3-4 weeks.

MTT was used for staining to identify viable clones. The use of metabolizable dyes in conjunction with soft agar cell culture has been described previously [Alley and Lieber, 1984]. Briefly, stock MTT was prepared fresh at concentration of 5 mg/ml PBS. The optimal staining of colonies was achieved by applying 1 ml of MTT solution to the surface of each culture, followed by reincubation at 37 °C for at least 4 hours. Viable colonies of approximately 50 cells could be counted easily.

For standardization, selected colonies were isolated and teased apart to confirm the number of cells in a given-sized colony, both by phase contrast microscopic examination and following Giemsa staining.

### 2.4.2 Monolayer Clonogenic Assay

As in cytotoxicity assays, except that drug was not included in the protocol.

## 2.5 CELL LINE CHARACTERISATION

The species of origin of cell lines was confirmed by chromosome analysis and cross contamination excluded by isoenzyme analysis.

### 2.5.1 Lactate Dehydrogenase Isoenzymes Analysis

Lactate dehydrogenase (LDH) isoenzymes were separated by electrophoresis on an agarose gel. The area of enzyme activity was then

visualized histochemically, according to the following reactions.

$$\text{L[+] Lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH}$$

$$\text{NADH} + \text{PMS} \longrightarrow \text{NAD} + \text{PMS-H}$$

$$\text{PMS-H} + \text{TNBT} \longrightarrow \text{PMS} + \text{TNBT-Formazan}$$

The TNBT formazan which is coloured and insoluble, localizes in the electrophoretic zones of LDH activity.

NAD: Nicotinamide adenine dinucleotide.
NADH: Nicotinamide adenine dinucleotide, reduced.
PMS: Phenazine methosulfate.
TNBT: Tetranitroblue tetrazolium.

The assay can be divided into five parts: 1) preparation of the cell extract, 2) electrophoresis, 3) staining, 4) scanning, 5) and analysis.

For preparation of the cell extract, exponentially growing cell cultures were harvested by trypsinization to give approximately $1x10^7$ cells. The cells were washed three times by centrifugation in 10 ml 0.9% NaCl pH 7.1, containing $6.6x10^{-4}$M EDTA (saline-EDTA). The cell pellet was resuspended in 1 ml saline-EDTA solution, and lysed by freezing and thawing rapidly at least three times in liquid nitrogen or a dry ice-alcohol bath and a water bath at 37 °C. The lysate was then dispensed into Eppendorf tubes, and clarified by centrifugation at 8700g in a Microfuge B (MSE) for 5 min. The supernatant was collected and stored at -70 °C for subsequent assay.

Agarose gel (Corning Universal Electrophoresis Films) was equilibrated in LDH-Electrophoresis Buffer solution (50 mM tris, 12 mM citrate, 25 mM barbital) (Sigma) for 1 hour prior to electrophoresis. Gel was removed from the equilibrium solution and excess buffer was drained off. The area containing sample application wells was blotted gently using a filter paper, and two 1 $\mu$l aliquots of freshly thawed sample extract were applied to each application well. Separate disposable sample tips (Corning Medical Co.) were used for each sample. After absorption of the second aliquot of the sample, the loaded gel was placed in the electrophoresis cell cassette holder, with agarose side facing down, and run at 90 V (120 V/cm) for 30 min.

For staining the gel was removed from the electrophoresis cell, trimmed

to approximate size and stained in the chromogen at 37 $^{0}$C, in the dark for 30 min. The gel was then soaked in 200 ml of methanol acetic acid solution for 30 min, followed by another wash in 200 ml of distilled water for 30 min, and then dried in an incubator at 60-70 °C to give a transparent film, which was scanned on a densitometer, but usually it was possible to evaluate the patterns visually [see below, Plate 3A.2]. A sample of human LDH supplied with the Kit was used as a standard.

Results were recorded both in terms of the number and intensity of bands, and with reference to the distance of migration from the origin, compared to standard. Typical banding patterns for each cell line were also compared to the standard zymograms [Macy, 1978].

### 2.5.2 Chromosome Analysis

Adapted from Rothvels and Siminovitch [1958], Worton and Duff [1979] and Freshney [1987].

Cells in mid log phase of growth were exposed to colcemid (0.1 $\mu$M) for four hours, at 37 °C. Medium was replaced with hypotonic solution (0.04 M KCl, 0.025 M sodium citrate) for 20 min. Non-adherent cells were treated in suspension while adherent cells were collected by shake-off and pipetting. This procedure was useful to obtain maximum number of cells in metaphase. The cells were then spun at 100g for 3 min, fixed in ice cold acetic-ethanol (1 part glacial acetic acid + 3 parts ethanol) slowly with constant mixing on a vortex, and then centrifuged again at 100g for 3 min. The supernatant was discarded. The pellet was buzzed on a vortex mixer, and then fresh fixative was added, slowly with continuous mixing. This procedure was repeated three times. Finally the pellet was buzzed in 0.5 ml fixative to give the final dispersed cell suspension. Approximately 50 $\mu$l was then dropped onto a clean, cold glass slide and blown slightly. The preparations were air dried and examined under the microscope for optimal spread, and processed for G-banding.

The chromosome number was counted per spread for a minimum of 20-30 spreads, and the results presented as a histogram. The status of ploidy was defined i.e aneuploid, diploid, near diploid if countable, and polyploid if high and uncountable. Any other aberrations such as deletions, losses, were also recorded.

### 2.5.3 Chromosome Banding

Slides were incubated in 2x SSC (saline sodium citrate : equal volumes of sodium chloride (17.63 g/L) and sodium citrate (8.82 g/L), at 60 $^{0}$C for 2 hours.

They were then rinsed in saline and in distilled water, and dehydrated in graded alcohols (50%, 70%, 90%, 100%), 2 min in each, and allowed to dry. Slides were then treated with trypsin solution (1% trypsin in saline); test slides were used to determine optimum timing. Slides were rinsed in saline, and stained in 1 part Leishman to 3 parts buffer (Gurr's buffer tablets were dissolved in water and adjusted to pH 6.8, according to manufacturers instructions) for 3 min. Slides were subsequently rinsed in buffer and allowed to dry. Completely dried slides were used to examine under bright field, oil-emersion objectives without a coverslip. Finally the slides were soaked in xylol for 15 min and mounted. (Chromosome banding was done by Dr Elizabeth Boyd at Yorkhill Yospital, Glasgow).

## 2.6 BIOCHEMICAL AND RADIOIMMUNOASSAYS

### 2.6.1 Bombesin Radioimmunoassay

Radioimmunoassay (RIA), for bombesin-like immunoreactivity (BLI), was adapted from Moody *et al.*, [1983]; Cuttitta *et al.*, [1985]; and Bepler *et al.*, [1987]. BLI was measured both in cellular as well as in secreted fractions of the tumour cells in culture. Exponentially growing cultures were harvested by centrifugation at 200g for 10 min. Medium was retained for the assessment of secreted BLI. For the measurement of the intracellular bombesin content, the cells were washed twice in PBS, and the cell pellet was resuspended in 1 ml of acetic acid (2 M) (BDH), boiled in a water bath for 15 min, and homogenized. A fraction was saved for protein estimation. The remainder of the homogenate was clarified by centrifugation at 10,000g for 15 min. The supernatant was frozen, lyophilized, and stored below -70 °C until assayed.

For the assessment of secreted BLI, trasylol (Aprotinin, Sigma) 500 KIU; kallikrein inhibitor unit) per ml was added to the medium in which the cells were grown, immediately prior to centrifugation at 760g for 15 min. Acetic acid was added to the supernatant to give a final concentration of 2 M. The acidified samples were then vortexed, frozen, lyophilized, and stored below -70 °C.

Lyophilized extracts of the test samples were resuspended in 1 ml PBS, supplemented with bovine serum albumin (BSA), 0.25% w/v. The amount of BLI in the test samples was assayed by a commercial double antibody RIA Kit (Incstar Co. Ltd.), following the manufacturer's instructions. Briefly, BLI-RIA involved simultaneous addition of unknown sample (unlabelled bombesin), rabbit-anti-bombesin (specific primary antibody), and $^{125}$I-BN (labelled specific antigen), followed by an overnight (16-20 hours) incubation at 4 °C. During this

incubation BN in the unknown samples competed with the labelled-bombesin for the common antibody binding sites.

A pre-precipitated carrier, secondary antibody, and polyethylene glycol (goat-anti-rabbit precipitating complex, GAR-PPT) were then added simultaneously, and incubated for 20 min at 25 °C. The precipitated antigen-antibody complex was then separated by centrifugation at 760g, and decanted. The radioactivity in the precipitate was counted using a gamma scintillation counter (Nuclear Enterprices, NE1600), for 4 min, and the amount of BLI in the unknown sample calculated from a standard curve included in each test.

Using the standard curve, the BLI equivalent of the cell homogenate peptide was determined and expressed as pg BLI per mg total cellular protein in the homogenate. The results were the mean of triplicate determinations, with duplicate counts for each determination.

### 2.6.2 Neuron-Specific Enolase Radioimmunoassay

Cell cultures in late log growth phase were harvested and centrifuged at 200g for 5 min. The cell pellet was washed twice in PBS, then resuspended in 2.5 volumes of Tris-phosphate buffer (20 mM) containing $MgSO_4$ (2 mM), and lysed by freezing and thawing three times, using Drikold-methanol and a water bath at 37 °C. Having confirmed that the lysis was complete, a fraction of the lysate was saved for protein estimation, and stored frozen below -70 °C. The remaining lysate was centrifuged at 100,000g for 1 hour in an ultracentrifuge (Model L5-50, Beckman & Co., U.S.A.), in a Ti 50 rotor. The supernatant was stored frozen below -20 °C until assayed.

For the measurement of secreted NSE, medium from exponentially growing cells was collected, centrifuged, and the supernatant frozen immediately, and stored at -20 °C prior to assay.

NSE was measured using the Pharmacia NSE-RIA test (Pharmacia Diagnostic AB, Uppsala, Sweden) as described by Cooper *et al.* [1985]. The Pharmacia NSE-RIA test is a double antibody RIA. Briefly NSE in the sample (test or standard) competes with a fixed amount of $^{125}$I-labelled NSE for the binding sites of the specific antibody (rabbit IgG). The antibody is available only in limited amount. Bound and free NSE were separated using a second antibody (sheep anti-rabbit IgG), covalently bound to sepharose particles. After addition of decanting suspension i.e, sepharose antibody derivatives (sepharose-anti-rabbit IgG raised in sheep), the mixture was incubated for 30 min at room temperature, and centrifuged at 1500g for 10 min. The supernatant with free NSE was then

separated from the sepharose pellet, containing the bound NSE, by decanting for 5 min. The radioactivity in the pellet was then determined using a gamma counter as above. The radioactivity in the sample is inversely proportional to the quantity of NSE.

Each sample was assayed in triplicate and total cellular protein determined for each replicate. Computer calculations were performed using the WHO Immunoassay Processing Program (version 5.1) software. Assays were repeated at least twice to get reproducible results. NSE values are expressed as ng NSE/mg cellular protein.

### 2.6.3 Creatine Kinase Assay

To determine total creatine kinase (CK) activity the cell cultures were harvested in late log phase, and centrifuged at 200g for 5 min. The cell pellet was washed three times with ice cold PBS, and finally resuspended in 1 ml borate buffer (Borax 0.025 M + $KH_2PO_4$ 0.05 M, pH 7.6). The cell suspension was lysed as before by freezing and thawing 3 times. Complete cell disruption was confirmed by trypan blue test. The lysate was mixed thoroughly and aliquots were saved for protein estimation for each sample. The remainder of the lysate was centrifuged at 8000g for 5 min. The supernatant was saved, and stored frozen below -70 °C until assayed.

Total CK activity in the test sample was determined using a commercial Kit (Sigma Co. Ltd.) following the manufacturer's instructions. Briefly, the fraction of test sample for CK analysis was added to CK reagent preincubated at 30 °C, mixed by inversion, and placed again at 30 °C for sufficient time (4-5 min) to get a linear trace at 340 nm against water as a reference, in a spectrophotometer (Gilford 250). The rate of reaction was then determined from the trace. The rate of change in absorbance was proportional to formation of NADH. The enzyme activity was expressed as units per milligram cellular protein, where one unit was defined as the amount of enzyme which produced one $\mu$mol of NADH per min under the conditions of the assay.

The CK isoenzymes were analyzed electrophoretically, according to manufacturer's instructions (Corning Diagnostic Ltd). The isoenzymes were separated by electrophoresis for 20 min in a preformed 1% w/v agarose gel film at 9 v/cm, loaded in a cassette electrophoresis cell containing 3-(N-morpholino)-2-hydroxypropane sulfonic acid (MOPSO) buffer (0.05 M, pH 7.8). Following electrophoresis the film was overlaid with CK substrate solution (MES buffer, pH 6.2). The film was incubated at 37 °C for 20 min in the dark. During this period

of incubation NADH was formed. Following incubation, the agarose film was dried and visualized under UV light at 340 nm. NADH produced was quantified using a scanning fluorimeter (Helena) at an excitation wavelength: 365 nm, and emission wavelength: 460 nm, against a human CK standard.

CK-1 (CK-BB) was the fastest migrating fraction; CK-2 (CK-MB) migrated in the position of an alpha-2 globulin, and CK-3 (CK-MM) remained at the site of application in the mid gamma globulin position. The percentage of each isoenzyme was calculated from total CK activity. For quantitative results the percentage of each isoenzyme was multiplied by total CK activity to get isoenzyme concentration in IU/mg protein.

### 2.6.4 DOPA Decarboxylase Assay

The assay was adapted from Laduron and Belpaire [1968]; Fonnum [1969]; Emson *et al.* [1974], and modified by Murray [1989].

DOPA decarboxylase (DDC) in the presence of pyridoxal 5-phosphate converts L-DOPA to dopamine, with loss of carbon dioxide. The enzyme activity can be estimated by the rate of formation of $^{3}$H-dopamine from L-$^{3}$H-DOPA. Radioactive dopamine (product) is separated from unchanged L-DOPA by heptane-3-one extraction in the presence of tetraphenylboron. The amount of product formed is directly proportional to the enzyme activity.

The extraction method was same as for CK assay. The incubation mixture for enzyme reaction was prepared as below, omitting pyridoxal 5'phosphate.

| | | |
|---|---|---|
| Borate buffer | Borax (0.025 M) + $KH_2P0_4$ (0.05 M, pH 7.6), (BDH) | |
| pyridoxal 5'phosphate | 100 $\mu$M | (Sigma) |
| Nialamide | 125 $\mu$M | (Sigma) |
| EDTA | 1.5 mM | (BDH) |
| L-DOPA | 1200 $\mu$M | (Sigma) |
| (L-$\beta$-3,4- dihydroxyphenylalanine) | | |
| $^{3}$H-L-DOPA | 30 $\mu$Ci/ml (20-40 Ci/mmol) | |
| (Dihydroxyphenylalanine L-3,4-[ring-2,5,6-$^{3}$H] | | (Du Pont) |

Reaction mixture (45 $\mu$l) was aliquotted into 15 ml glass stoppered centrifuge tubes, and incubated at 37 °C for 5 min. The cell extract (200 $\mu$l) was added to 100 $\mu$l of pyridoxal 5-phosphate (1200 $\mu$M), and vortexed. To start the reaction, 15 $\mu$l of this mixture was added to the appropriate tubes containing

reaction mixture. Samples were incubated at 37 °C for 1 hour. The reaction was stopped by adding 5 ml ice cold sodium phosphate buffer (10 mM, pH 6.5), and samples transferred to ice.

Dopamine was separated from unchanged L-DOPA by liquid cation exchange with sodium tetraphenylboron according to the method of Fonnum [1969]. One ml of heptane-3-one (BDH) containing 25 mg sodium tetraphenylboron (BDH) was added to each sample tube. Samples were mixed gently by inversion for 1 min. Subsequently the two phases were separated by centrifugation at 1500g for 5 mins at 4 °C. The aqueous phase was removed, using a Pasteur pipette, and discarded. The organic phase was washed again with 2 ml of sodium phosphate buffer (10 mM, pH 7.4), containing 1 mg tetraphenylboron. The samples were centrifuged at 1500g for 5 min at 4 °C. Finally a 0.5 ml aliquot was removed from the organic phase and 10 ml of Ecoscint [National Diagnostic, Sumerville, NJ, U.S.A] were added. The radioactivity in the sample was counted for 10 min, using a scintillation counter (Packard).

For determination of the extraction efficiencies, percentage recoveries of both $^{3}$H-dopamine (5-15 Ci/mmol) (Amersham UK) and $^{3}$H-DOPA were calculated for each assay. The values range from 50-75% in case of $^{3}$H-dopamine, and less than 1% percent in case of $^{3}$H-DOPA.

Controls included were a reagent blank (incubation mixture without enzyme) and a sample blank (enzyme added at the end of the incubation). The blank values were subtracted from the sample values to account for non-specific or non-enzymatic decarboxylation of DOPA. The results were expressed as $\mu$IU/mg cellular protein, where one $\mu$IU was defined as one $\mu$mol of substrate converted into the product per minute under the conditions of the assay.

### 2.6.5 Protein Assay

The cellular protein was solubilised in 0.3 N NaOH (BDH) prior to protein assay. Samples were stored at -20 °C for subsequent assay. The estimation of total cell protein was carried out according to the method of Lowry *et al.* [1951].

## 2.7 ANIMAL EXPERIMENTS

Nude mice (MF-1/NuNu/OLAC/HSD) were produced in sterile isolators by mating heterozygous (Nu/+) females with homozygous (Nu/Nu) males, or purchased from Olac (Bicester, Oxon). The mice were housed in sterile

transparent MTI polycarbonate cages in vertical Bassair laminar flow hoods, and sterile grade 18 wood chips were used as bedding. The surrounding temperature was maintained at 27 °C, with a humidity of 40-60%. A cycle of 12 hrs light and 12 hrs dark was provided. Pre-sterilized food Special Diet Services (SDS) No. 3, (Essex) and drinking water were freely available.

### 2.7.1 Tumour Growth and Maintenance

Studies were performed on both the primary (implant) and the secondary (transplant) tumours in nude mice.

The primary xenografts were generated by injecting a minimum $1x10^6$ cells subcutaneously into the caudal flank of each animal. Following inoculation mice were examined twice weekly and tumour growth and condition of animals recorded.

Growing tumours were maintained in nude mice as stocks by serial passage in a laminar flow hood at intervals of 4-6 weeks. A tumour bearing mouse was killed by cervical dislocation. Tumour was removed, obvious fat, connective tissue, or necrotic tissue was separated. Usually the outer rim of the tumour contained the healthy viable tissue, which was carefully dissected out, and divided into small pieces. The tumour fragments were then used either for serial passage (2x2 mm) or stored frozen in liquid nitrogen at -196 °C. For tumour transplantation, animals were anaesthetized, by intraperitoneal injection of 0.1 ml anaesthetic; 1 part Hypnorm, (Janssen Pharmaceutical Ltd., Oxford), to 1 part of Hypnovel (Roche Products Ltd., England), diluted in sterile distilled water at 1:2 dilution. Alternatively ether by inhalation was used as anaesthetic.

Animals were assigned to experimental groups in a random manner. A group of 4-6 animals was prepared by placing them with their dorsal surfaces facing upwards. With a small surgical incision, a hole (4-5 mm) was made in the skin of the right flank. The scissor tip was inserted subcutaneously, and widened slightly to open the incision. Using fine tipped forceps, a piece of tissue was inserted into the wound, well away from the site of the incision. The wound was then closed with sterile autoclips. The animals were then placed in sterile cages in the laminar flow hood, and allowed to recover. Clips were removed once the wound had healed.

### 2.7.2 Analysis of Tumour Growth

The tumour take was defined as formation of a tumour which grew after implantation and could be serially passaged.

The latent period was defined as the time between inoculation and the appearance of a palpable tumour at the site of injection, measuring approximately ≥ 3x3 mm. This was confirmed histologically.

The tumour take rate was defined as the number of successful implants or transplants, expressed as the percentage of the total number of the implants or transplants.

The volume was calculated from the two diameters of the tumour, on the assumption that the tumour was ellipsoid in shape, from the following mathematical expression [Fergusson *et al.*, 1986].

$$V = \pi \times Dd^2/6$$

Where V = Tumour volume in $mm^3$

$\pi$ = 3.14

D = largest diameter across the tumour

d = smallest diameter across the tumour

Tumour doubling time, and growth rate were also determined for each tumour. The growth rate of subcutaneously implanted tumours decays from exponential with increasing volume and is designated as Gompertzian [Poulsen *et al.*, 1982]. Tumour doubling times were therefore estimated during the early growth phase approximating to exponential and later when the rate had decreased. Actual time of measurement depended on individual curves and is described later. The tumour doubling time was calculated by slight modifications in the methods adapted by Steel *et al.* [1983]. The tumour volume was plotted against time scale, and the tumour doubling time was determined by interpolation on a semilogarithmic plot, and from the formula given below. The growth rate was assessed from tumour doubling time.

$$Dt = T/Dn$$

Where

Vi = tumour volume ($mm^3$) at any given time Ti

Vf = tumour volume ($mm^3$) after time Tf

V = change in volume between Vi and Vf

T = time between Ti and Tf in days

Dn = number of doublings in tumour volume (V), in time (T).

Dt = tumour doubling time in days

Tumours were also evaluated for total duration of observation i.e interval between inoculation and death of animal, minimum tumour bearing period i.e duration of observation minus latency period. Any gross lesions e.g ulceration were also recorded.

## 2.8 HISTOLOGICAL METHODS

Tissues were fixed in 10% neutral buffered formalin (NBF), or Bouin's fixative, and embedded in paraffin wax. Sections 4 $\mu$m thick were cut, and processed for haematoxylin & eosin, periodic acid-Schiff alcian blue, and immunohistochemical staining [Figure 2.2].

Where appropriate, fresh tissue was snap frozen in liquid nitrogen and stored below -70 °C. Frozen sections, 3 $\mu$m thick were cut onto slides coated with 3-aminopropyl triethoxysilane (Sigma Co. Ltd.), and air dried overnight. These were fixed in acetone for 10 min, and air dried before staining.

Cell suspensions in PBS were centrifuged onto silane coated glass slides. The cytospin preparations were fixed in methanol, acetone, or 10% NBF, and processed for immunohistochemistry.

Cells suspensions in a plasma clot or 2% agar were fixed and processed for paraffin sections. Plasma was replaced by agar for immunohistochemical staining. Cells were also grown on glass coverslips, Lab-Tek slides (Miles Scientific, USA).

## 2.9 IMMUNOHISTOCHEMICAL TECHNIQUES

Immunohistochemical staining was performed using the indirect immunoperoxidase, peroxidase anti-peroxidase (PAP) [Sternberger, 1979], avidin-biotin complex (ABC) [Hsu, 1981; Guesdon *et al.*, 1979], or indirect immuno-alkaline phosphatase (AP) techniques [Cardell *et al.*, 1984].

Controls for all techniques included omission of primary antibody, substitution of non-immune rabbit serum or an inappropriate antibody, or monoclonal control as appropriate, and where possible incubation of the sections with antibody preincubated with excess antigen. Known positive controls were used to establish optimal dilutions in all antibodies, and an appropriate positive control was included in each run.

### 2.9.1 Indirect Immunoperoxidase

For paraffin sections, appropriate numbers of test and control sections were cut, and brought to water through graded alcohols. Endogenous peroxidase

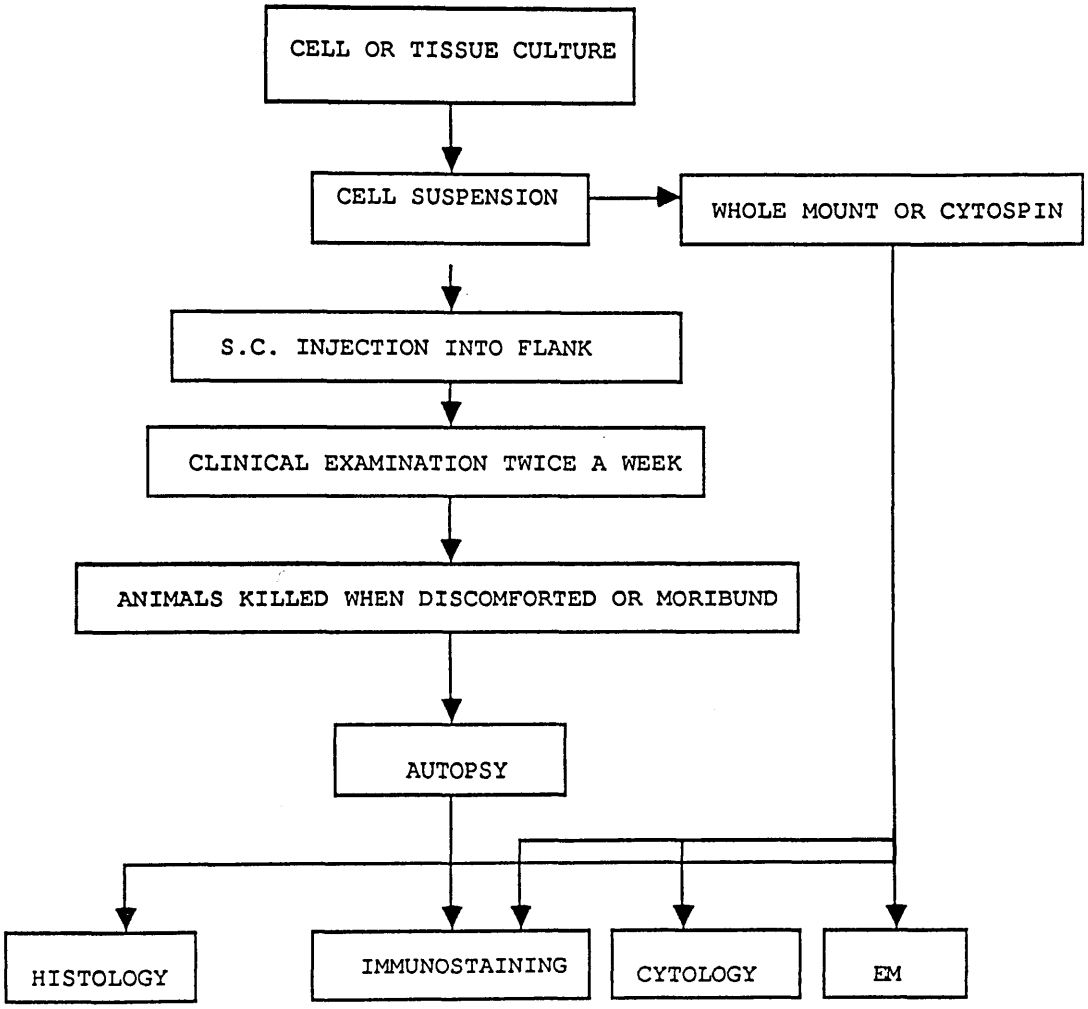


Figure 2.2:- Schematic representation of working strategies for histopathological studies.

activity was blocked by incubating the slides in a solution of 0.5% hydrogen peroxide and 0.2% hydrochloric acid in methanol, for 30 min. Slides were rinsed briefly in water, and then in tris-saline. Sections were trypsinized as appropriate, using a solution containing 0.1% trypsin, and 0.1% $CaCl_2$ in tris buffer pH 7.8 at 37 °C for 10 min, then washed in two changes of tris-saline, 5 min each wash. Sections were incubated with non-immune serum, from the species supplying the second antibody at a dilution of 1:5 in tris buffer for 15 min. This step was omitted in the case of monoclonal antibodies. The primary antibody was applied at standard established dilutions, usually for 30 min in case of polyclonal and 2 hours with monoclonal antibodies. Slides were rinsed three times in tris-saline, 5 min each. Horseradish peroxidase (HRP)-conjugated secondary antibody was then applied for 30 min and the slides washed in tris-saline for three changes of 5 min each. The colour reaction was developed using diaminobenzidine tetrahydrochloride dihydrate (DAB) solution for 10 min. Slides were counterstained in haematoxylin, dehydrated, and mounted.

For frozen sections tumour was removed immediately after sacrificing the animal. Tissue blocks, 3x3 mm, were embedded in OCT (Miles Scientific, USA) snap frozen in liquid nitrogen, and stored below -169 °C until sectioned. Four micron thick sections were mounted on slides coated with silane, air dried overnight, and fixed in acetone for 10 min at room temperature. They were rehydrated in water then in tris-saline and processed as above.

For cytology, monolayer whole mounts and cytospin preparations were fixed in methanol at 4 °C for 20 min, 1% paraformaldehyde or in acetone for 10 min at room temperature, and rehydrated before staining as above.

### 2.9.2 Peroxidase-Anti-peroxidase

Steps for the PAP technique were as for indirect immunoperoxidase until the application of the unconjugated secondary antibody which was applied for 30 min. Then peroxidase-antiperoxidase complex (Dakopatts) was applied at appropriate dilutions in tris-buffer for 30 min.

### 2.9.3 Avidin-Biotin Complex

As indirect immunoperoxidase until the application of the secondary antibody. Biotinylated antibody was applied for 30 min, and avidin-biotin peroxidase complex; ABC Kit (Vector Labs.) for 45 min. Mouse ABC Kit was used for monoclonal antibodies, and rabbit ABC kit for polyclonal antibodies.

### 2.9.4 Immuno-Alkaline Phosphatase

An indirect AP technique was applied. The sections were processed as for indirect immunoperoxidase until the application of the secondary antibody. An AP-conjugated antibody was applied at 1:20 dilution for 45 min. Freshly prepared substrate solution-A was applied for 20 min to develop a red coloured end product. Cryostat sections were incubated in substrate solution-A containing 1 mM levamisole (Sigma) to inhibit endogenous alkaline phosphatase. Slides were washed twice in distilled water, counterstained lightly with haematoxylin without differentiating in acid alcohol, dehydrated rapidly, and water mounted in glycerine gelly.

## 2.10 ELECTRON MICROSCOPY

### 2.10.1 Transmission Electron Microscopy

Material for transmission electron microscopy (TEM) was obtained either from cell cultures or from the xenografts. Tissue was fixed in 4% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in epoxy resin. At least two blocks were selected from each specimen. Semithin sections were cut and used to locate the areas for ultrathin sections. Silver/gold sections were cut on an Ultratome-III at a thickness of 500-700 °A, mounted on copper grids, and stained in 2% uranyl acetate and lead citrate. The grids were scanned on a Philips E.M 301 transmission electron microscope, at 80 Kv. Electron micrographs were made using either Kodak plates (type EM-5, Kodak electron microscope cut films, 6.5x9 cm) or Ilford plates (Ilford technical films, 6.5x9 cm). Prints were made using Ilfospeed paper grade 1-5.

### 2.10.2 Scanning Electron Microscopy

The specimens were fixed as described above, and processed according to the standard schedule. The critical point dried specimen was mounted on mounting copper stubs in carbon mounting medium (carbon conducting cement), and air dried, spatter coated with gold particles in an atmosphere of argon (polaron), and an SEM coater unit (E5000M, BIO-RAD, Polaron Division). All specimens were examined using a Jeol 1200 EX TEM-SCAN, at 60 KV gun potential. Electron micrographs were made on Kodak 35 mm roll films. Prints were made as above.

## 2.11 PHOTOGRAPHY

Growing cell cultures were photographed under phase contrast on Ektachrome 160 ISO tungston film. Fixed cultures were photographed using a Polyvar microscope [Reichart] with a 35 mm camera attachment on Ektachrome 35 mm ASA 160 film.

## 2.12 STATISTICAL METHODS

In experiments where the structure was simple (i.e there was no blocking factor such as varying times of analysis) non-parametric tests were used. For multiple group comparisons the Kruskal-Wallis test was adopted. A level of $P \leq 0.05$ was considered significant. In the event of significance, two group comparisons were carried out using the Mann-Whitney U-test and the Bonferoni adjustment. Differences were considered significant at $P \leq 0.05/n$, where n= number of pairwise comparisons. If the Kruskal-Wallis test indicated a difference but none was evident using the Bonferoni adjustment, then the root significant pairwise comparison was used.

In experiments with more complex structure, analysis of variance was applied. Where appropriate, data were transformed before analyses. Two group comparison were carried out using the two independent sample t-test. The Bonferoni method of multiple comparison was again applied. Significant levels were as above. Computations were undertaken using a statistical package (Systat version 4.0).

# CHAPTER THREE

## (A)

# ESTABLISHMENT AND CHARACTERIZATION OF A MODEL FOR THE STUDY OF SPONTANEOUS PHENOTYPIC TRANSITIONS IN HUMAN SMALL CELL LUNG CARCINOMA.

**This chapter deals with the origin, heterogeneity, and interrelationship of lung cancers. A SCLC cell line and two NSCLC-like derivative cell lines were used to analyze phenotypic characteristics relevant to the transition from SCLC to NSCLC. The data suggest a common origin for both types of lung cancer.**

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## 3A.1 INTRODUCTION

SCLC is a clinicopathological distinct entity of pulmonary carcinoma, characterised by more rapid tumour cell proliferation [Straus, 1974], early metastatic potential [Matthews *et al.*, 1973], and greater response to both chemotherapy and radiation therapy [Cohen, 1975]. It also has characteristic morphological [Cohen & Matthews, 1978], biochemical [Baylin *et al.*, 1980], ultrastructural [Hattori *et al.*, 1972], and cytogenetic [Whang-Peng *et al.*, 1982] markers. Although this apparent distinction suggests a separate histogenesis of SCLC, a significant number of SCLC cases appear to be admixed with or convert to other histological types of lung cancer during the course of disease [Gazdar *et al.*, 1981; Minna *et al.*, 1982;].

Heterogeneity in lung cancer particularly in SCLC is well recognised. The classic histological description of SCLC by Azzopardi [1959] identified tubular structures, squamous nests, rosette formation, giant cells, and mucin secretion. The presence of foci of squamous carcinoma or adenocarcinoma in SCLC are well recognised entities by the WHO classification of lung cancer [1982]. Patients presenting with mixed histology have a significantly worse prognosis, compared to pure SCLC [Radice *et al.*, 1982]. Histological changes in SCLC are frequently observed after drug treatment. In a clinical study of patients diagnosed as SCLC on biopsy material, Abeloff *et al.* [1979] showed that at postmortem 12% had non-small cell and 15% had mixed small cell-large cell histology. Minna *et al.* [1981] have shown that approximately 35% patients who died of SCLC show mixed histology at postmortem, and these patients are less responsive to chemotherapy. Goodwin & Baylin, [1982] have shown that the transition from small cell to non-small cell phenotype is associated with resistance to radiation therapy.

SCLC derived cell lines sometimes show a similar transition *in vitro* [Gazdar *et al.*, 1981; Goodwin & Baylin, 1982; Bepler *et al.*, 1987] and in SCLC xenografts in athymic nude mice [Goodwin *et al.*, 1983]. This phenotypic conversion in SCLC *in vitro* is accompanied by a change to NSCLC-like morphology and loss or reduction of neuroendocrine cell markers. SCLC cell lines with NSCLC-like properties have been classified by some investigators as variant cell lines [Carney *et al.*, 1985; Gazdar *et al.*, 1985]. There is also clear evidence that some of the neuroendocrine and cytogenetic markers are also expressed by NSCLC. Significant levels of L-dopa decarboxylase activity have been detected in other types of lung cancer [Baylin *et al.*,

1980; Gazdar *et al.*, 1988], bombesin-like immunoreactivity has been found in about 17% of NSCLC [Yamaguchi *et al.*, 1983], and 3p deletion, a lesion previously thought to be SCLC specific, is now also found in about 25% of NSCLC [Brauch *et al.*, 1987]. Immunohistochemical studies utilizing specific monoclonal antibodies have shown that most of the antigens are expressed by all types of lung cancer. Waibel *et al.* [1987] have shown that common antigens are expressed by SCLC and other lung cancer type (e.g. LAM-2). MOC-1 a monoclonal antibody raised against a human SCLC cell line was found to react with all major histological types of lung cancer including SCLC, adenocarcinoma, and in mixed adeno-squamous tumours [de Leij *et al.*, 1985]. Others [Olsson *et al.*, 1984] have shown phenotypic heterogeneity in clones of human lung cancer cell lines.

The origin of SCLC is uncertain. Initially it was considered to be an undifferentiated tumour associated with a variety of paraneoplastic syndromes caused by ectopic hormone secretion. Later on in late 1960s, recognition of neurosecretory granules in these tumours [Bensch *et al.*, 1968; Hattori *et al.*, 1968] led to a new concept that different forms of lung cancer originate from different specialised normal cells, and according to this hypothesis SCLC was thought to arise from the normal neuroendocrine or APUD (Kulchitski) cells of the lung [Bonikos & Bensch, 1977], which were originally supposed to be neural crest origin [Pearse & Takor, 1979]. However, more recent observations (above paragraph) have led to the common stem cell theory of lung carcinoma histogenesis [Gazdar *et al.*, 1981]. According to this theory all lung cells arise from a common stem cell of endodermal origin and different histological tumour types represent different routes of malignant differentiation regulated by the nature of the genetic lesion(s) and the effects of cellular microenvironment.

Although these phenotypic conversions in lung cancer may have significant implications for prognosis and response to therapy of lung cancer, the mechanism of phenotypic changes in SCLC is as yet unknown. This is in part due to lack of readily available material for the study of sequential changes in the development of tumours in natural host, and also due to the fact that relevant *in vitro* models have not been developed. The present study is an attempt to develop an *in vitro* model for the study of phenotypic transition in SCLC *in vivo*. A classic SCLC cell line (H69) and its two derivative cell lines (H69V & H69VZ) have been used as a model, because of the potential relevance to clinical progression of lung cancer. The

parental cell line was originally established from a pleural effusion of a patient with relapsed SCLC [Gazdar *et al.*,1980], and characterised as a classic SCLC cell line with typical morphological and biochemical profiles [Carney *et al.*, 1985; Gazdar *et al.*, 1985]. However, drug resistant variant cells have been reported in this cell line [Twentyman *et al.*, 1986; Mirski *et al.*, 1987]. Heterogeneity in culture morphology of the cell line is often found with some cell sticking to the substrate having epithelioid morphology, while the bulk of cells grew in suspension. Characterization of these phenotypic shifts from SCLC cells (H69) to NSCLC-like cells (H69V & H69VZ) *in vitro* probably will better reflect the clinical behaviour of lung cancer progression.

Specific objectives were: 1). To exclude cross-contamination and confirm a common origin of the parental and the derivative line, by cytogenetic analysis, isoenzyme studies, study of H69 clones, antigenic marker expression, and ultrastructural features. 2). Characterization of the derivative cell lines, and comparison with SCLC (parental) and NSCLC cell lines, for growth, morphology, NE-cell marker expression, invasion, metastasis both *in vitro* and *in vivo*, and for response to chemotherapy, radiotherapy and oncogene expression.

## 3A.2 MATERIALS AND METHODS

### 3A.2.1 Cell Lines

Cell lines used in this Chapter belonged to three groups [Table 3A.1] : (1) the SCLC cell line NCI-H69; (2) derivative cell lines of the SCLC cell line; (3) and NSCLC cell lines. The SCLC cell line H69 was originally derived from a male patient who had previously received multidrug therapy including adriamycin. It was obtained at passage number 33, from Dr Desmond Carney, NCI, Bethesda, Md. It grew in suspension, forming tight cellular aggregates. The cell stocks were stored frozen, and all experimental work on this line was conducted between passage 33-45 in culture. Two adherent sublines (H69V and H69VZ) were derived from H69 cell line in culture (see below). Two NSCLC cell lines (WIL and A549) were also included for comparison. The WIL cell line obtained from ICR, Sutton, was derived from an adenocarcinoma of lung, and grew as monolayer of epithelioid cells (see Chapter 5). Cell line A549 derived originally from human pulmonary adenocarcinoma [Giard *et al.*, 1972] grew as monolayer.

**Table 3A.1:- Cell lines.**

| Cell line | Source | Tissue of origin |
|---|---|---|
| [1]NCI-H69 | NCI Bethesda | Human SCLC |
| [2]MOG-H69V | Med. Oncol. Glasgow | NCI-H69 |
| [2]MOG-H69VZ | Med. Oncol. Glasgow | NCI-H69 |
| [3]WIL | Haddow Labs. Sutton | Human lung adenoca. |
| [4]A549 | ATTC, Maryland | Human alveolar cell carcinoma |

[1] Carney et al., [1985]. [2] Khan et al., [1990]. [3] Unpublished. [4] Giard et al., [1972].

### 3A.2.2 Cytotoxic Drugs

Adriamycin [Farmitalia], and vincristine [Sigma] were used in this study. Stock solutions of the drugs in distilled water were stored frozen at -20 °C. Drugs were diluted in complete culture medium before use. For longer duration of treatment fresh drug was used after every 24 hours.

### 3A.2.3 Isolation of Adherent Sublines of H69

The parental cell line H69 was grown in 175 $cm^2$ flasks (Falcon) for 3 weeks, by which time cells had formed large sized aggregates which settled down on to the substrate. Subsequently, cells from the aggregate grew out as adherent cells. Once the monolayer was formed, cells in suspension were removed, and the adherent cells were washed, and re-incubated to form a complete monolayer, which was then trypsinized and subcultured at $10^5$ cell/ml in 75 $cm^2$ flasks. Cultures were passaged weekly, and mass cultures were derived after 8-10 passages for frozen stock. Two adherent lines (H69V and H69VZ) have been derived from the parental cell line H69 by this technique at different occasions.

### 3A.2.4 Isolation and Expansion of H69 Clones

A single cell suspension was cloned in agar. Growing colonies were isolated, dispersed mechanically, and plated in 24-well plates containing 2 ml RPMI1640 containing 10% FBS. These cells were expanded in culture, stored and used to get mass cultures for isolation of monolayer cells.

### 3A.2.5 Radiation Sensitivity Assay

Cell cultures were prepared in 96 well plates, as for the MTT assay. Exponentially growing cultures were irradiated using a Mobaltran Cobalt-60 gamma unit, operating at a dose rate of 140 cGy per minute, at room temperature, and with a source-axis distance of 80 cm, using full built-up and backscatter. Following radiation cells were fed with fresh medium, for at least three cell doublings.

### 3A.2.6 Other Methods

The details of primary antibodies used in immunohistochemical methods are shown in Table 3A.2. Other methods employed in this Chapter have been described in General Methods.

**Table 3A.2:- Primary antibodies.**

| Antibody | [1]BLI | NSE | CG | SP | CAM5.2 |
|---|---|---|---|---|---|
| Source/ Supplier | (see below) | Dako | Atlantic antibody | Dako | Becton Dickinson |
| Species raised in | Rabbit | Rabbit | Rabbit | Mouse | Mouse |
| Mono-/or Poly-colnal | Poly | Poly | Poly | Mono | Mono |
| Dilution | 1:50 | 1:300 | 1:5 | 1:20 | 1:10 |
| Incubation | 16 hrs | 16 hrs | 16 hrs | 16 hrs | 2 hrs |
| Specificity | Neural | Neural | Neural | Neural | Epithelial |
| Positive control | SCLC cells Fetal lung | Pancreas SCLC cells | Pancreas Pituitary | Pancreas Pituitary | Skin |
| Additional treatment | Blocking | Blocking | Blocking | Blocking Trypsin | Trypsin |

BLI: Bombesin-like immunoreactivity, NSE: Neuron specific enolase, CG: Chromogranin A, SP: Synaptophysin. [1]Anti-bombesin antibody was a kind gift from Prof. J. Polak of Hammersmith Hospital London.

## 3A.3 RESULTS

### 3A.3.1 Establishment of Model

The H69 cell line consists of an apparently homogeneous cell population [see below, Plate 3A.4]. However, in a dense culture, a few cells adhere to the substrate and grow out to form monolayer [Plate 3A.1]. Two adherent sublines (H69V and H69VZ) have been isolated from the H69 cell line.

### 3A.3.2 Confirmation of The Origin of Cell Lines

LDH isoenzymes and chromosome analyses confirmed the human origin of the cell lines. Both the parental and the sublines showed human specific lactate dehydrogenase isoenzyme profiles [Plate 3A.2; Figure 3A.1]. The cell lines were aneuploid and heteroploid with similar modal chromosome numbers as shown in Figure [3A.2].

Karyotypic analysis of derivative lines carried out by Dr Stephen Merry in association with the Dept of Medical Genetics Yorkhill Hospital and Dr Jeffery Trent showed that the H69V cell line did in fact contain many of the identifiable markers which had previously been found in H69, including 1P deletion, a marker involving 10P, and 11q, and a translocation 9:19 [Plate 3A.3].

### 3A.3.3 Lineage Identification

Various markers including cytokeratin (CAM5.2) expression, ultrastructural features such as desmosomes, and intermediate filaments, and an epithelial morphology in monolayer culture were consistent with an epithelial origin of both parental and the derivative cell lines (see below).

### 3A.3.4 Morphological Characterization *In Vitro*

***Culture Morphology***

The parental line grew as tightly packed floating cellular aggregates, spherical as well as irregular in outline, amorphous, and without central necrosis [Plate 3A.4a]. Individual cells appeared small and rounded, with clear cytoplasm, and small nuclei. The sublines grew as a monolayer of multipolar epithelioid cells, with a clear amorphous cellular outline. Individual cells appeared larger in size, with large clearer cytoplasm and a small granular nuclear zone with indistinct outline and small

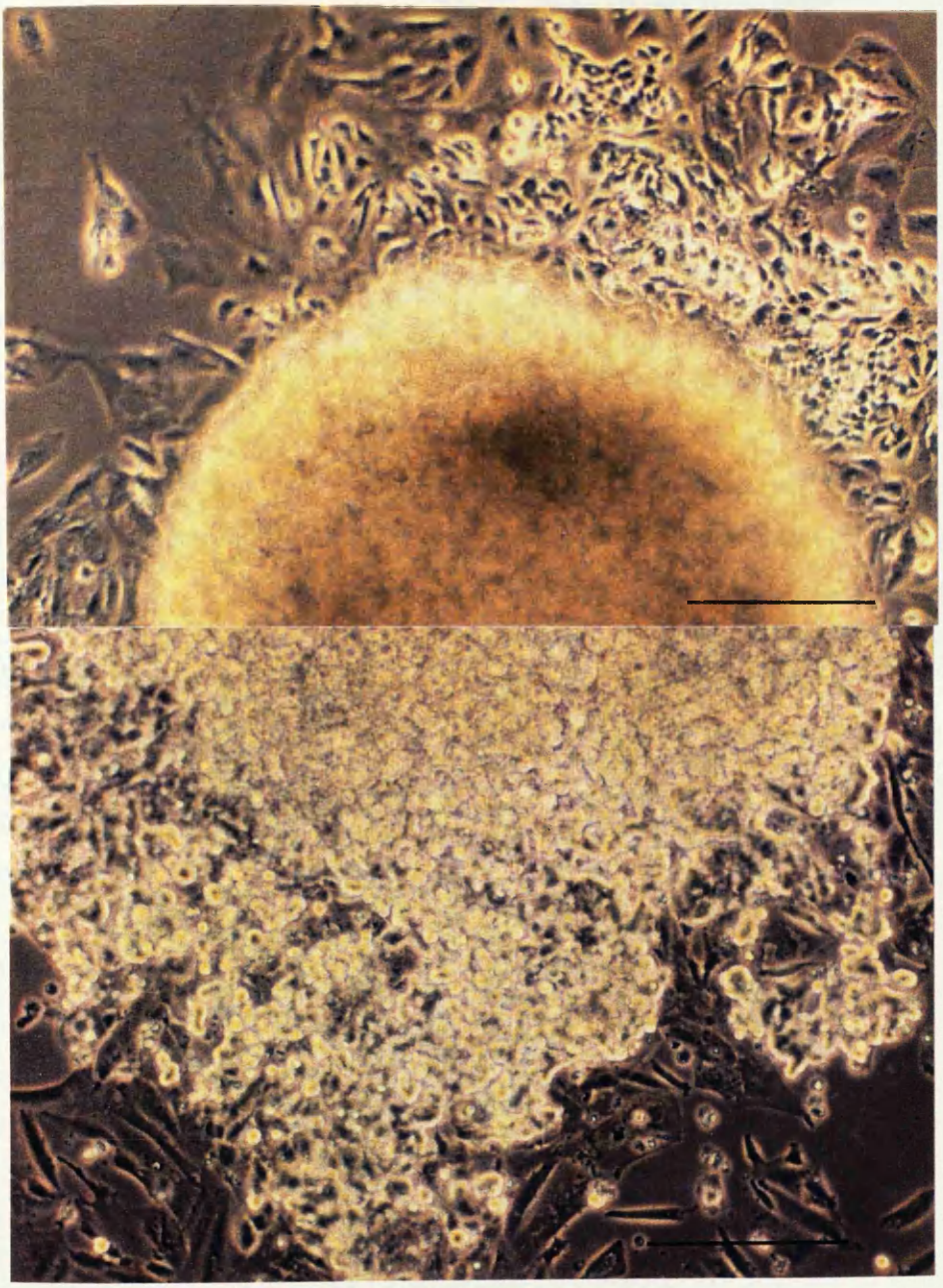


Plate 3A.1:-**Derivation of adherent cells from NCI-H69 non-adherent cell line.**

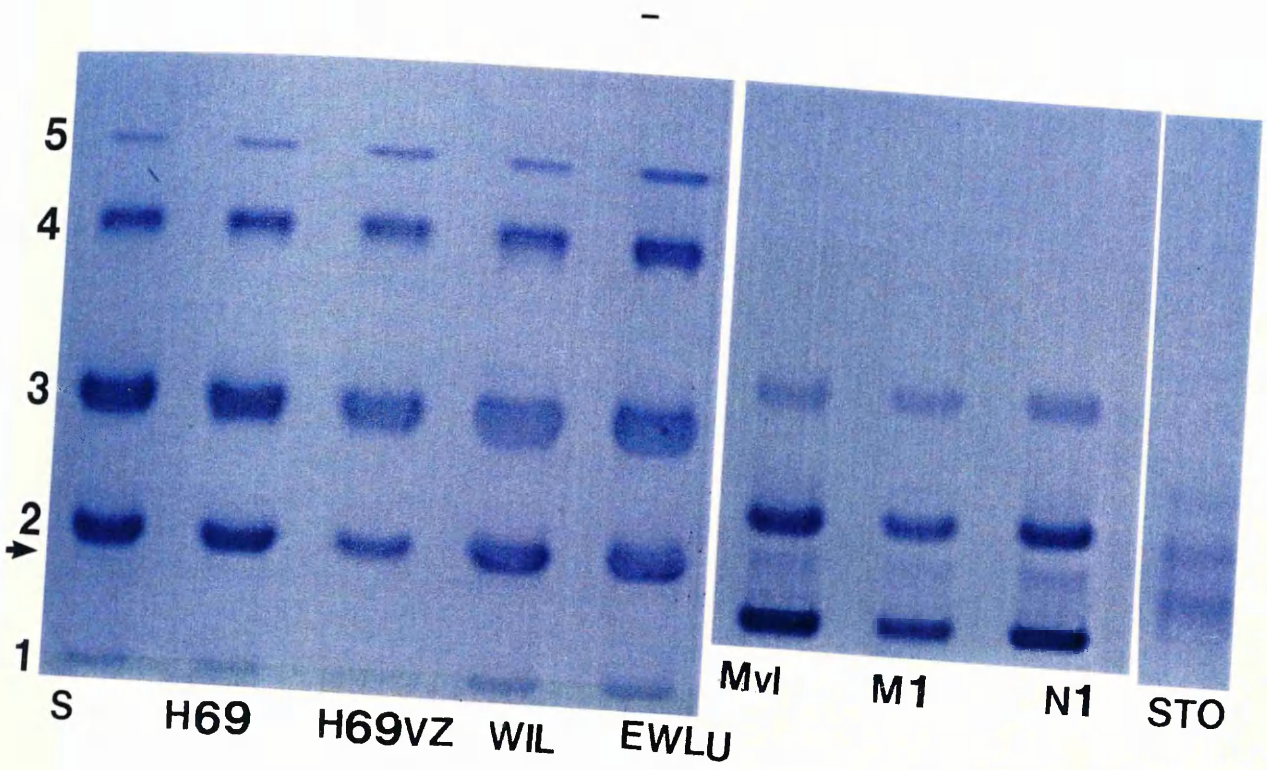
H69 cells after 4 weeks in culture, showing large sized aggregates, both rounded (above) and irregular (below) shaped, attached to the bottom of the flask. Adherent cells are seen growing out from the H69 aggregate, forming a monolayer of cells, different from the parental line (X 20 objective, phase-contrast, bar = 100 $\mu$m).

**Plate 3A.2 :- Electrophoretic fractionation of lactate dehydrogenase isoenzyme profile from the cell lines of different species.**

All human cell lines (H69, H69VZ, WIL, EWLU) show five distinct lactate dehydrogenase (LDH) isoenzyme bands corresponding to the human standard (S). Mink (Mvl, M1, N1) and mouse (STO) cell lines show LDH isoenzyme bands at different levels, compared either to the standard or the human cell lines. Numbers 1-5 refer to the LDH isoenzyme bands present in the human LDH standard. Arrow indicates the site of sample application. All samples were electrophoresed and stained as outlined in General Methods.

**Figure 3A.1:- Electrophoretic scan of the lactate dehydrogenase isoenzymes.**

The scan shows the typical pattern of five human LDH isoenzymes with total LDH activity within normal range. [Sigma].


-
5
4
3
2
1
S
H69
H69VZ
WIL
EWLU
Mvl
M1
N1
STO
+

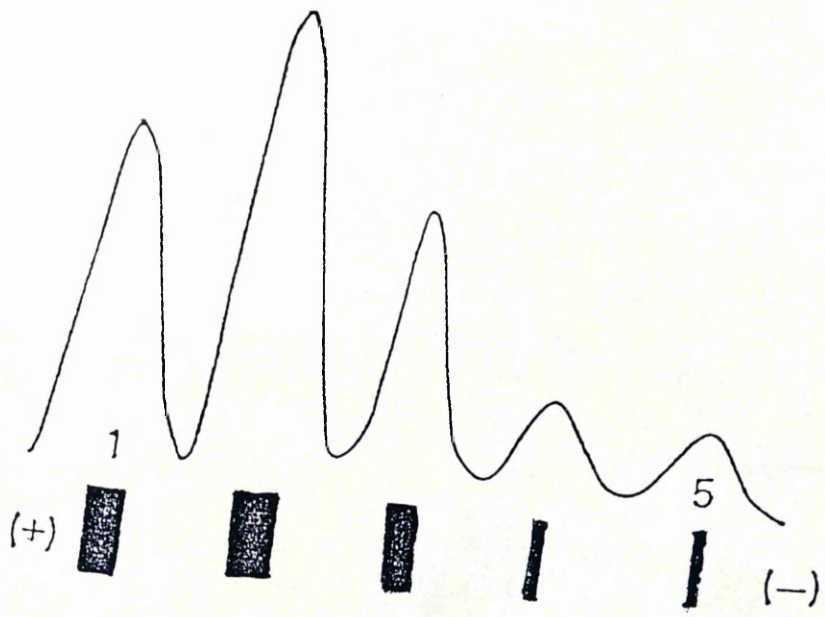
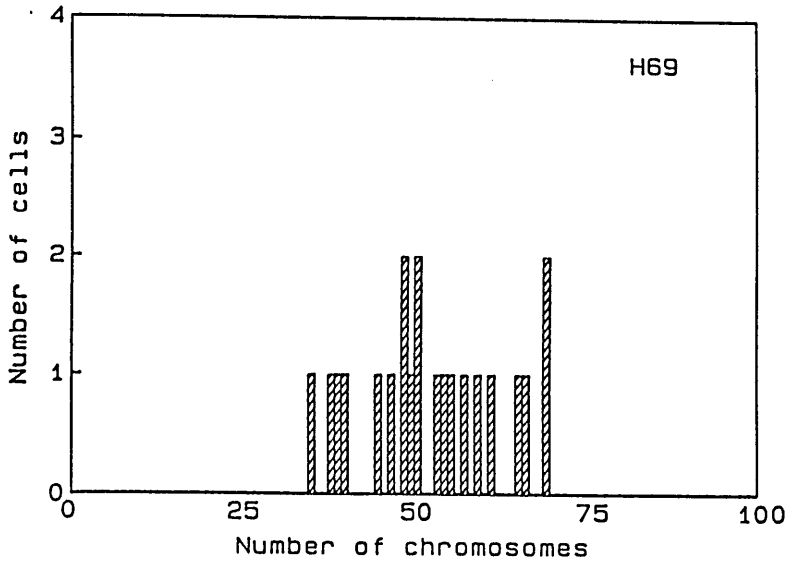
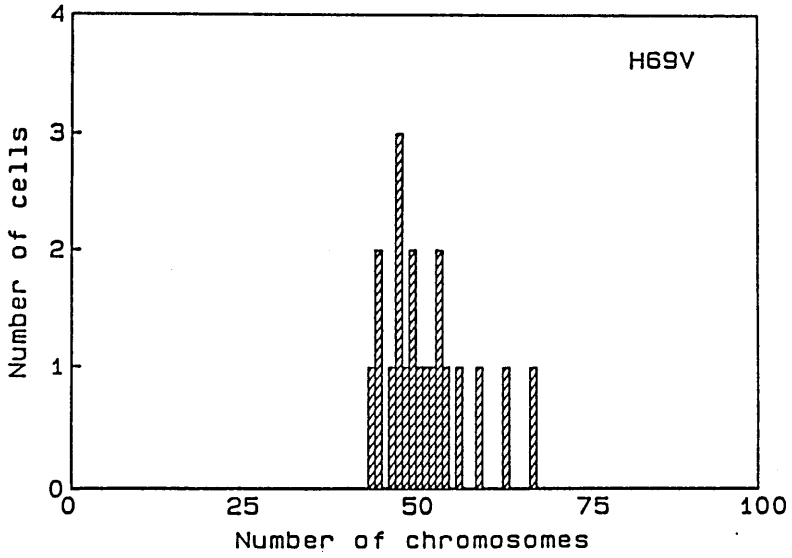

1
5
(+)
(–)

**Figure 3A.2:- Histiogram showing distribution of chromosomes in H69, H69V, and H69VZ cell lines.**

Exponentially growing cells were incubated with colcemid for 4 hours, exposed to hypotonic solution for 30 min, and metaphases collected, fixed and spread on slides by drop technique. Air dried, Giemsa stained preparations were examined under oil immersion lens x 100. Chromosome number was determined from at least 20 spread. All cell lines are aneuploid with chromosome number ranging from 35 to 70.


H69
Number of cells
Number of chromosomes
0
1
2
3
4
0
25
50
75
100


H69V
Number of cells
Number of chromosomes
0
1
2
3
4
0
25
50
75
100

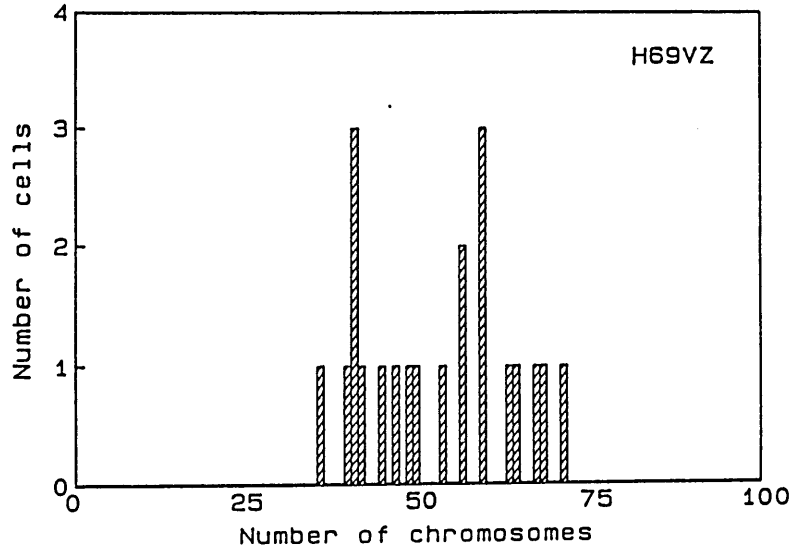

H69VZ
Number of cells
Number of chromosomes
0
1
2
3
4
0
25
50
75
100

**Plate 3A.3:- Karyotype of H69V cell with 60 chromosomes.**

G-banded karyotype of H69V cells showing 1p deletion, a marker involving 10p and 11q, a translocation 9:19, and other lesions, found in SCLC [from Dr Stephen Merry].

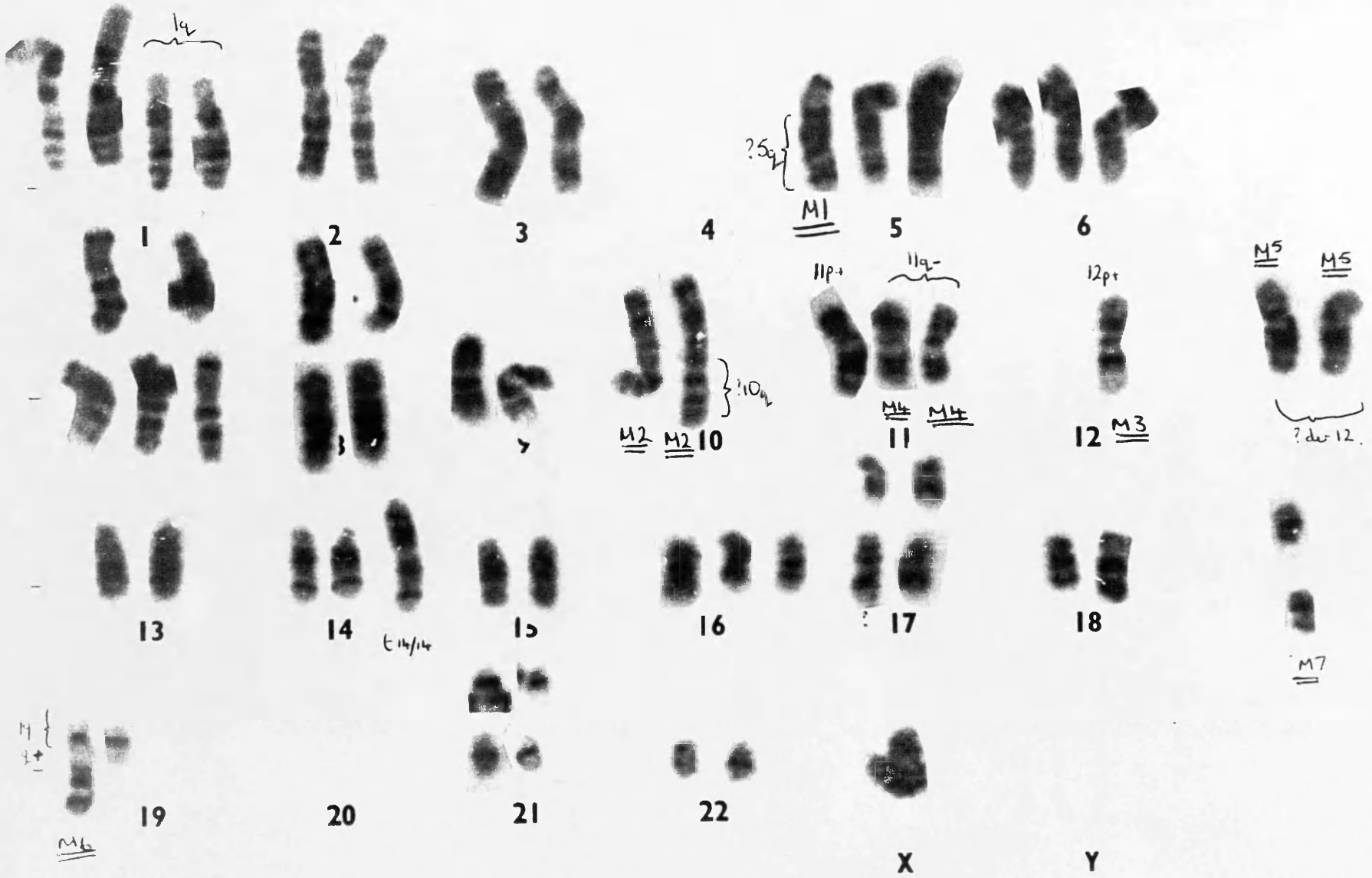

1q
1
2
3
?5q
M1
4
5
6
11p+
11q-
12p+
M5
M5
M2
M2
?10q
M4
M4
M3
10
11
12
? del 12.
13
14
t 14/14
15
16
?
17
18
M7
M6
19
20
21
22
X
Y

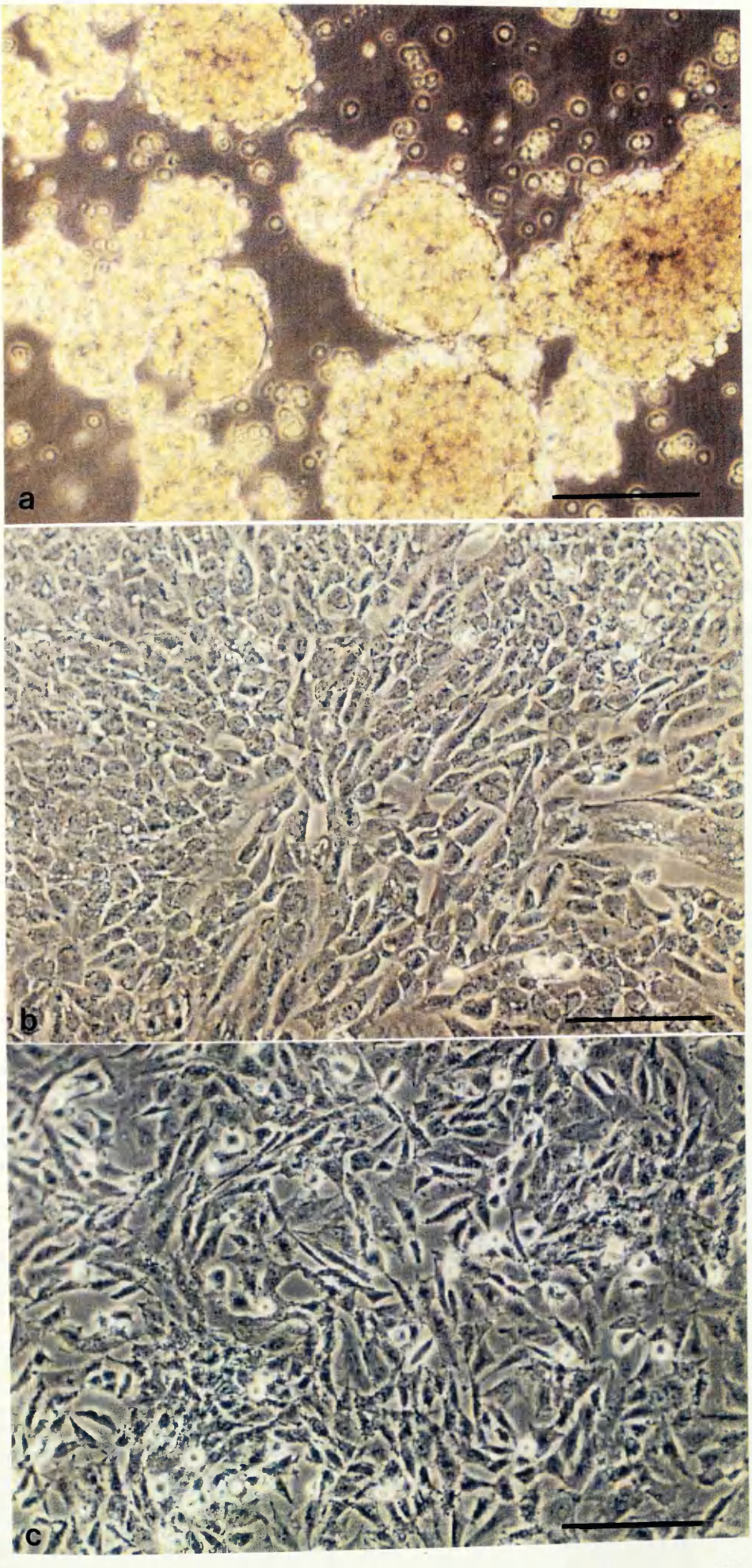
**Plate 3A.4:- Culture morphology of H69, H69V, and H69VZ cell lines.**

(a) Morphology of H69 cells growing in RPMI 1640 medium as free floating cellular aggregates with amorphous outline, and showing two types of aggregates; aggregates with an irregular outline and others having rounded appearance.

(b) H69V cells derived from H69 cells, grow as monolayer of epithelioid and spindle-shaped cells.

(c) H69VZ cells derived from H69 cells, grow as monolayer of spindle-shaped epithelioid cells similar to (b).

(x 10 objective, phase-contrast, bar= 200 $\mu$m) .


a
b
c

nucleoli [Plate 3A.4 b & c]. These features were distinct from the NSCLC cell line WIL [see below, Plate 5.1 a], which had a pavement-like appearance.

*Cytological Features*

The parental cell line, H69, showed small rounded cells, with deeply staining nuclei, granular chromatin, nuclear moulding, clearer and scanty cytoplasm [Plate 3A.5 a]. The NSCLC cell line, WIL, showed large cells with abundant cytoplasm [Plate 3A.5 b]. The sublines, H69V & H69VZ, showed large cells with moderate amount of palely staining cytoplasm, inconspicuous nucleoli and perinuclear clearing [Plate 3A.5 c & d].

### 3A.3.5 GROWTH CHARACTERIZATION *IN VITRO*

*Growth rate*

The parental cell line grew slowly, with a population doubling time of around 2 days [Table 3A.3], and a lag period of around 2 days [Figure 3A.3]. The population doubling times decreased in both sublines, to 34 hours. Both the derivative lines reached plateau after approximately 10-12 days, when plated at initial cell concentration of $1x10^4$ cells/well, in 24 well plates, however H69 cells with initial seeding concentration of $1x10^5$ cells/well did not reach plateau. The growth of adherent cells was faster than the parental line.

*Plating Efficiency in Monolayer*

The plating efficiencies (PE) of both derivative lines in monolayer culture were not significantly different, but lower than the NSCLC line WIL [Table 3A.3]. Both the derivative lines had similar values.

*Colony Forming Efficiency in Soft Agar*

Table 3A.3 shows the mean cloning efficiencies (CE) of the cell lines in suspension. These were significantly higher in NSCLC cell lines, than in the SCLC line and intermediate in both sublines. The CE of both sublines were approximately the same and both NSCLC lines had similar values. The H69 cell line formed diffuse colonies with irregular margins.

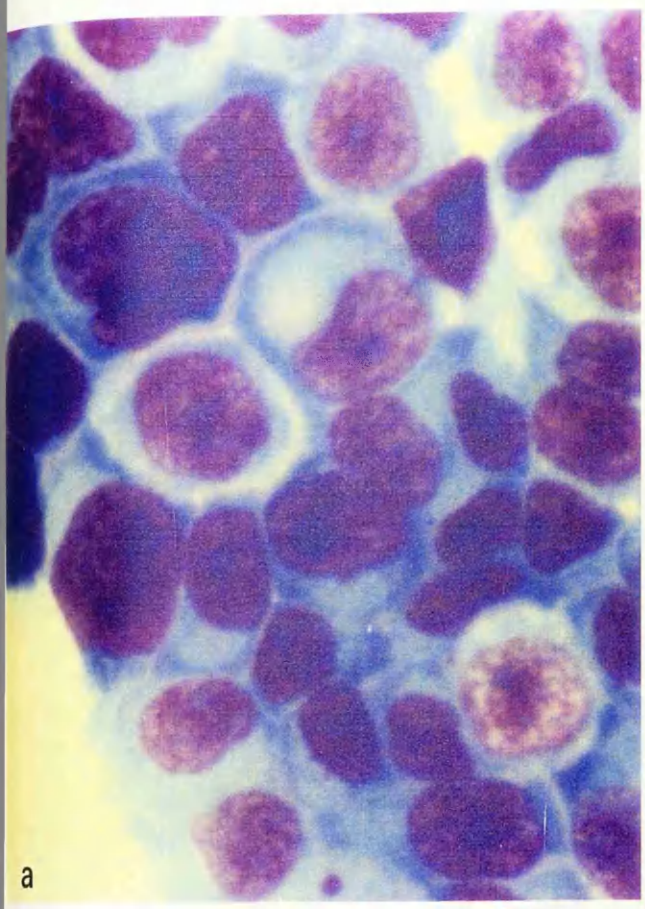
**Plate 3A.5:- Cytological characteristics of the cell lines.**

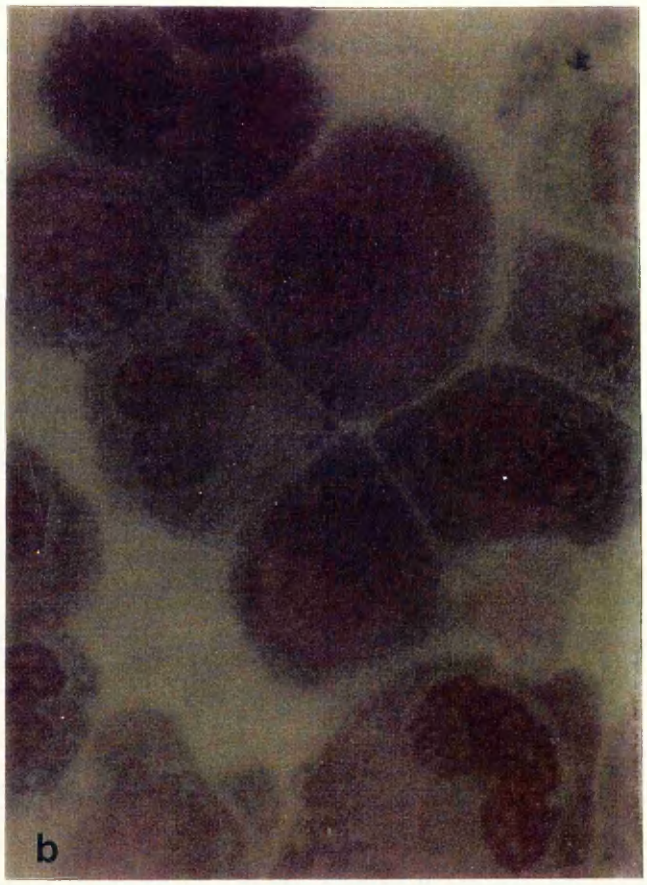
(a) SCLC cell line, H69, shows small rounded cells, with clearer and scanty cytoplasm, deeply staining nuclei, granular chromatin, and one or two darkly staining nucleoli. Acetone fixation, Papanicolaou stain, X 900.

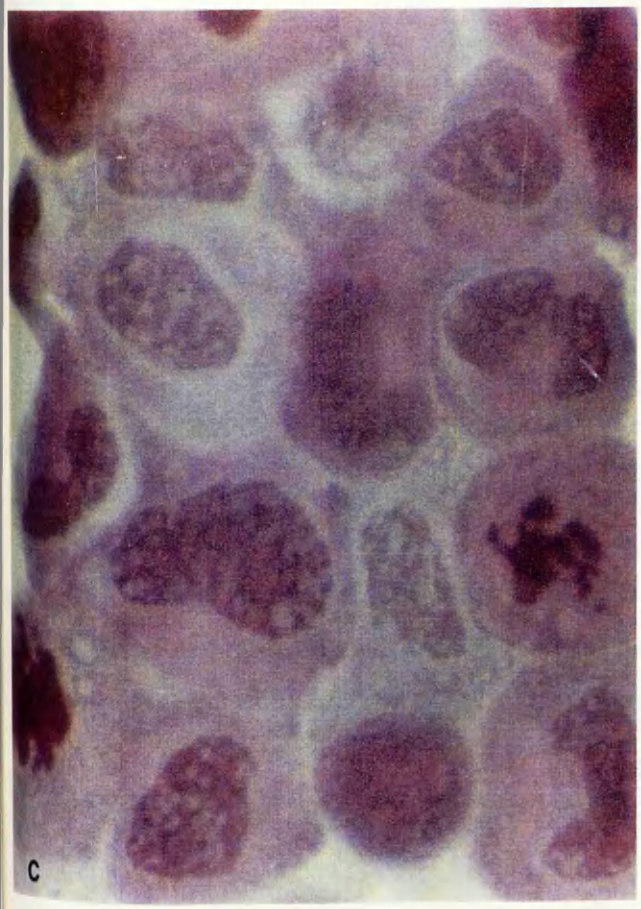
(b) NSCLC cell line, WIL, showing larger cells with polygonal outline, abundant cytoplasm, granular chromatin, and inconspicuous nucleoli. Acetone fixation, Papanicolaou stain, X 900.

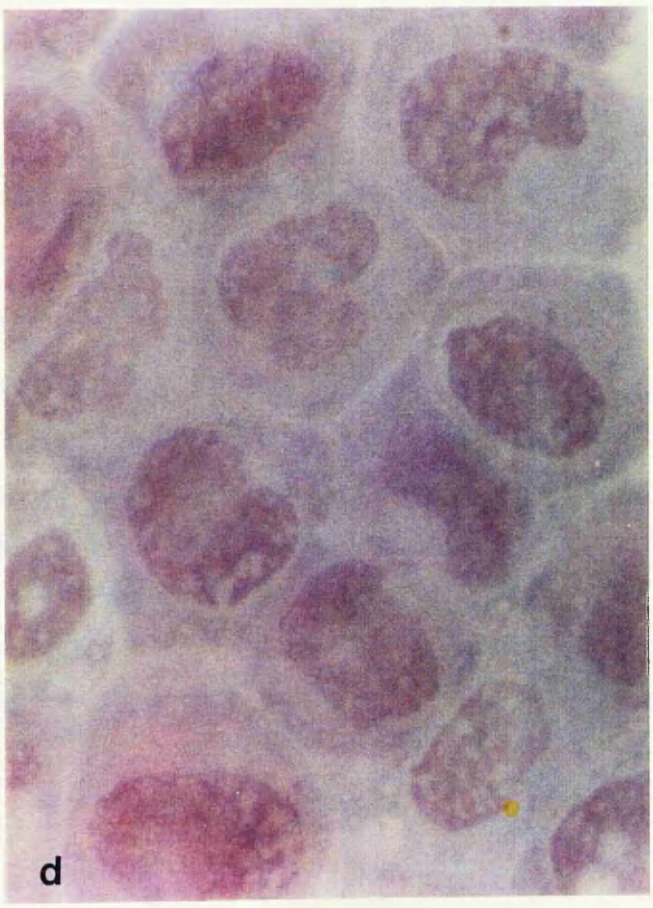
(c) H69 derivative cell line, H69V, shows larger cells with abundant amount of palely staining cytoplasm, evenly distributed granular chromatin, perinuclear clearing, and inconspicuous nucleoli. Acetone fixation, Papanicolaou stain, X 900.

(d) H69 derivative cell line, H69VZ, shows features very similar to the H69V cell line (c). Both the derivative lines show cytological features similar to the NSCLC cell line, WIL. Acetone fixation, Papanicolaou stain, X 900.


a


b


c


d

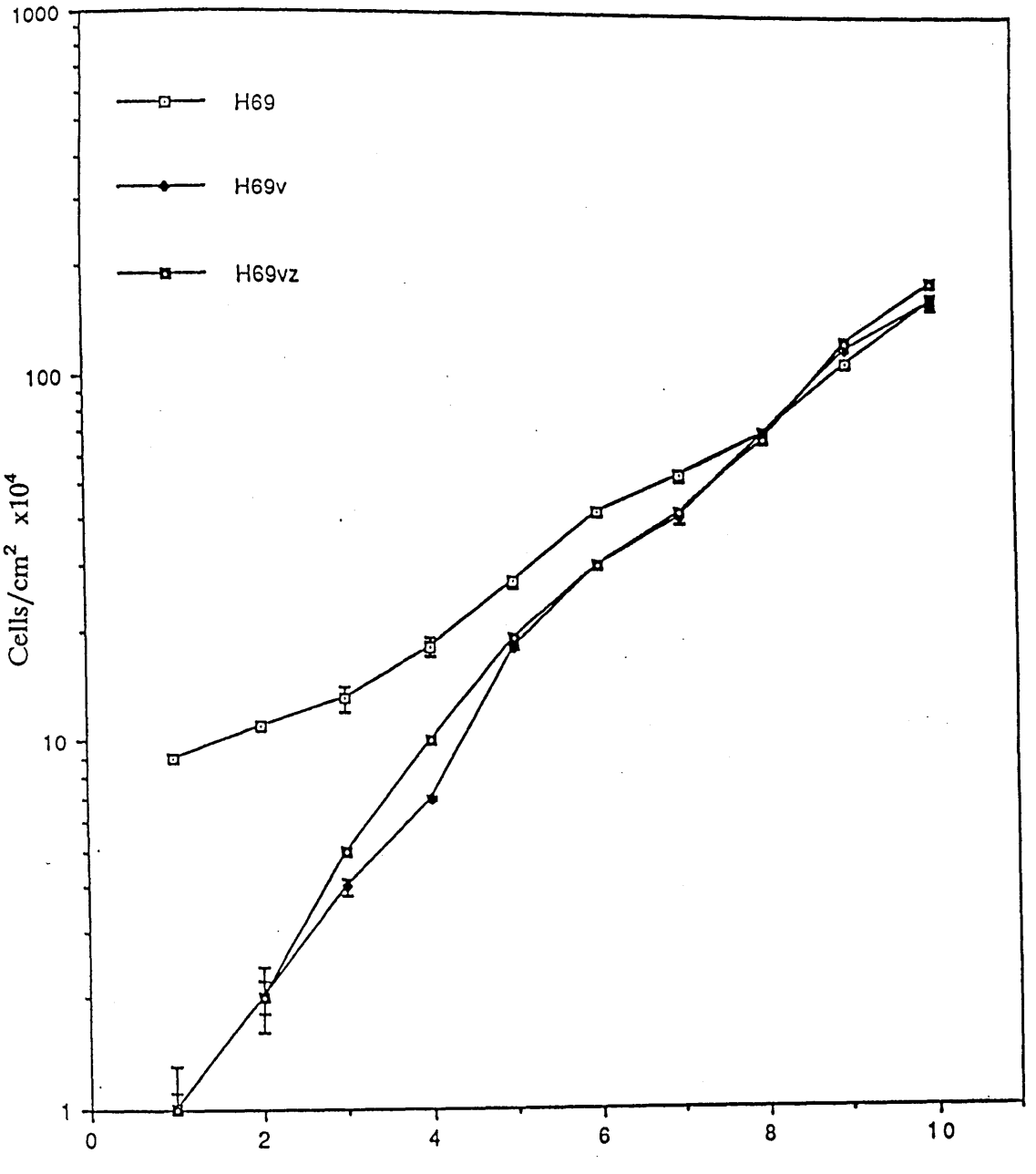



**Figure 3A.3:- Growth curves of H69 and its derivative cell lines in vitro.**

A semi-logarithmic plot for the cell lines, grown in 24-well plates. Cultures for the parental cells were set up at $1x10^5$ cells/$cm^2$, while for the derivative lines were at $1x10^4$ cells /$cm^2$, on day 0. The cells were fed on alternate days during the first 5 days, and daily thereafter. Cells from at least three replicate wells were trypsinised and counted daily. Each point represents the mean ± SEM (bars).

**Table 3A.3:- *In vitro* growth characteristics of cell lines.**

| Cell line | Doubling time (hours) | Cloning Efficiency (%) | |
|---|---|---|---|
| | | Monolayer | Suspension |
| H69 | 56.0 | NA | 7.5 |
| H69V | 34.0 | 21.5 | *14.0 |
| H69VZ | 34.5 | 22.5 | *14.5 |
| WIL | 35.5 | 45.5 | *24.5 |
| A549 | 34.5 | 36.5 | *25.5 |

Plating efficiency (PE) was determined by monolayer cloning. Cells (500) were seeded in 60 mm petri dish and in 5 ml culture medium. Colonies were fixed after 3 weeks. Cloning efficiency (CA) was determined by plating $10^4$ cells per ml 0.3% agar, over 1% agar underlayer. Data are mean of at least two separate experiments. NA: non-adherent. The cloning efficiencies of both the derivative and NSCLC cell lines were significantly higher than the H69.

*$P<0.001$ (analysis of variance, and Bonferoni adjustment).

*Colony Morphology*

The H69 cell line formed diffuse, asymmetrical, colonies with irregular margins, and bizarre morphology. Both derivative and NSCLC lines formed more spherical colonies of regular size.

*Isolation and Expansion of H69 clones*

Preliminary experiments showed that H69 clones can give rise to a heterogeneous cell population in culture, as found in the parental line. Clones were grown in 0.3% agar in RPMI 1640 medium with 10% FBS. After 6 weeks colonies were transferred to 24 well plates, and fed regularly. After another 3-4 weeks period cells were transferred to flasks and allowed to grow to confluence, which was reached in further 4-5 weeks. Adherent cells appeared in the confluent cultures of H69 clones, with morphology similar to H69V and H69VZ.

*Growth Media and Serum Requirements*

All cell lines showed growth in serum free conditions for up to 2 weeks. However, after this, cultures deteriorated with partial necrosis which was not complete by 4 weeks.

### 3A.3.6 NEUROENDOCRINE MARKER EXPRESSION

*Biochemical Analysis*

Dopa decarboxylase (DDC) activity was measured during the late log phase of the cell growth cycle, where the activity has been shown to be steady [Murray, 1989]. The numerical data are shown in Table 3A.4. The DDC levels were approximately 7-fold lower in both derivative lines, than in the parental line. The NSCLC lines were 10-fold lower than H69. There was no significant difference between H69V and H69VZ.

For CK analysis all experiments were carried out during late log phase of growth. The creatine kinase BB isoenzyme (CKBB) was the predominant form, and H69 cells were found to express the highest CKBB activity among all the lines investigated [Figure 3A.4, Table 3A.5]. The activity of the enzyme was below the level of detection both in NSCLC cell lines and in the derivative lines.

The BLI levels were significantly higher in the parental line compared to both the derivative lines and the NSCLC cell lines [Table 3A.6]. The activity was similar

**Table 3A.4:- Expression of L-DOPA Decarboxylase (DDC) activity in H69, its derivative cell lines H69V & H69VZ, and 2 NSCLC cell lines.**

| Cell line | [1]DDC activity [uIU/mg protein] | [2]Difference (%) |
|---|---|---|
| NCI-H69 | 2853 + 335 (n=9) | 100 |
| H69V | 408 + 116 (n=6) | *14 |
| H69VZ | 333 + 61 (n=6) | *12 |
| WIL | 276 + 68 (n=6) | *10 |
| A549 | 203 + 121 (n=6) | *7 |

[1]The DDC activity is expressed as micro international units (uIU) per mg cellular protein. Where 1 uIU is equivalent to one u mole of product formed per minute.
[2]Difference in percentage from classic SCLC cell line NCI-H69, taken as 100%. Data are mean + SEM of the number given in the parentheses, from at least 2 independent experiments.

*$p<0.0001$ (analysis of variance, and Bonferoni adjustment)

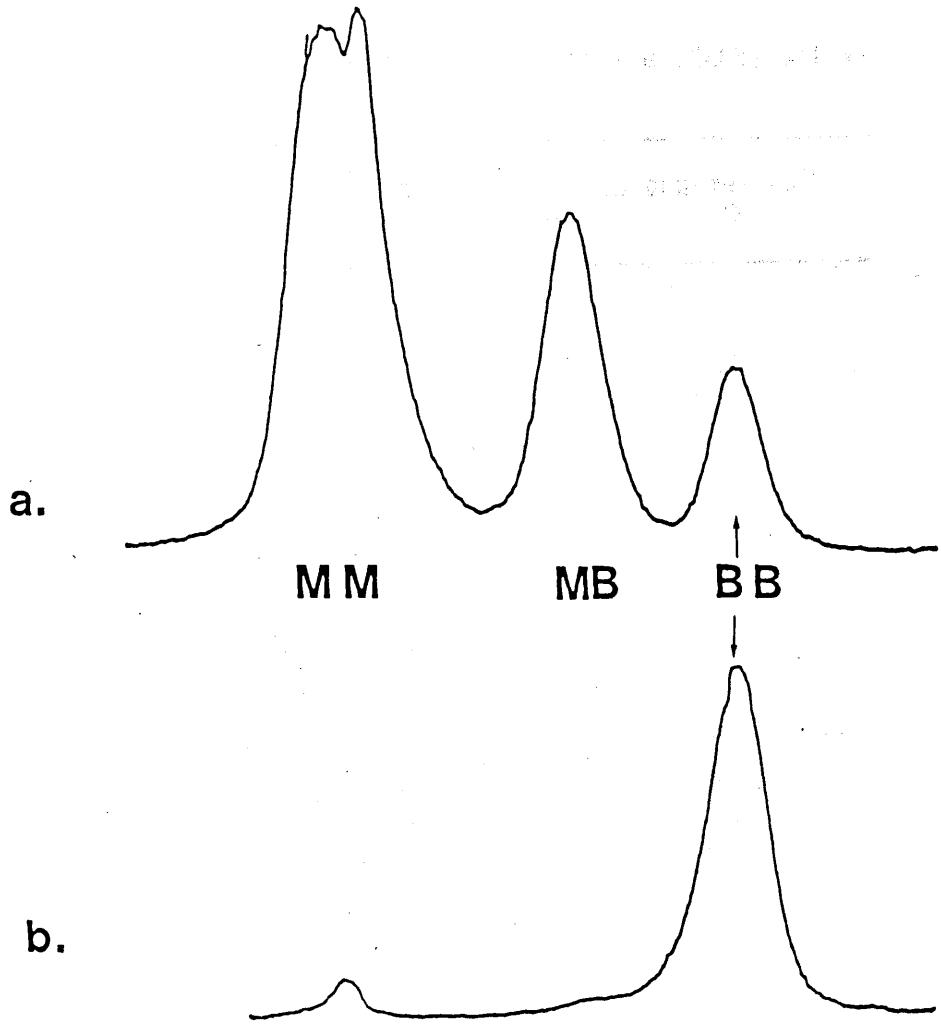



**Figure 3A.4:- Electrophoretic scan of the human creatine kinase isoenzymes activity.** (a). The scan shows the result of electrophoretic separation of the three creatine kinase isoenzymes in the standard; the first peak represents the MM isoenzyme which is the slowest of all, followed by MB isoenzyme which is intermediate, and lastly the BB isoenzyme is fastest migrating fraction. (b). The human SCLC cell line NCI-H69 shows a significant increase in BB isoenzyme (arrows).

**Table 3A.5:- Creatine kinase brain isoenzyme (CKBB) activity of the cell lines.**

| Cell line | [1]CKBB activity [IU/mg protein] | Isoenzymes (%) BB | MM | MB |
|---|---|---|---|---|
| NCI-H69 | 3.0 ± 0.3 | 95.2 ± 0.9 | 4.9 ± 1.0 | 0 |
| H69V | 0 | 0 | 0 | 0 |
| H69VZ | 0 | 0 | 0 | 0 |
| WIL | 0 | 0 | 0 | 0 |
| A549 | 0 | 0 | 0 | 0 |

[1]The CKBB activity is expressed as international units (IU) per mg cellular protein. Where 1 IU is equivalent to one umole of NADH produced per minute. Data are mean ± SEM from 3 separate experiments.

**Table 3A.6:- Expression of bombesin-like immunoreactivity (BLI) in cell lines.**

| Cell line | BLI [pg/mg protein] | | Relative Value (%) | |
|---|---|---|---|---|
| | Cellular | Secreted | Cellular | Secreted |
| NCI-H69 | 0.480 ± 0.040 | 0.672 ± 0.080 | 100 | 100 |
| H69V | 0.037 ± 0.003 | 0.091 ± 0.0.030 | *8 | *13 |
| H69VZ | 0.036 ± 0.007 | 0.079 ± 0.027 | *7 | *12 |
| WIL | 0.006 ± 0.001 | 0.001 ± 0.000 | *1 | *0.1 |
| A549 | 0 | 0 | 0 | 0 |

Data are mean + SEM of at least 6 values from at least two independent determinations. The secreted acivity was determined in the conditioned medium following 48 hours incubation with test cells.

*$P<0.0001$ (analysis of variance, with Bonferoni adjustment).

in both the derivative lines, with the secreted levels slightly higher than the cellular. The activity was very low in WIL, while there was no detectable activity in the A549 cell line.

The H69 cell line expressed the highest levels of NSE activity [Table 3A.7], ranging from 689 to 818 ng/mg protein. The activity was significantly lower in the derivative lines, with an approximately 14-fold difference from the parental line. NSE expression in WIL was significantly lower, with a difference of approximately 30-fold from H69, but only 2-fold from sublines of H69. The secreted NSE level in H69 was approximately 8-fold lower than the cellular level. The secreted fraction was collected after 48 hours cellular incubation. The overall secreted activity of the sublines was in the same range as the parental line, but there was no significant difference in the secreted and cellular enzyme activity between the sublines.

*Immunohistochemistry*

H69 cells showed intense granular positivity for BLI in over 70% of cells [Plate 3A.6 a] compared to the derivative lines which showed only occasional positive cells. There was no positive staining of NSCLC cell lines.

The H69 cells stained 80% positive with intense and uniform staining for NSE [Plate 3A.6 b]. Both the derivative lines stained only focally, while there was no specific staining in NSCLC lines.

Chromogranin staining was only focal with approximately 25% cells showing positive staining. This pattern was seen in parental line as well as in sublines. Staining was always more focal in derivative lines. There was no staining of NSCLC cell lines.

### 3A.3.7 Invasion Studies *In Vitro*

When the test cells (H69, H69V & H69VZ) were confronted with embryonic chick heart fragments, attachment between test cells and the heart fragment was observed in all the cell lines within 24 hours. However, there was no evidence of invasion, and cells were found at the periphery of the heart fragment [Plate 3A.7 a, T1]. The parental cells had invaded the heart fragment by day 2, singly and at different sites. Invading cells could be observed in the centre of the fragment, in haematoxylin and eosin stained sections by day 4 [Plate 3A.7 a, T4]. A progressive growth and replacement of the heart fragment by the invading cells was observed by

**Table 3A.7:- Neuron specific enolase (NSE) activity of cell lines.**

| Cell line<br>Cell line | NSE [ng/mg protein] | | Relative Value (%) | |
|---|---|---|---|---|
| | Cellular | Secreted | Cellular | Secreted |
| H69 | 741.3 + 40.4 (n=9) | 90.6 + 5.2 (n=9) | 100 (control) | 100 (control) |
| H69V | 52.3 + 10.9 (n=9) | 55.8 + 10.4 (n=9) | *7 | *58 |
| H69VZ | 56.9 + 5.2 (n=9) | 90.7 + 16.6 (n=9) | *8 | 100 |
| WIL | 25.7 + 5.4 (n=9) | 25.7 + 5.4 (n=9) | *3 | *28 |

Results are mean + SEM of number in parentheses, from 3 separate experiments. The secreted activity was determined in the conditioned medium collected after 48 hours incubation with cells

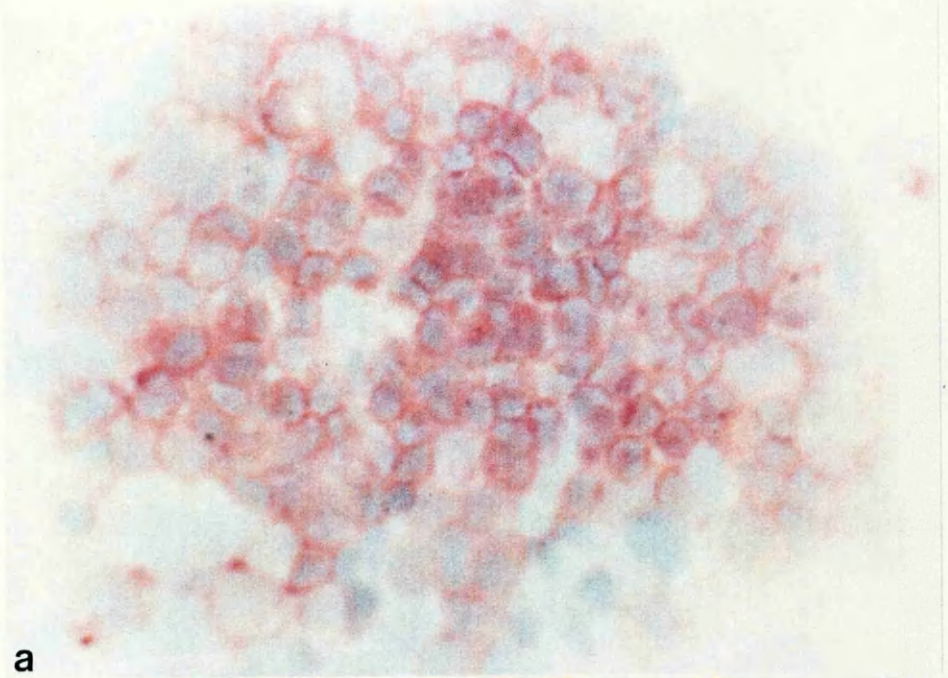
*$P<0.001$ (analysis of variance and Bonferoni adjustment).

**Plate 3A.6:- Immunostaining of the H69 cells for neuroendocrine markers.**

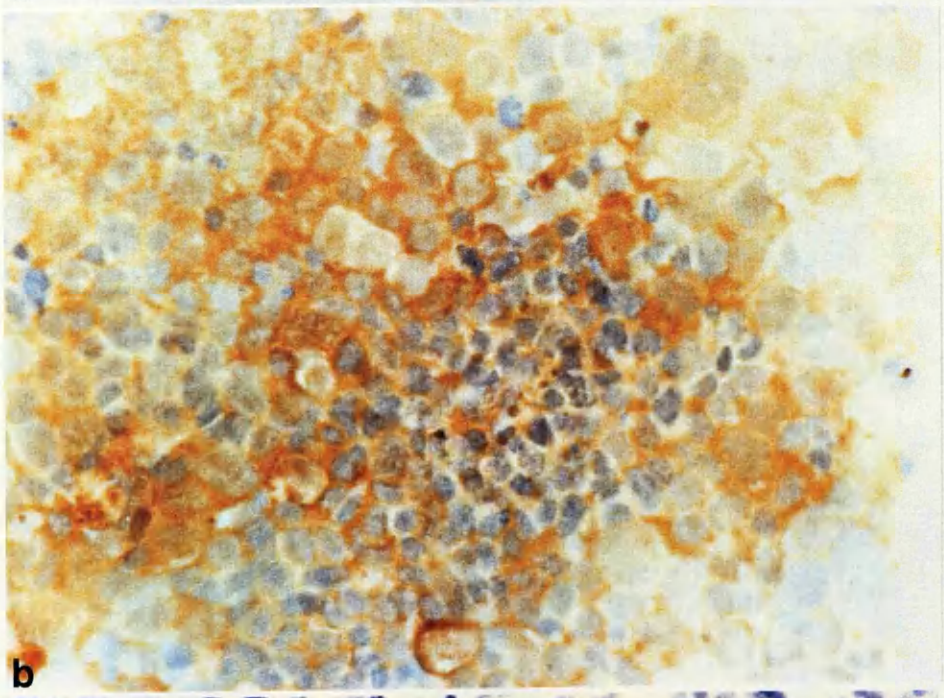
(a) H69 cells, stained for bombesin-like immunoreactivity, by the indirect alkaline phosphatase method. The majority of cells are immunopositive (x 247, indirect AP).

(b) H69 cells, stained for NSE by PAP method. Most cells are immunopositive (x 247, PAP).

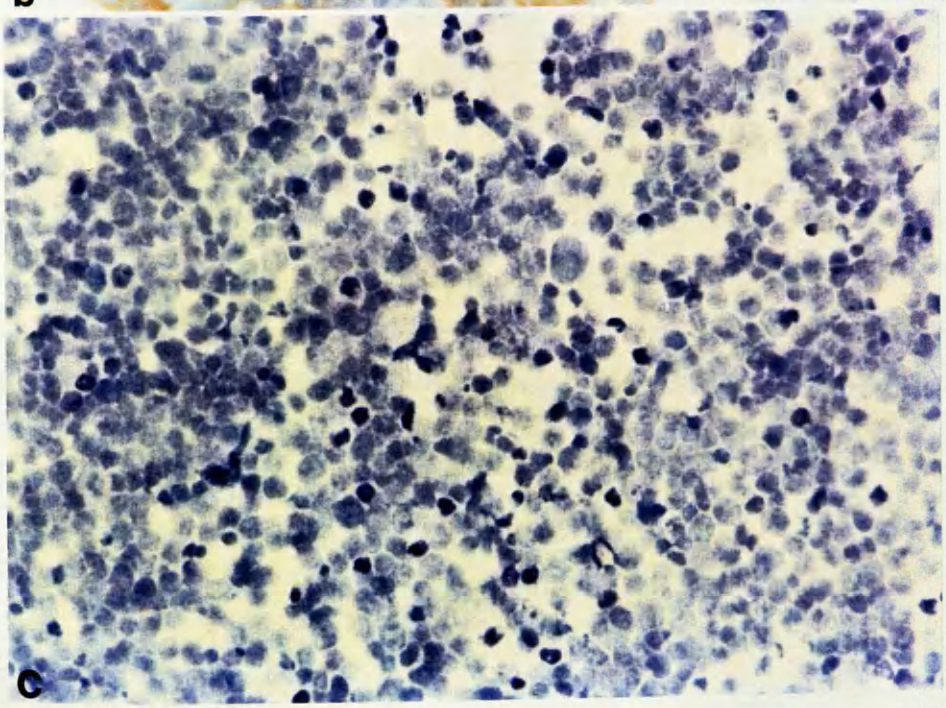
(c) Control H69 cells, stained as (b) except that the primary antibody (anti-NSE) was replaced by preabsorbed normal rabbit serum, show no staining.



a



b



c

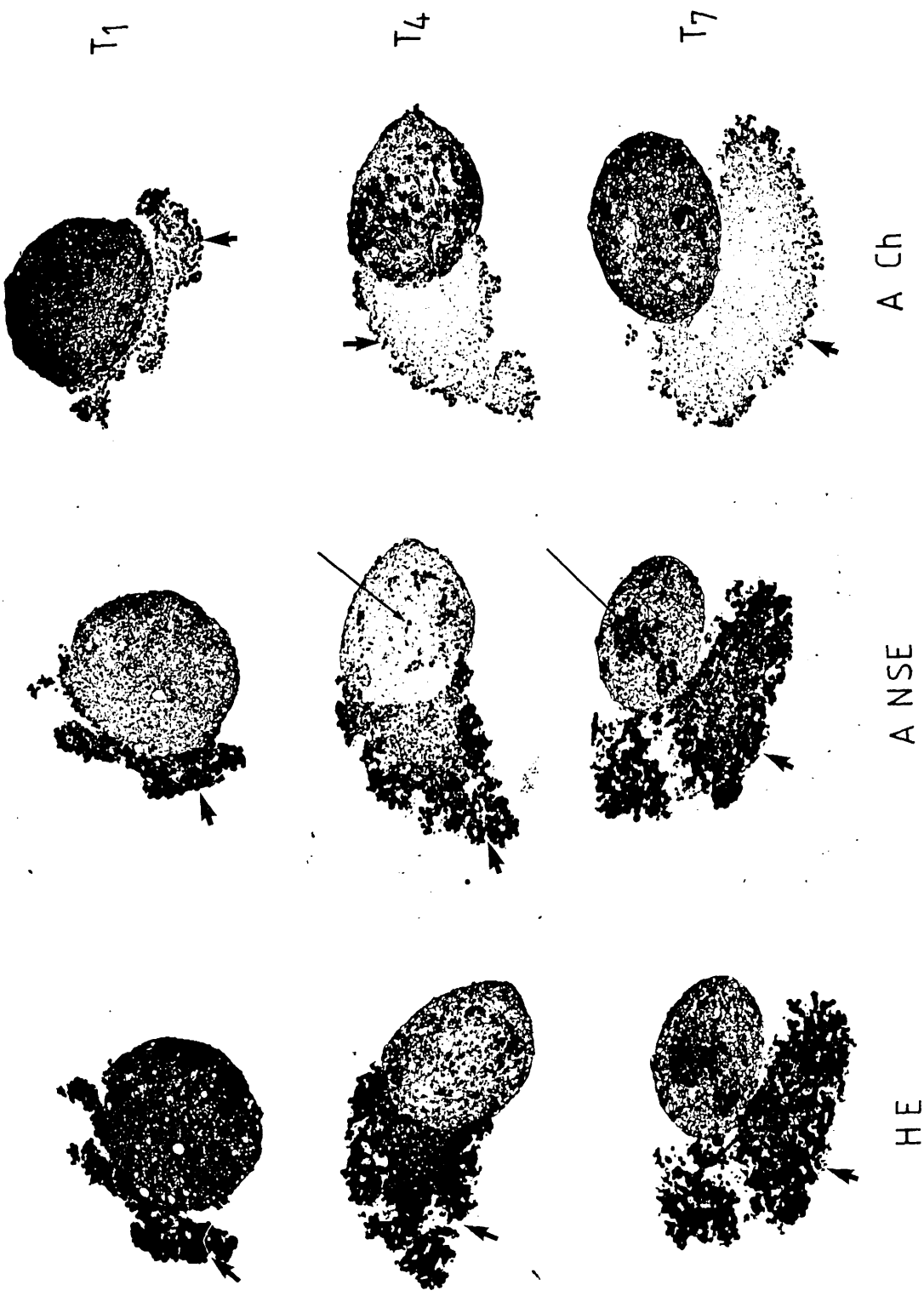
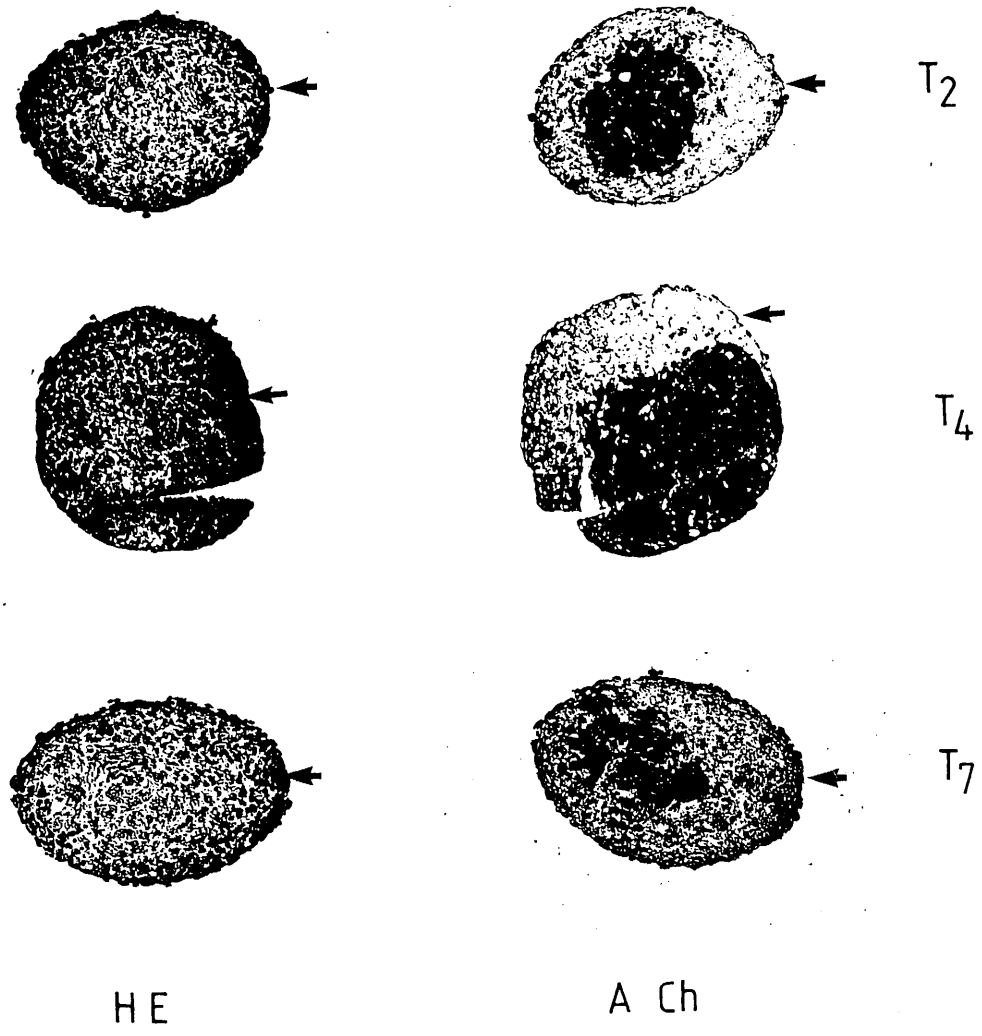
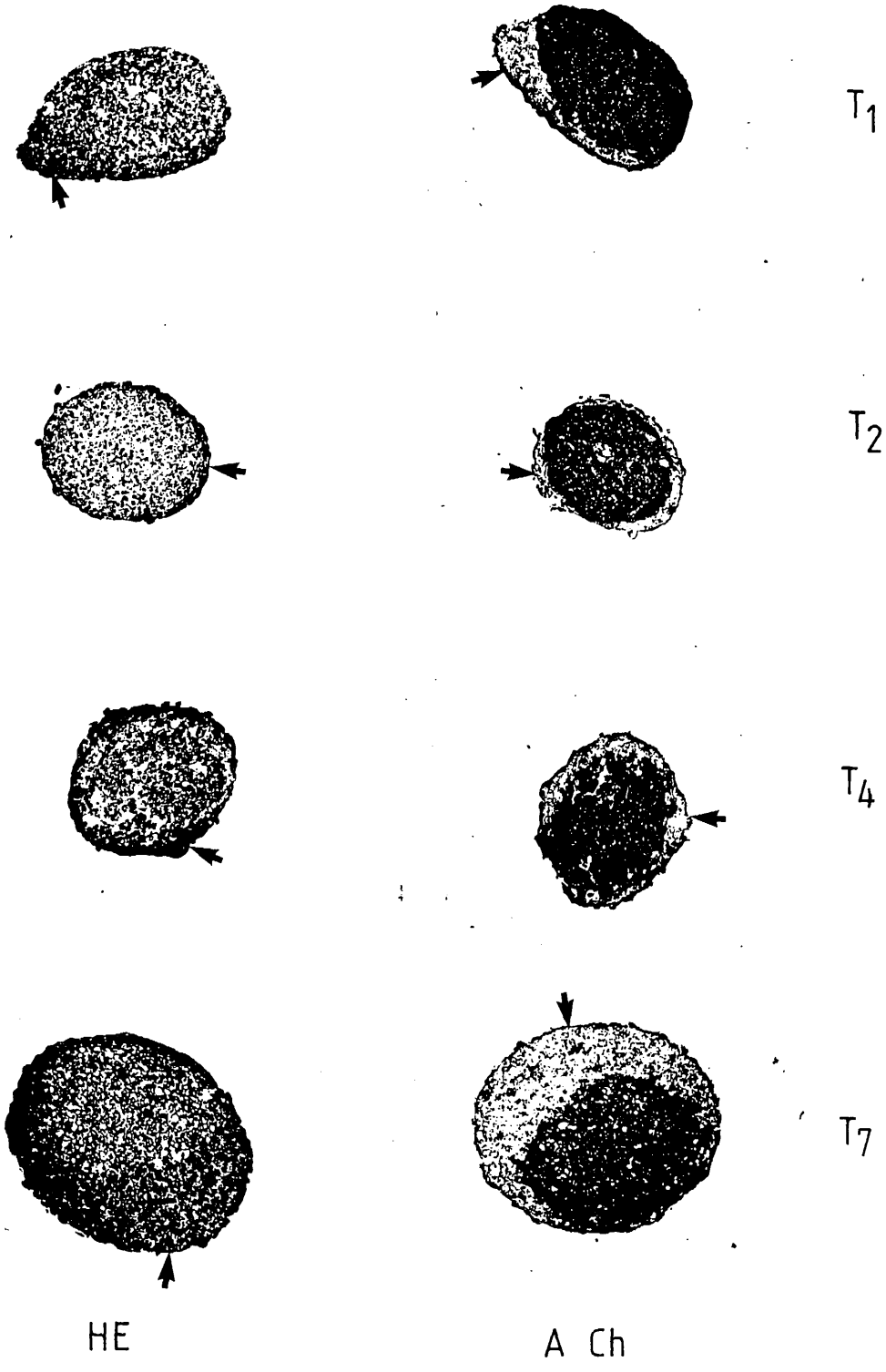



Plate 3A.7:- ***In vitro*** **invasion assay.**

(a). H69 cells co-cultured with chick heart show invasion within 48 hours. Tumour cells (short arrows) are identified in the centre of the heart fragment (long arrows), by haematoxylin & eosin (HE) staining, and confirmed by immunoperoxidase staining using anti-neuron specific enolase (A NSE), and anti-chick (A Ch) antibodies. Samples at day 1 (T1), 4 (T4), and 7 (T7), after co-culture are shown (X 250, H&E and IP).




(b). As (a) except that the H69V cells are identified by H&E and anti-chick (A Ch) antibody, at day 2 (T2), 4 (T4) and 7 (T7). The cells (arrows) grow outside the heart fragment, using it as substrate, but invasion of individual cells is not identified.




(c). As (a) except that the H69VZ cells are stained with H&E and anti-chick antibody, at day 1 (T1), 2 (T2), 4 (T4), and 7 (T7) after co-culture. The tumour cells (arrows) grow around the heart fragment as H69V cells, and single cell invasion is not identified.

day 7 [Plate 3A.7 a, T7]. Invasion was confirmed by both tumour cell specific antibody (anti-NSE), and anti-chick antibody. Cells did not completely replace the heart fragment by day 10. Tumour cell proliferation was only moderate inside the fragment.

H69V cells co-cultured with chick heart showed greater proliferation than H69 and engulfed the heart fragment with progressive destruction and replacement of heart tissue [Plate 3A.7 b]. In contrast to the parental line, single cell invasion was not found. H69VZ showed a similar growth pattern around the heart fragment to H69V [Plate 3A.7 c]. It appeared as if both the derivative lines used the heart fragment as a substrate; attached to it they grew fast, with consequent degradation of the confronting normal heart tissue.

### 3A.3.8 Effects of Stromal Interaction

H69 cells grown with fetal lung fibroblasts (EWLU) in a 50:50 ratio, showed formation of small colonies, both floating and attached, to the fibroblast layer. Cells could be seen growing out as a monolayer from the H69 colony and running parallel to the fibroblasts [Plate 3A.8].

### 3A.3.9 GROWTH STUDIES *IN VIVO*

*Tumorigenicity*

The parental, as well as its derivative lines, produced tumours in nude mice with 100% take rate [Table 3A.8]. The growth curves of tumours are shown in Figure 3A.5. The parental lines grew moderately after a latent period of approximately three weeks, with a doubling time of around 4 days. Tumours reached a volume of 2 $cm^3$ in 3 weeks. Derivative lines yielded exponentially growing tumours with a shorter latency period, and reached maximum size in a shorter time than the tumours produced by the parental line.

*Morphological Features of Xenografts*

Grossly, the parental tumour appeared as a small nodule, at the site of inoculation, and grew slowly with well defined edges, a smooth surface and prominent vasculature. There was no ulceration. The tumour was attached firmly to the surrounding host tissues, and was firm in consistency. On the cut surface there was no evidence of necrotic tissue. The tumour showed evidence of gross

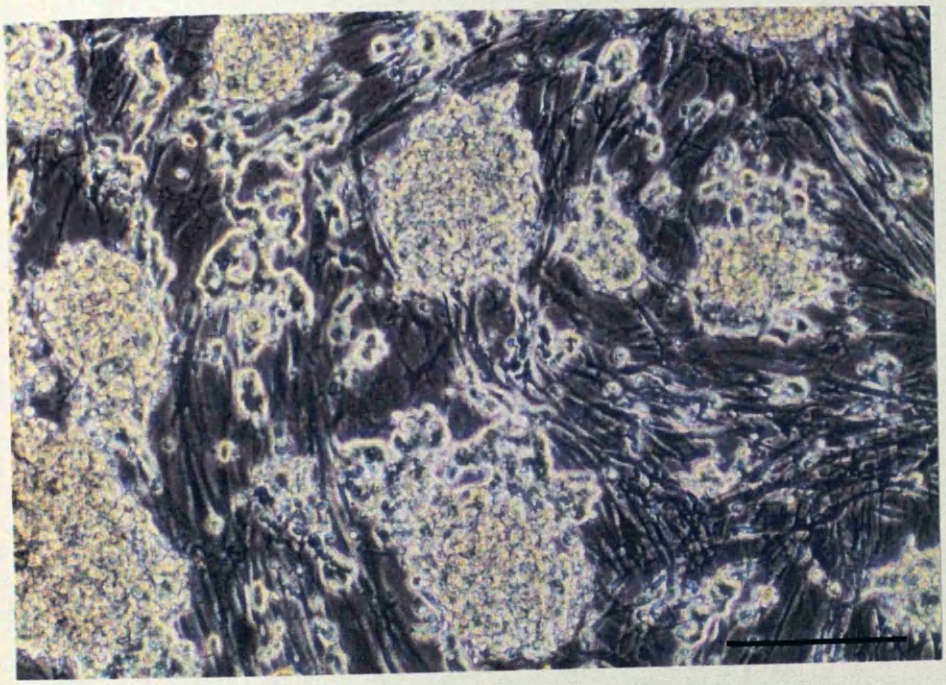


Plate 3A.8:- **Effect of stromal interaction on the morphology of H69 cells.**

H69 cells grown in 50:50 ratio with EWLU cells in 25 $cm^2$ flasks, at $10^4$ cell/ml concentration, in RPMI1640 medium, at day 10 after subculture. The cells form small sized aggregates, firmly attached on top of the fibroblast layer. Cells can also be seen growing out from aggregates, apparently mixing with the fibroblasts (x 10 objective, phase-contrast, bar= 200 $\mu$m).

**Table 3A.8:- In vivo growth characteristics.**

| Cell line | [1]Tumorigenicity | [2]Latent period (days) | Duration of observation (days) | Tumour bearing time (days) | Tumour doubling time (days) | Maximum tumour volume ($cm^3$) |
|---|---|---|---|---|---|---|
| H69 | 8/8 | 20 ± 3 | 43 ± 1 | 23 ± 1 | 4.0 ± 0.2 | 2.1 ± 0.4 |
| H69V | 8/8 | *14 ± 1 | 43 ± 1 | 29 ± 1 | 3.7 ± 0.1 | **9.7± 0.8 |
| H69VZ | 8/8 | *13 ± 1 | 43 ± 1 | 30 ± 1 | 3.4 ± 0.4 | **7.6± 0.6 |

[1]Number of tumours produced/Number of animals inoculated. [2]Time between injection of cells into the animal and appearance of tumour at the site of injection, with an approximate volume of >33 $mm^3$. Data are mean ± SEM of at least 7 values, from at least 2 independent experiments.

*$P<0.008$, **$P<0.001$ (Mann-Whitney test, and Bonferoni adjustment).

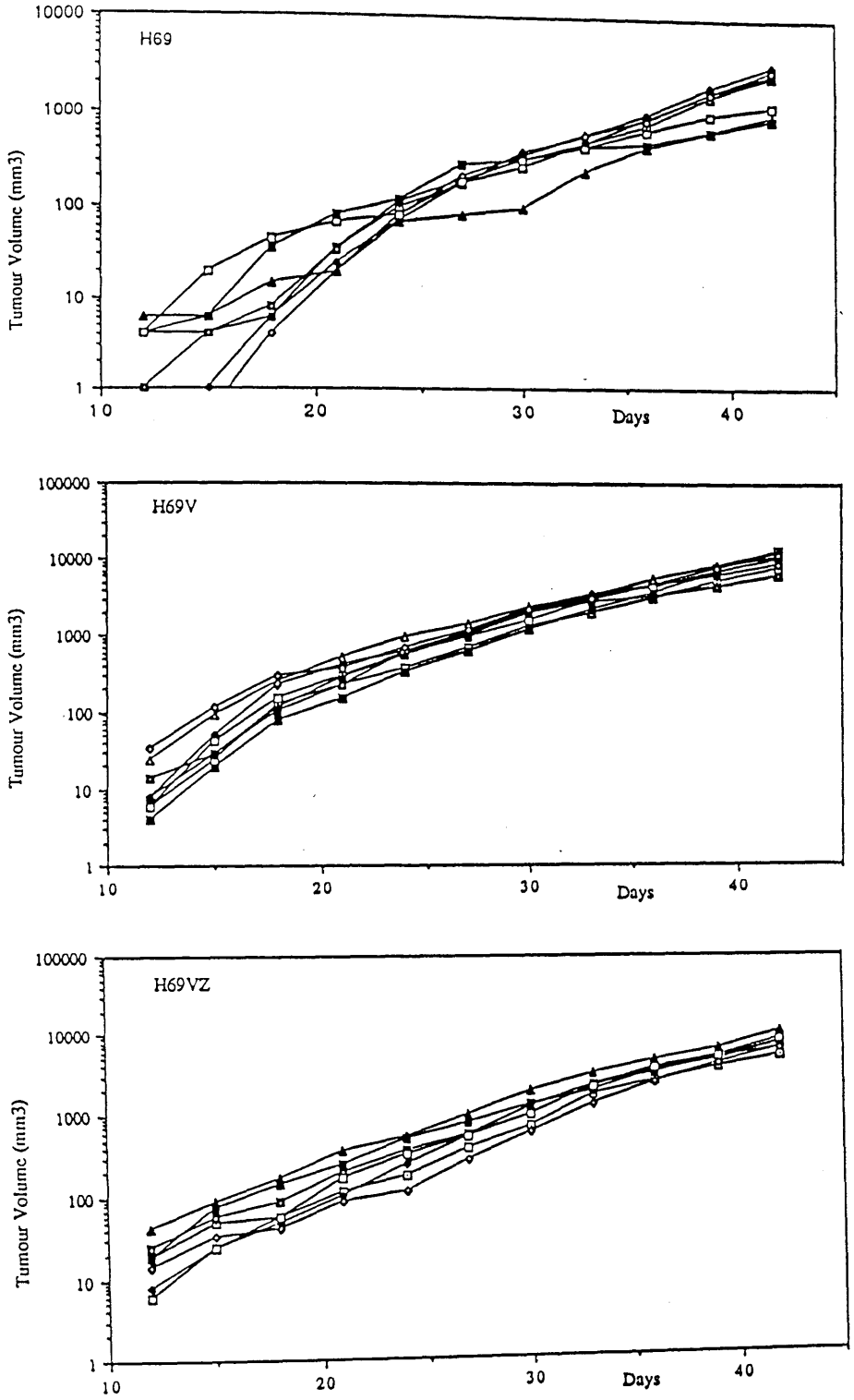



**Figure 3A.5:- Growth curves of xenografts derived from H69 and its derivative lines in nude mice.**

Each mouse received a s.c innoculation of $1x10^7$ cells in 0.5 ml PBS on day 0. Each line represents tumour growth in an individual mouse.

infiltration into the surrounding tissues. Microscopically, the tumour was anaplastic [Plate 3A.9 a], with a high incidence of abnormal mitotic and apoptotic figures [Table 3A.9].

Tumours produced by the derivative lines appeared as small nodules at the site of inoculation, with a shorter latency period. Growth was gradual, and tumours showed circumscribed margins. They were not firmly attached to the surrounding host tissues. There was no ulceration, and vasculature was not as prominent as in the parental tumour. Tumours appeared as isolated nodules during dissection, and the cut surface did not show gross evidence of necrosis. Microscopically, tumours of both H69V and H69VZ [Plate 3A.9 b] were poorly differentiated, pleomorphic, and showed moderate numbers of mitotic and apoptotic bodies. Tumours also showed focal areas of large spindle shaped cells, and spaces filled with amorphous eosinophilic material that stained positive with PAS/alcian blue [Plate 3A.10]. These features were not found in the tumours of the parental cell line. These findings were confirmed by more than one pathologist, independantly in blind fashion.

### 3A.3.10 Invasion and Metastasis *in Vivo*

The parental cell line produced invasive tumours. Local tumour cell infiltration was found in skin and its appendages, subcutaneous fat, and deep into muscles [Plate 3A.11]. Both the derivative lines showed no evidence of invasion into deep structures, although, occasional fat infiltration was found.

Metastases were found in lungs [Plate 3A.12 a] and in liver [Plate 3A.12 b] of all the animals, bearing H69 tumour. Metastases were not found either in lungs or livers of animals with tumours produced by the derivative lines.

### 3A.3.11 Ultrastructural Studies

While the parental line showed clusters of dense core granules of approximately the same size, in the cell cytoplasm [Plate 3A.13 a], they were absent from both the derivative lines [Plate 3A.13 b] and the NSCLC cell line [see below, Plate 4.4]. Other ultrastructural features such as desmosomes, intermediate filaments, tonofilaments, were also found in all the cell lines, which were consistent with their epithelial origin.

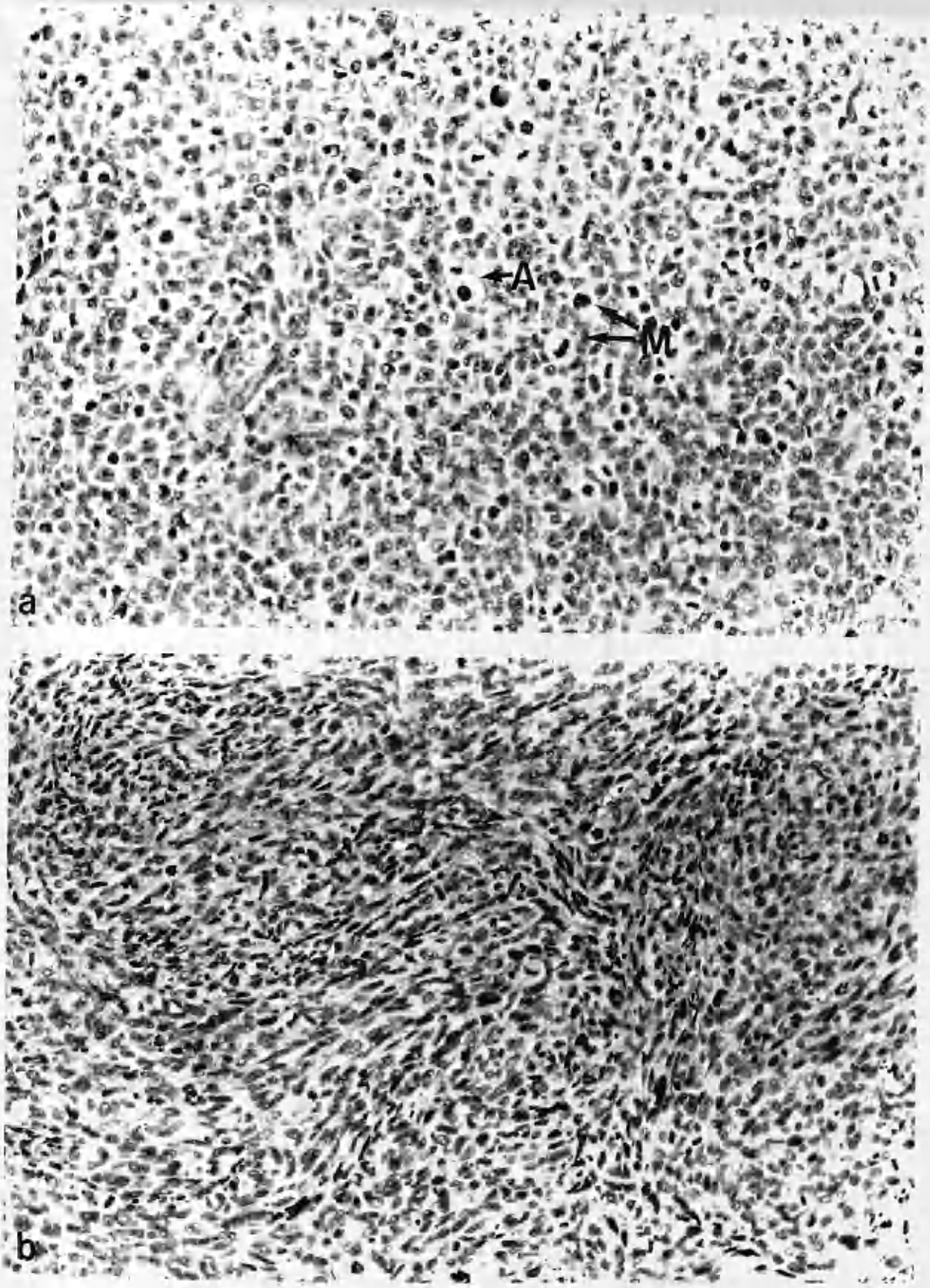



Plate 3A.9:- **Histology of xenografts.**

(a). H69, showing pleomorphic, anaplastic tumour with abundant mitoses (M) and apoptotic (A) figures (X 247, H&E). (b). H69VZ, showing poorly differentiated tumour, with a more spindle cell morphology than H69. There is cellular and nuclear pleomorphism, but fewer mitoses and apoptotic figures (X 247, H&E).

**Table 3A.9:- Invasion and metastasis.**

| Cell line | In Vitro invasion | In Vivo invasion | [1]Metastasis | [2]Mitotic index |
|---|---|---|---|---|
| H69 | + | + | ++ | 15.4 ± 0.04 |
| H69V | +/- | +/- | NF | *7.7 ± 0.02 |
| H69VZ | +/- | +/- | NF | *7.8 ± 0.03 |

[1]To liver and lungs. NF (not found). [2]For each tumour the mean of mitotic figures in 12 high power fields (X 40) per slide were determined from at least 6 slides of tumours of each cell line, grown in more than one experiments.

*$P<0.0001$ (analysis of variance, and Bonferoni adjustment).

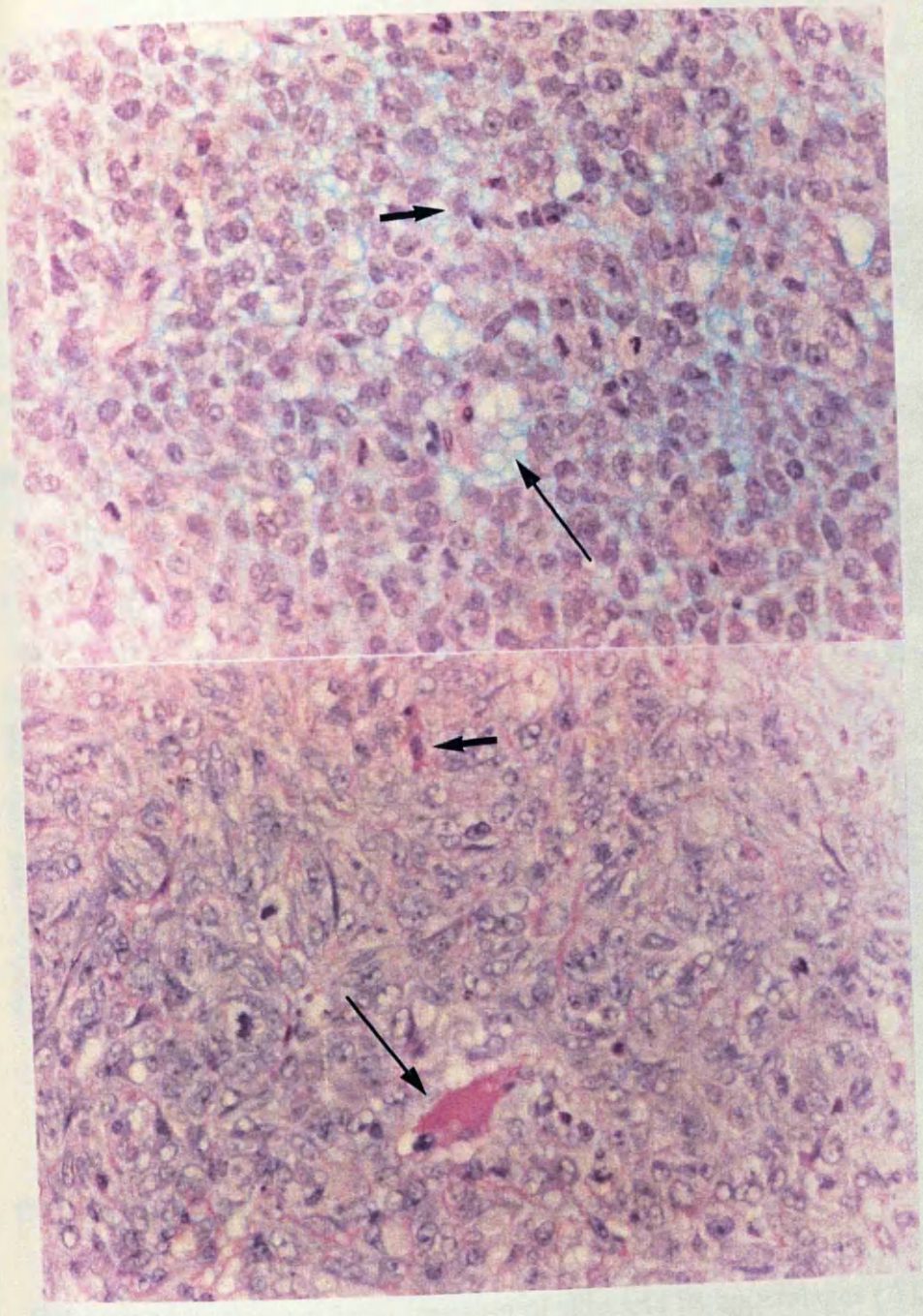


Plate 3A.10:- **Demonstration of mucin in H69VZ tumours.**

Tumour derived from H69VZ cells shows positivity for both acid (blue) and neutral (pink) mucins. Staining is seen in spaces between cells (long arrows) and also within individual cells (short arrows) (x 280, PAS-diastase/Alcian blue).

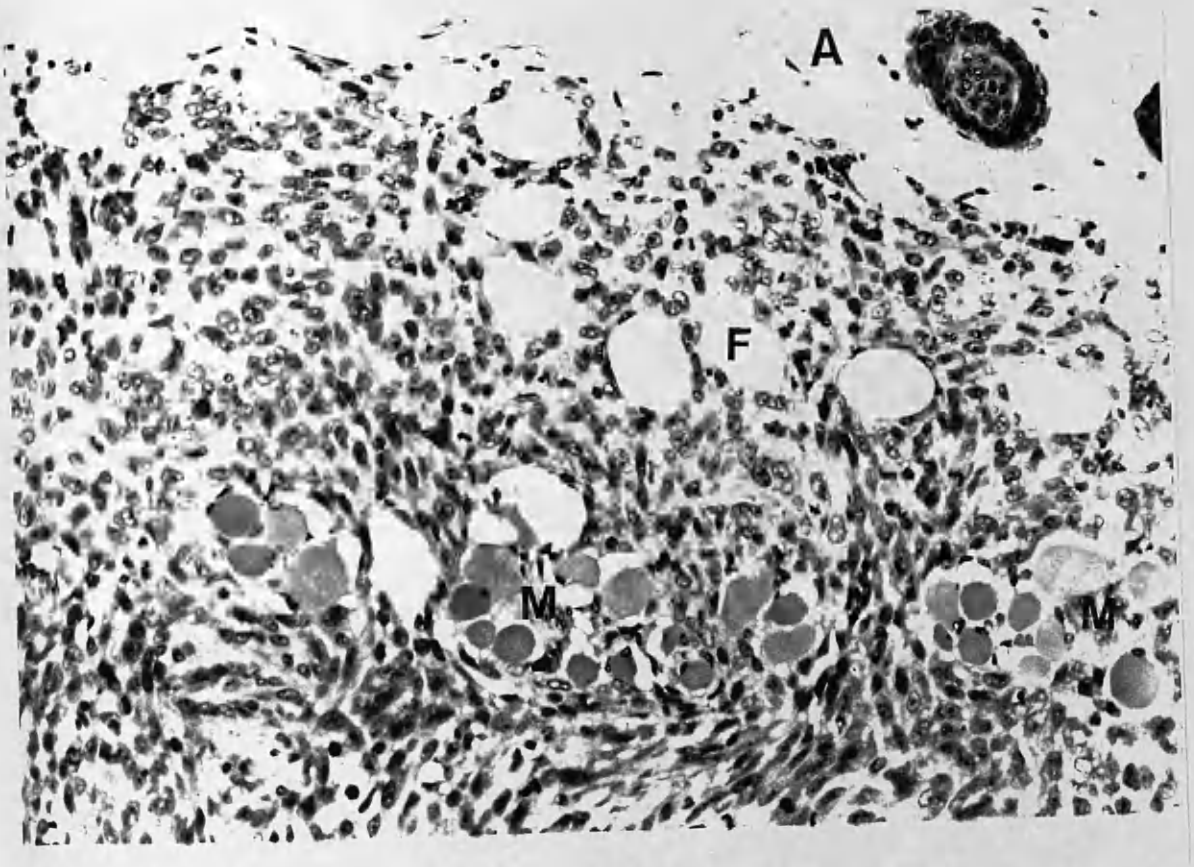



Plate 3A.11:-**Local invasion *in vivo* by H69 tumour cells.**

H69 xenografts showed local invasion of skin and its appendages (A), subcutaneous fat (F), and muscles (M), with evidence of tumour cell proliferation and destruction of the invaded host tissues (X 247, H&E).

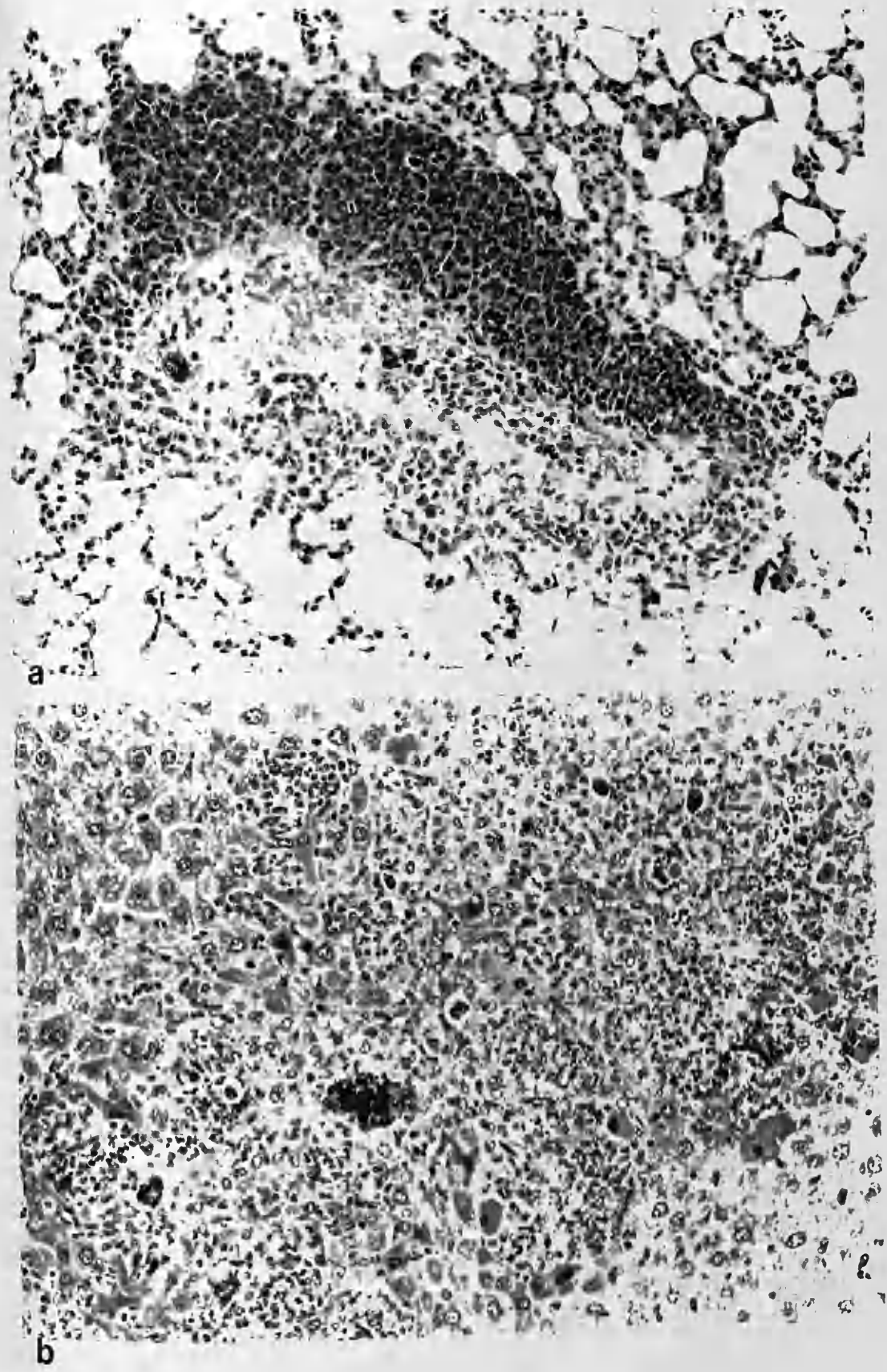



Plate 3A.12:- **Metastases of H69 tumour.**

Tumour derived from H69 cells shows metastases both to lung (a) and liver (b). (X 247, H&E).

**Plate 3A.13:- Electronmicrographs of the H69 and the H69VZ tumours.**

(a) Clusters of dense core neurosecretory granules (long arrow) can be identified in the cytoplasm of the H69 cell. Cell-cell relationships and the presence of desmosomes (short arrows) are typical of epithelial differentiation (Uranyl acetate, and lead citrate stain; X 24500).

(b) H69VZ cell line, showed no evidence of specific neurosecretory granules, as illustrated in these three adjacent cells. The cells showed features of epithelial differentiation as above (Uranyl acetate, and lead citrate stain; X 24500).

a
b

### 3A.3.12 Xenograft Immunohistochemistry

Parental tumours stained positive for neuroendocrine markers, used in culture cell lines, however, the staining was not intense compared to culture cells. The derivative lines stained focally, as did their cells in culture (see above).

### 3A.3.13 Sensitivity to The Cytotoxic Drugs

The parental line showed approximately 4-fold higher $IC_{50}$ values to adriamycin compared to its both derivative lines [Table 3A.10]. Both the derivative and NSCLC cell lines were more sensitive to the drug. The parental line showed around 3-fold lower $IC_{50}$ values for vincristine, compared to its sublines and NSCLC cell lines.

### 3A.3.14 Radiation Sensitivity

The parental cell line was significantly more sensitive (2-fold) compared to both its sublines [Figure 3A.6] and NSCLC cell lines [Table 3A.11].

### 3A.3.15 Myc Oncogene Expression

The c-myc expression was found in both H69 and the derivative cell lines and also the tumours produced by them, by immunohistochemical methods using monoclonal antibody 9E10. Staining was focal in parental line (5-10%), while the derivative lines showed more generalised and intense staining (25-30%), both cytoplasmic and nuclear [Plate 3A.14].

## 3A.4 DISCUSSION

The objective of this study was to establish whether the parental cell line (SCLC) and its derivative cell lines could be a valid model for the phenotypic transition in SCLC, *in vivo*. These data have shown that while the parental line expressed features of a classic SCLC, both the derivative cell lines had features similar to NSCLC, suggesting this was a potentially interesting model of *in vivo* tumour progression.

There are a number of possibilities for the origin of adherent cells in the parental cell line: 1) Cross contamination. 2) Polyclonal carcinogenesis. 3)

**Table 3A.10:- Sensitivity of the cell lines to cytotoxic drugs.**

| Cell line | Adriamycin $ID_{50}$ ($\times 10^{-8}$M) | Vincristine $ID_{50}$ ($\times 10^{-10}$M) |
|---|---|---|
| H69 | *14.0 ± 4.0 | **1.6 ± 0.7 |
| H69V | 1.8 ± 0.3 | 5.5 ± 0.5 |
| H69VZ | 4.0 ± 0.8 | 3.5 ± 0.6 |

For non-adherent cells, $10^4$ cells in 100 ul medium were plated per well in 96 well, round bottom plates, while $5\times10^3$cells were plated per well in 200 ul medium for adherent cells. Drug was added to non-adherent cells after 3 hours incubation at 37 $^{o}$C in a volume of 100 ul at 2x final concentration. In adherent cultures drug was added after 24 hours at final concentration in culture medium. Cells were exposed to drug for 48 hours, and fresh drug was added after 24 hours. A recovery period of 3 days was allowed following drug removal.

Results are Mean ± SEM, from 4 experiments.

*P<0.02, **P<0.05 (Mann-Whitney test, with Bonferoni adjustment).

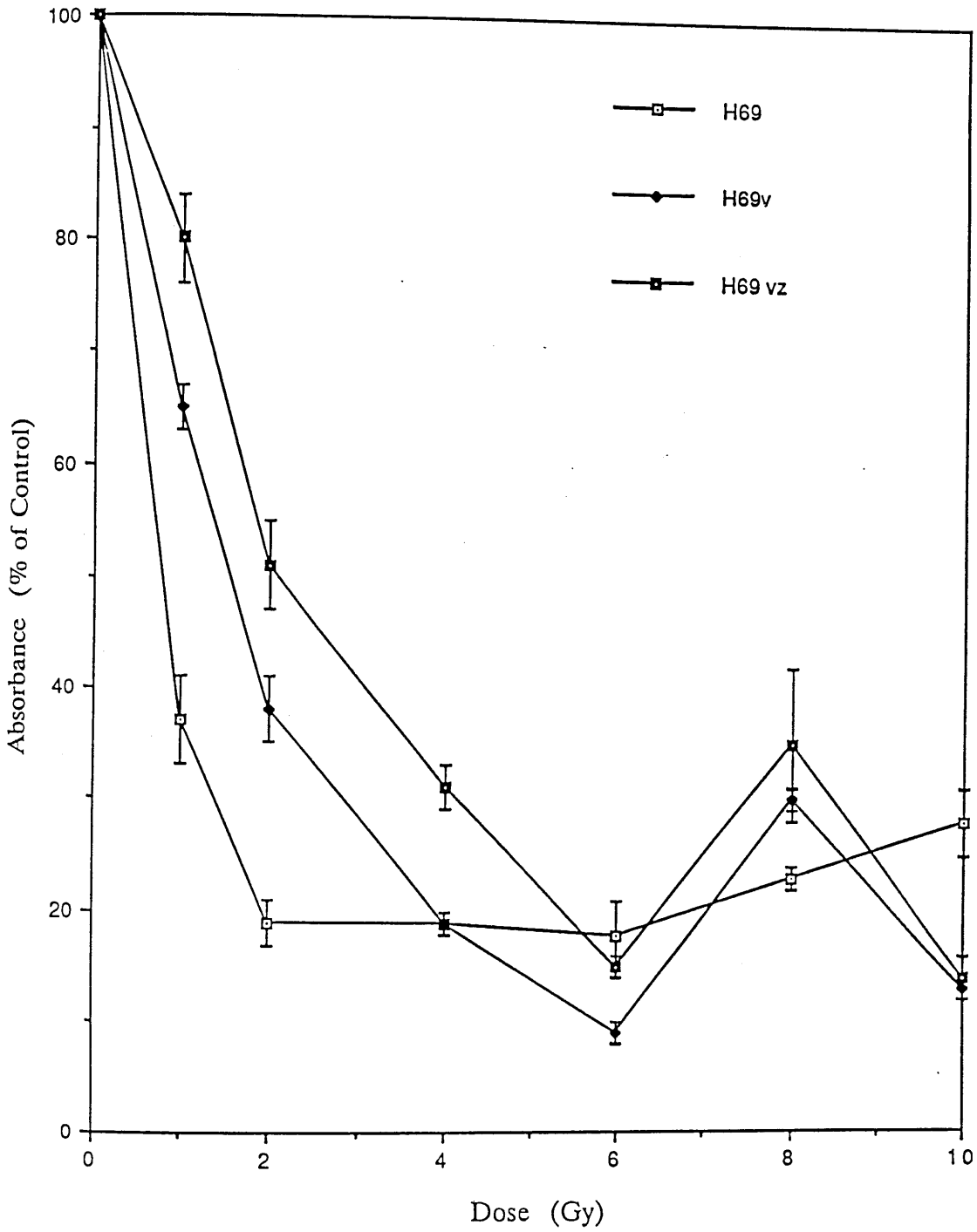



**Figure 3A.6:- Radiation survival curves of the cell lines.**

The cells were grown in 96 well plates. Exponentially growing cultures were exposed to increasing doses of radiation, and allowed to recover for at least three cell generations. Cell survival was determined by MTT assay. Results are from one experiment similar results were obtained in a second experiment. Each point represents mean ± SEM (bars).

**Table 3A.11:- Radiation sensitivity of the cell lines.**

| Cell line | $ID_{50}$ (cGy) |
|---|---|
| H69 | 96.3 ± 2.4 |
| H69V | *157.5 ± 4.8 |
| H69VZ | *198.8 ± 4.3 |
| WIL | *182.5 ± 3.3 |

Cells were plated as in drug sensitivity assays (see above, Table 3.11). A recovery period was allowed for the equivalent of 2-3 cell doublings. Data are mean ± SEM from two experiments.

*$P<0.05$ (Mann-Whitney test).

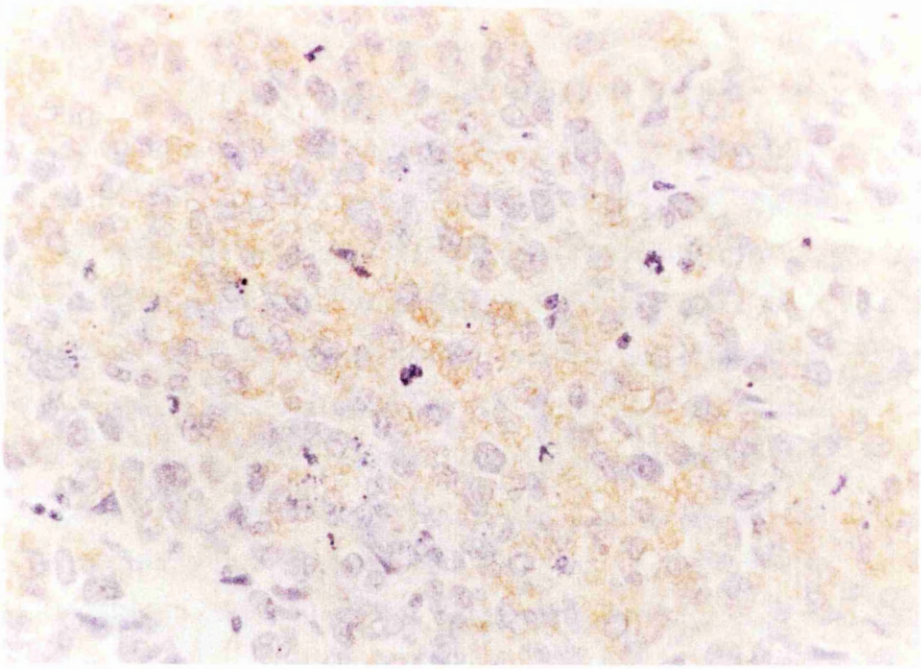


Plate 3A.14:- **The c-myc expression in H69VZ cell line.**

Immunoperoxidase staining for c-myc protein product is present in many cells. While the majority show cytoplasmic staining, a few also show nuclear positivity (x 360, Avidin biotin method; 9E10 antibody (anti-c-myc protein).

Interconversion of SCLC into NSCLC. 4) Existence of a pluripotent stem cell subpopulation in the parental cell line.

Since the parental cell line was established from a pleural effusion of a patient with SCLC, it could be argued that the line was contaminated with other cells such as those of mesothelial origin, during establishment of the line, or the established line could have been cross contaminated in the laboratory. It was possible to rule out accidental interspecies cross-contamination by LDH-isoenzyme and cytogenetic analysis. Both the parental and the derivative cell lines showed typical human specific LDH isoenzyme bands. The chromosome number showed that cell lines were aneuploid and heteroploid. The karyotypic analysis of derivative line (H69V) indicated that it was derived from H69 [personal communication, Dr Jeffery M. Trent, Arizona Cancer Centre, The University of Arizona via Dr Stephen Merry]. The presence of a Y chromosome in the derivative line H69V supported a common origin of both the parental and the derivative lines, since the parental cell line was established from a male patient [Carney *et al.*, 1985].

H69 clones isolated and expanded to mass culture, showed the morphological heterogeneity found in the parental line suggesting the existence of a pluripotent stem cell subpopulation in the parental line, as shown by clonogenic assay (5-7% of total cell population). It was however not possible in the time to characterize these clones fully, or expand the adherent cell lines to examine whether they behaved like H69V and H69VZ. However, the preliminary observations indicated similar behaviour and therefore favoured a common origin of the parental and the derivative cell lines. In addition, all lines expressed cytokeratin intermediate filaments, and adhesion specialisations such as desmosomes, suggesting a link between parental and the derivative lines. From these data it can be concluded that both H69 and its derivative lines share a common origin, probably from a stem cell sub-population in the parental cell line. Further confirmation of a common origin of the lines could be carried out by fingerprinting, and isoenzyme analysis.

The presence of a heterogeneous stem cell population in established SCLC cell lines has been demonstrated by Olsson *et al.* [1984], with sublines varying in tumorigenicity in nude mice, colony forming efficiency, reactivity with monoclonal antibodies, and radiosensitivity. Others, however, did not find significant differences in these properties among sublines of established SCLC cell lines [Morstyn *et al.*, 1983; Gazdar *et al.*, 1985]. In the present study characterization *in vitro* and *in vivo*

of cell lines derived from a classic SCLC cell line has been described, with a view to establishing a model of phenotypic transition in SCLC, and its implications for *in vivo* tumour progression. The cell lines have been characterized morphologically, for growth characteristics, neuroendocrine marker expression, invasion and metastasis, clonogenicity in soft agar, tumorigenicity in nude mice, chemosensitivity and radiation sensitivity, and myc oncogene expression.

Morphological differences between the parental and the derivative cell lines were apparent in living culture, cytology, and xenograft histology. Significant morphological differences have been found in culture between H69 and the derivative lines. While the parental line grew as densely packed free floating aggregates, the sublines grew as monolayers of epithelioid cells. Though not identical, morphologies of the derivative lines were similar to those of NSCLC cell lines. This morphological change was one of the most obvious differences between the parental and the derivative cell lines. As the culture conditions were identical, the differences must be intrinsic to the cells.

Altered cell surface configuration may lead to an altered interaction of cells with their microenvironment. Each cell is thought to have a particular combination of cell-surface molecules that enables it to bind in its own characteristic way to other cells and to the extracellular matrix. It is possible that H69 cells may have increased cell-cell adhesion molecules, while the derivative cells may have an increased number of extracellular matrix receptors e.g a decreased expression of N-CAM, and an increased expression of integrins. Gazdar *et al.* [1981] have shown a change in morphological appearance of cells from SCLC to large cell morphology. Similar changes have been found in SCLC cell lines *in vitro* by Goodwin and Baylin [1981]. The change in SCLC into large cell type was associated with an increase in growth rate and cloning efficiency. There was also an accompanying loss of some of the NE-cells properties, including L-dopa decarboxylase, and neurosecretary granules. In another study Goodwin and Baylin [1982] established a cell line OH-1 from a patient with SCLC. After 16 months in culture the cells changed into a large cell phenotype with loss of NSG, DDC, and emergence of radiation resistance, suggesting a link between loss of neuroendocrine markers, acquisition of a NSCLC type morphology, and the resistant phenotype as commonly found in clinical studies of SCLC. The phenomenon of transition from SCLC to other lung cancer types has also been observed by Watanabe *et al.* [1988]. They have established a cell line

SCLC-MO, which expressed phenotypic features of transitional cell type, between a classic and variant, such as low levels of DDC, high levels of NSE and CKBB, and a classic SCLC morphology, and an intermediate cell type histology in xenograft. After 9 months in culture this cell line showed some adherent cells. A continuous monolayer was established from these adherent cells after about an additional 8 months in culture, two sub-lines (MOA1, MOA2) were established from this monolayer with different antigenic properties. The MOA1 cell line was characteristic of variant SCLC, it lacked DDC but expressed NSE and CKBB, grew as a monolayer, with small cell type, and gave rise tumours with an intermediate cell type histology, while the MOA2 cell line expressed features characteristic of NSCLC as it expressed very low DDC activity, lacked NSE, CKBB, and grew as a monolayer with large cell type morphology, and formed tumours in nude mice with large cell type histology. The further characterisation of these lines for antigenic markers and chromosomal analysis confirmed the origin of clones from one parental cell type. Bepler *et al.* [1987] established two cell line (SCLC-H21, & SCLC-H22) from different metastatic sites of a patient with histopathologically proven SCLC of oat cell type. Following *in vitro* characterization of lines it was concluded that the cell lines showed a transition from pure SCLC to large cell phenotype, suggesting a link between SCLC and other lung cancer types.

The cytology of cytospin preparations of the cell lines in this study showed that both the derivative cell lines had large nuclei with evenly distributed granular chromatin, inconspicuous nucleoli, moderate amount of cytoplasm, and a lower nucleo-cytoplasmic ratio, features similar to NSCLC cells, while the parental line revealed small cell characteristics with scanty cytoplasm, and inconspicuous nucleoli. The tumours produced by both the derivative lines showed NSCLC type features e.g. mucin production, more spindle and large cell histology and lower mitotic figures. These features were absent from the parental tumour, suggesting a transformation from SCLC towards NSCLC morphology.

Morphological changes were also observed following co-culture of H69 with embryonic fibroblasts. The purpose was to investigate whether any change in H69 cells could be induced by the stromal environment. It has been shown that stroma can exert its effects on tumour cells both by physical as well as by chemical interactions [Post & Smith, 1984]. Embryonic fibroblasts were used because of the potential relevance between tumour phenotype (undifferentiated or quasi-embryonic)

and embryonic stroma. Secondly, although adult stroma may have some effects on the tumour cell phenotype and could have been worth pursuing, all tumours contain adult stroma which is apparently unable to control differentiation in the tumour. H69 cells co-cultured with fibroblasts showed a phenotypic shift, as they attained a more epithelioid and spindle morphology. It was not possible in the time, to take this work any further, however, it would be interesting to isolate the morphological variants following stromal interaction, to compare with H69V and H69VZ, and establish whether similar or different phenotypic changes are induced in H69 cells by the different microenvironment.

The growth of derivative cell lines in monolayer culture was fast compared to the parental line, with doubling times around 35 hours, similar to NSCLC lines. Similarly, lower serum requirements and higher cloning efficiencies of derivative lines and the NSCLC lines were in approximately the same range, compared to the parental line. While the growth of SCLC is rapid in patients compared to NSCLC, the reverse is true in culture. It is possible that this difference is due to alterations in expression or distribution of cell surface receptors. Adherent cells may have greater access to the medium compared to cells in tight aggregates, but it is also possible that a different type of autocrine control is expressed in the derivative lines such as an alteration in the production of autocrine growth factors; e.g a decrease in bombesin or an increase in TGF-$\alpha$ or IGF-1. Furthermore with an altered surface morphology two cells may behave differently under similar growth conditions, where one cell can utilize a certain growth factor present in growth medium but another cell cannot due perhaps to the expression of, or access to, different receptors. Further investigations are required to analyze this difference, by looking at synthesis of growth factors and the expression and distribution of growth factor receptors.

The derivative lines expressed very low levels of NE-cell markers compared to the parental line. The DDC activity was 7-9 fold lower in the derivative lines while it was 10-14 fold lower in the NSCLC cell lines, compared to the H69 cell line. CK-BB activity was undetectable, in both derivative and NSCLC lines, while the parental line expressed high levels of enzyme. Previously it has been shown that morphological variants expressed CK-BB, though other features of APUD-cells were lost [Gazdar *et al.*, 1985], but in present study the morphological changes were accompanied with gross reduction or loss of NE-cell markers, including total absence

of CK-BB. This was a major difference in the present study compared to the previous observations. Continued expression of high levels of CK-BB after morphological change is the basis of classification of SCLC cell lines into classic and variant [Carney *et al.* 1985; Gazdar *et al.*, 1985] indicating that H69V & H69VZ do not fit into the variant category. This phenotypic shift is more NSCLC-like rather than a variant type, in agreement with previous observation by Watanabe et al. [1988], they have isolated a cell line MOA2 from a variant SCLC cell line and MOA2 cell line had all features of NSCLC, it lacked NSE and CK-BB, expressed only minimal levels of DDC, grew as monolayer with large cell morphology, and formed tumours in nude mice with large cell histology. It could be that the phenotypic changes are gradual, from stem cell to differentiated tumour cell, and the studies so far have only dissected individual links of this continuous spectrum.

SCLC cell lines have been found to contain higher levels of BLI than other lung cancers [Moody *et al.*, 1981]. While H69 expressed BLI, both the derivative lines and the non-small cell lines expressed only minimal activity. Carney *et al.* [1982] have shown that high levels of NSE are expressed by SCLC cell lines. While significant levels of NSE were found in the parental line in agreement with previous observations [Gazdar *et al.*, 1985], the derivative lines had significantly lower levels with around 12-fold difference from the parental line. NSCLC cell lines had only minimal activity. Previously it has been shown that a change in SCLC from non-adherent to adherent morphology was accompanied with a loss of neuroendocrine markers except CK-BB and NSE [Gazdar *et al.*, 1985]. However, in contrast to the previous observations, it has been found in the present study that both CK-BB and NSE were either grossly reduced or lost in the derivative line.

The parental line stained positively for immunohistochemical markers including NSE, BLI, and chromogranin A. Both sublines and NSCLC cell lines stained only focally with NSE, and staining was absent for BLI and chromogranin A. Thus both derivative lines showed either reduced or complete loss of NE-cell markers as determined either by immunohistochemical or biochemical techniques.

Neurosecretory type dense core granules in SCLC cells have been shown by others [Bensch *et al.*, 1968; Sidhu , 1982]. It has been suggested that they are the only criterion that can differentiate SCLC from NSCLC with certainty [Dingemans & Mooi, 1984; Mooi & Dingemans, 1986]. A good correlation was found in the present study for the expression of different markers biochemically,

immunohistochemically and ultrastructurally. The parental line expressed all markers and also showed increased numbers of DCG, while the derivative lines either expressed only low levels of markers or did not express them at all and had only occasional single neurosecretory type DCG. NSCLC lines did not show any significant marker expression and had no DCG at all.

Marker studies indicated that both the derivative cell lines express features different from H69, but similar to NSCLC cell lines. Therefore, the change in phenotype of the derivative lines is not of variant type, but a NSCLC type. Though NE-cell marker data have shown that both the derivative lines had significantly reduced levels compared to the parental line, there is an indication that the loss is not as complete in some of the markers (DDC, BLI, NSE), as seen in NSCLC lines. This may be due to the fact that all or some of the cells are still in the process of transition and are not fully transformed to NSCLC, and are still expressing some of the properties of the parental line. Some heterogeneity has been found in immunocytochemical staining of the derivative cells. These data indicate that further studies are required to investigate whether, with continued culture, the derivative lines would lose the marker expression completely or keep expressing markers at a low level. It would also be interesting to see whether expression of markers could be modulated by experimental manipulation e.g chemical induction with differentiation inducing agents, as will be discussed with H69 alone in the following chapter.

Though *in vitro* invasion was found in all cells lines, the pattern and the degree was different between H69 and the sublines. While the parental line grew more slowly attached to heart fragments, it was significantly more invasive, and solitary invasion was found from different fronts as observed by histological and immunohistochemical staining of the invading cells. Both the derivative cell lines showed proliferation around the heart fragment and engulfed it from all sides. It seemed as if tumour cells used the heart fragment as a substrate, and proliferated attached to it, and the replacement of the heart fragment was secondary to their local growth. It is also consistent with the preferential growth of the derivative cells in monolayer. It may be that invasion is a selective or programmed event in SCLC, where an individual cell is capable of invading without any apparent degradation of the invaded tissue. These findings suggest that a single SCLC cell may have had all the necessary mechanisms involved in invasion and metastasis, whereas invasion and

metastasis may be the events secondary to local degradation of tumour and host tissue in case of NSCLC. It may reflect the clinical behaviour of SCLC and NSCLC tumours, since most of the SCLC show early metastatic spread [Matthews *et al.*, 1973], compared to NSCLC.

The derivative lines were more proliferative in culture, but they were less invasive. Although tumour growth and cell proliferation are associated with invasion there is evidence that invasion is not dependent on cell proliferation *in vitro* [Mareel *et al.*, 1982, Thorgeirsson *et al.*, 1984] and *in vivo* [Disttelmans *et al.*, 1985]. Drugs such as 5-Flourouracil (5-FU) that completely inhibited cell proliferation did not block invasion [Bracke *et al.*, 1984, Storme *et al.*, 1985]. The present data tend to support these findings and suggest that invasion is not controlled by the rate of cell proliferation.

All lines were tumorigenic in nude mice. The parental tumours were more irregular in outline, had a higher mitotic rate and an increased single cell death, which might have been responsible for a slightly smaller final tumour volume in spite of a relatively high mitotic rate. There was macroscopic evidence of invasion in the parental line, but the sublines were better demarcated and had less infiltration into the surrounding tissues. H69 tumours invaded skin, subcutaneous tissues, and deep structures, e.g muscles, while tumour cell infiltration was only found in subcutaneous fat in the case of the derivative cell lines. These features were consistent with the metastatic behaviour of the cell lines, with the parental cell line tumours producing metastatic deposits both in liver and lungs, while metastasis was not found in these organs from animals bearing tumours of the derivative lines. These data suggest that the parental line is both invasive and metastatic, while the derivative lines are mildly invasive, but non-metastatic under these conditions.

It may be possible that tumours were removed earlier than their required period for metastasis, as NSCLC are metastatic, but at relatively later stages of disease, whereas SCLC is metastatic from early stage disease. The data from both *in vitro* and *in vivo* invasion showed good correlation.

It is possible that the interplay of matrix adhesion receptors and cell adhesion molecules suggested as having a role in the altered growth characteristics in culture, are also related to changes in metastatic behaviour. A SCLC cell may be programmed for reduced homotypic adhesions at the primary site, but increased in the circulation, and increased heterotypic adhesions at secondary site. This can be

possible simply by expression and deletion of cell receptors or ligands for matrix receptors. Homotypic adhesion is necessary for tumour cells to break in lumps or aggregates and to survive the long journey in the blood or lymphatic channels, and also to help their arrest in a capillary embolism. Heterotypic adhesion is essential for tumour cell-stromal interactions at different levels of invasion and at metastatic sites. Homotypic adhesion may be reduced, but heterotypic adhesion may be increased in metastatic tumour cells.

The parental cells formed bizarre colonies in agar with irregular margins and a poor outline compared to the colonies of derivative lines which were evenly rounded. These cells also form irregular aggregates in culture. A correlation can be established between the bizarre colony morphology and the metastatic phenotype. The parental cell line formed irregular diffuse colonies in agar, and showed wide spread metastases, while both the derivative lines formed spherical colonies and did not show metastases. Cifone [1981] has shown that the frequency of irregular shaped colonies was increased in cell lines that developed from the cells of highly metastatic clones growing in agar, and these colonies rarely developed from cells of clones with a low metastatic frequency, and when tested for *in vivo* experimental metastasis, the cells from such colonies were highly metastatic. This finding has significant implications; the diagnosis of the metastatic phenotype takes a long time in existing *in vivo* tumorigenic assays, and it would be a useful alternative assay for *in vitro* evaluation of metastatic potential of a tumour cell in a relatively shorter time period. The mechanism of irregular colony formation is unknown, but is probably due to variations in homotypic cell adhesion. Another possibility could be that the irregular colony formation was a result of a heterogeneous cell population in the colony of stem cells capable of generating cells with different growth rates. Further studies, particularly of the expression of integrins and CAMs, are required to establish the role of these alterations in cell adhesion, invasion and metastasis. The characterization of this model would indicate that it would be a good one for further study. Clonogenicity in soft agar and metastases formation in nude mice may be compared using cell lines of both low and high metastatic and clonogenic potential e.g. SCLC and NSCLC cell line.

The parental line was more sensitive to vincristine compared to the derivative or the NSCLC cell lines. This is in keeping with the common observation that SCLC is more sensitive to chemotherapy, but this was not the case with adriamycin,

where the parental line was more resistant. The possible explanation could be that the H69 was established from a relapsed tumour after chemotherapy including doxorubicin, vincristine, nitrosourea, and methotrexate, without any prior radiotherapy [Gazdar *et al.*, 1980; 1985; Carney *et al.*,1985], and it may be that resistance to adriamycin is inherent, but why this should not have been the case for vincristine is not clear. It implies that the resistance mechanism for adriamycin operating in these cells is not MDR as this would have included vincristine. Mirski *et al.* [1987] have isolated adriamycin resistant variants without MDR phenotype and no P-glycoprotein expression, suggesting that other mechanisms of drug resistance (e.g. expression of glutathione-S-transferase, and DNA repair) may also be involved.

The radiation experiments showed that the parental line was more sensitive than either the derivative or the NSCLC cell lines, suggesting a clinical correlation. It has been shown by others [Goodwin and Baylin, 1981; Carney *et al.*, 1983] that morphological variants of SCLC cell lines *in vitro* were more resistant to radiation compared to classic SCLC cell lines. It has been proposed that the morphological alterations with accompanied loss of neuroendocrine markers, and increased resistance to radiation may have clinical significance, since a poorer response to therapy has been reported in patients with relapse following chemotherapy or radiation treatment of primary tumours. It may be that neuroendocrine markers are expressed at a certain stage of tumour progression from stem cell to a more differentiated tumour. The coexpression of NE markers and radiation sensitivity may be coincidental. Alternatively, SCLC may represent a more undifferentiated phenotype, and therefore may be more vulnerable to radiation, and as the tumour progresses and its cells attain the NSCLC phenotype with differentiation, they acquire the capacity to become more resistant to radiation. A terminally differentiated cell is not capable of self renewal, and therefore less prone to potential risks of radiation compared to an undifferentiated, intermediate, highly proliferative cell.

Although the difference in chemosensitivity could have been due to variations in growth rates and growth fractions of the cell lines. This seems unlikely as vincristine and adriamycin sensitivities altered in different ways. Other factors such as variations in the assay systems may also be important. In this study the parental line grew in suspension, while both the derivative cell lines and the NSCLC cell lines grew as monolayer. It would have been possible to trypsinize the cells just before

irradiation or drug exposure, but it was not attempted because H69 cells do not grow well following trypsin treatment, suggesting some adverse effects on growth probably by surface changes caused by proteases. The differences in adriamycin sensitivity could also be due to the variations in penetration as the aggregate mode of culture may reduce access to the centre of a group of cells. However, this was apparently not true for vincristine, suggesting that the intake and distribution of two drugs may be under different types of control.

An increased expression of c-myc was observed in both the derivative lines using immunohistochemical techniques, which correlated well with an increase in growth rate, loss of neuroendocrine markers, and an epithelioid morphology of the cells. However, further studies, perhaps using gene transfection or anti-sense oligonucleotides, are required to correlate oncogene expression with phenotypic alterations in SCLC cell lines. The myc gene, its message and expression in the parental and the derivatives may also be investigated by southern, northern, and western blot analyses respectively. It has been suggested [Bepler *et al.*, 1987; Watanabe *et al.*, 1988] that myc amplification in variant cell lines might be responsible for a more aggressive malignant behaviour of these cells *in vivo* where a change occurs in relapsed SCLC with an epithelioid morphology, rapid growth, loss of neuroendocrine properties, drug and/or radiation resistant phenotype, either spontaneously or following therapy. Johnson *et al.* [1986] have shown that the transfection of a classic SCLC cell line with c-myc oncogene resulted in a change associated with large cell morphology, increased growth rate and high cloning efficiency. However, there was no significant difference in the expression of neuroendocrine markers including DDC, BLI, suggesting that c-myc expression may not be associated with biochemical markers, but rather with general morphology and growth potential of cells. It has been shown [Khan *et al.*, 1990 ] that c-myc oncogene transfection of an immortalised lung epithelial cell line MvlLu increased the growth of the transfectants both *in vitro* and *in vivo*. It has been shown that a down regulation of c-myc expression results upon induction of differentiation [Filmus & Buick, 1985], and a blocked of differentiation in the case of persistent c-myc expression [Prochownik & Kukowska, 1986].

There is now an emerging body of data suggesting a considerable overlap of various properties among different lung cancer types. Although lung cancer can be divided into SCLC and NSCLC with their characteristic biological and clinical behaviour, it is now evident that all lung cancers are derived from a common stem

cell [Carney & de Leij, 1988]. According to this theory a stem cell undergoes progressive differentiation, through morphologically undifferentiated intermediate stages committed to specific pathways of differentiation. The fully differentiated cells are end stage cells, and therefore, incapable of further division. A pluripotent precursor cell may differentiate along more than one pathway depending on environmental conditions and this could be altered or determined by a carcinogenic stimulus. Cells may even switch from one pathway to another, probably due to genetic alterations (e.g oncogenic activation, suppression of anti-oncogenes). Pulmonary NE-cells with amine precursor uptake and decarboxylation (APUD) cell properties may be one type of differentiation. It is possible that APUD-cell properties develop in this cell with the onset of endocrine differentiation or else these properties develop in all intermediate cells, and are then selectively retained only in endocrine cells [Baylin and Mendelsohn, 1981]. Transforming events occur in undifferentiated cells, since fully differentiated cells are not capable of replication, the differentiation status of the transformant may depend on the level of differentiation at which transformation occurs or progresses. According to this concept SCLC are regarded as partly differentiated along the endocrine pathway. Large cell carcinoma may represent the stem cells or undifferentiated tumour. This explains the presence of tumours having more than one morphological component, and also the APUD-cell markers in NSCLC. These cell types may represent intermediate stages in the spectrum of lung cancer progression.

The tumour cell population is propagated by a core of undifferentiated stem cells capable of a large number or infinite divisions, some of the progeny differentiate, while others remain relatively undifferentiated, and maintain the stem cell pool. The stem cell fraction in a tumour may appear morphologically different. It has been demonstrated that most SCLC cultures contain a very small percentage of large cells [Gazdar *et al.*, 1981] which may be stem cells. According to this theory, a changing tumour morphology can be explained by progressive failure of the stem cells to undergo differentiation. Depending on the severity of differentiation failure, a pure small cell or mixed small cell-large cell tumour or pure NSCLC may be found in an originally SCLC with a very small stem cell population. The stem cell theory also explain the co-existence of SCLC with other histological types of lung cancer such as squamous and adenocarcinoma. If all lung cancers arise from a single stem cell, conversion from one differentiated cell type to another can occur,

by maturation down one particular pathway, resulting either a pure squamous, adeno or SCLC or even a mixed population of SCLC with any of other lung cancer types.

The stem cell concept of lung cancer has also been supported by data derived from clonogenic assays both in the present and previous studies which indicate that only a small percentage of SCLC is capable of giving rise to progressively growing colonies with heterogeneous cell population, but that many cells are capable of limited replication. Carney *et al.*, [1980] have shown similar results in support of this concept. In addition, in mixed small cell-large cell tumours, cell kinetic studies indicate greater thymidine uptake by the large cell components, suggesting a greater replicating ability of the latter.

The present data have shown that the H69 cell line contains cells of both small and non-small cell like phenotypes. It is possible that a pluripotent stem cell sub-population may exist in the parental line capable of generating cells with different phenotypes, and some of them are selected according to the culture conditions. In light of the previous and the present data it can be concluded that all lung cancers develop from a common stem cell, capable of differentiating into various lung cancer types depending upon the stage of differentiation at which the genetic and epigenetic forces exert their influences. H69 and its derivative lines may provide a suitable model system for the study of phenotypic transition in lung cancer *in vivo*. In future studies H69 clones might be investigated for heterogeneity. Cloning of H69V and H69VZ cell lines and investigation for heterogeneity and signs of reversion to SCLC would determine whether the process is reversible. It is not known whether a stem cell population in a given clone of adherent cells would behave like a common stem cell capable of generating different lung cancer types. Further studies are also required to investigate whether some genetic changes e.g new oncogene expressions or deleted suppressors, are responsible for these phenotypic shifts in lung cancer both *in vitro* and *in vivo*. Therefore, it can be concluded from these data that the H69 and its derivative lines, H69V & H69VZ, may be used as a valid model for the study of phenotypic transitions in SCLC.

# CHAPTER THREE
# (B)

## INDUCTION OF PHENOTYPIC CHANGES IN HUMAN SMALL CELL LUNG CARCINOMA

Spontaneous phenotypic changes in a SCLC cell line (NCI-H69) have been described in the previous chapter. The phenotypic variations described above could be due to genetic selection, however, the expression of phenotype may be under regulatory control. This Chapter investigates the effects of various chemical agents on phenotypic expression of the cell line both *in vitro* and *in vivo*.

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## 3B.1 INTRODUCTION

Induction of the differentiated phenotype in malignant cells has been shown following treatment with differentiating agents [Spermulli & Dexter, 1984; Freshney, 1985]. Induction of the differentiated phenotype can be monitored by the expression of differentiation associated markers and suppression of malignancy related properties, e.g an increased haemoglobin production and suppression of tumorigenicity in erythroleukemia [Reuben *et al.*, 1976; 1980], suppression of PA, clonogenicity in agar, and tumorigenicity and induction of surfactant production in human alveolar cell carcinoma cell line [Speirs & Freshney, 1990]. The mode of drug action differs considerably depending upon the nature of the agent used and the system it is employed in. A phenotypic inducer may act via a number of pathways, such as alteration of cell surface proteins by expression or deletion, by phosphorylation and glycosylation of membrane and cytoplasmic proteins, resulting in altered cellular pathways. They also act on nuclear targets such as DNA, histones, resulting in expression or suppression of a particular gene.

To investigate the effects of various phenotypic inducers on H69 cells the following markers were selected to represent SCLC phenotype: dopa decarboxylase (DDC), creatine kinase BB isoenzyme (CK-BB), bombesin-like immunoreactivity (BLI), neuron specific enolase (NSE), chromogranin-A. In addition to the neuroendocrine markers, a number of other parameters were selected for investigation to indicate a phenotypic shift in SCLC. These included: growth and morphological characteristics of cells in culture, cloning in soft agar, invasion *in vitro* using chick heart assay [Mareel *et al.*, 1979], tumorigenicity, histology of xenografts, and invasion *in vivo* in athymic nude mice.

The key APUD enzyme L-dopa decarboxylase or DDC (E.C. 1.1.28) has been demonstrated in lung tumours, lung tumour cell lines in culture, and xenografts of SCLC in nude mice [Baylin *et al.*, 1978; 1980]. The enzyme occurs free in the cytoplasmic fraction of the cell. It is found in many human tissues including kidneys, liver, neurons, and the tumours of neuroendocrine origin. The activity of this enzyme constitutes an important element of the NE-cell concept [Pearse, 1969].

Creatine kinase (CK), found normally in striated muscles, brain, bladder, and gastrointestinal tract [Gazdar *et al.*, 1981] has three isoenzymes. Each isoenzyme is a dimer composed of a combination of two distinct subunits, M and B. Skeletal

muscle contains isoenzyme MM, brain tissue contains isoenzyme BB, while the MB hybrid form is characteristic of myocardium. High concentrations of creatine kinase BB isoenzyme or CKBB (E.C. 2.7.3.2) has been found in serum, biopsy specimens, and cell lines in culture from patients with SCLC. CKBB activity in SCLC is quantitatively (10-100 fold higher) but not qualitatively different from that in normal lung and in other lung cancer types [Gazdar *et al.*, 1981]. Although morphological variants of SCLC in culture show considerable loss of many of neuroendocrine features e.g DDC, BLI, high levels of CKBB are maintained [Abeloff *et al.*, 1979]. The presence of this enzyme has also been widely used as a criterion for SCLC phenotype in lung tumours and tumour-derived cell lines.

Bombesin is a 14 amino acid peptide originally isolated from the skin of the frog *Bombina* [Anastasi *et al.*, 1971]. It is found in large quantities in brain [Moody & Pert, 1979], stomach and intestine [McDonald *et al.*, 1979] and fetal lung [Moody *et al.*, 1981]. The mammalian equivalent of bombesin is believed to be gastrin releasing peptide [GRP], a 27 amino acid peptide. In human lungs bombesin-like immunoreactivity has been demonstrated in bronchial and bronchiolar epithelia both in solitary NE cells and in NEB [Wharton *et al.*, 1978]. High levels of BLI have been found in full term fetal and neonatal lungs, but this is significantly reduced or even absent in adult lungs. A regulatory role for BLI in normal development of lung has been suggested on the basis of these findings [Ghatei *et al.*, 1983].

Measurements of intracellular and secreted BLI in cell lines of lung cancer has shown that only lung cancer cell lines of SCLC origin expressed high levels, while it was not detectable in NSCLC cultures [Moody *et al.*, 1981; 1983]. Bombesin receptors have also been found in SCLC [Moody *et al.*, 1985], suggesting an autocrine growth effect of bombesin on SCLC, and Carney *et al.* [1985] showed that monoclonal antibodies to bombesin could inhibit the clonal growth of SCLC *in vitro* and *in vivo*. Exogenously added bombesin and GRP stimulated colony formation in SCLC *in vitro* but had no stimulatory effects on NSCLC [Carney *et al.*, 1987].

Enolases are a group of glycolytic enzymes necessary for the anaerobic conversion of glucose to metabolites suitable for oxidation. The enzyme has three subunits designated $\alpha$, $\beta$, and $\gamma$, and occurs in five forms including three homodimers [$\alpha\alpha$, $\beta\beta$, $\gamma\gamma$] and two heterodimers [$\alpha\beta$, $\alpha\gamma$]. The alpha isoenzyme is the predominant form occurring in most adult tissues, also termed non-neuronal enolase (NNE). The beta form is found in muscle. The gamma form was thought

to occur only in neural tissues and was therefore termed neuron specific enolase (NSE) [Marangos *et al*, 1979]. However, NSE has also been detected in the NE-cells of gut, pancreas, lung and skin. [Marangos *et al.*, 1979; Schmechel *et al.*, 1978], in tumours with neuroendocrine properties [Odelstad *et al.*, 1982], in neuroblastoma [Ishiguro *et al.*, 1982], in small cell lung carcinoma [Carney *et al.*, 1982; Esscher *et al.*, 1985], and also at low levels in NSCLC [Baylin *et al.*, 1980]. The levels of NSE in the continuous cell lines of SCLC are found to be much higher than in NSCLC cells.

The family of proteins called chromogranins [Carmichael and Winkler, 1985] has emerged as a potential universal marker for neuroendocrine tissues and tumours [Wilson and Loyd, 1984; Lauweryns *et al.*, 1987]. It has also been found in the central and peripheral nervous system, in retinal photoreceptor cells, thymic epithelial cells, and cells lining sub-mandibular gland ducts [Nolan *et al.*, 1985]. The chromogranins have been divided into three classes, A, B, and C. They have similar properties, have overlapping but not co-localization in tissue, and are secreted with other biologically active peptides. Their major component, chromogranin A, was first discovered by Banks and Helle [1965] secreted by adrenal medulla chromaffin granules upon neural stimulation. It has been characterized and in chromaffin cells chromogranin A with a molecular weight of 75,000 daltons, is synthesized as a single precursor which is partly broken down by the action of proteases within chromaffin granules, and smaller proteins thus formed can be detected by antisera against chromogranin A [Falkensammer *et al.*, 1985]. Elevated levels of serum chromogranin-A have also been demonstrated in patients with SCLC [Sobol *et al.*, 1986]. Chromogranins may act by binding intravesicular calcium, and may have a role in growth control [O'Connor and Bernstein, 1984].

Though there are different treatment modalities for cancer, the treatment of choice in SCLC is chemotherapy alone or in combination with radiotherapy. However, generation of resistant variants in relapsed tumours is a major limitation of cytotoxic therapy. It is speculative whether resistance is inherent or acquired following treatment by the overgrowth of a mutant subpopulation derived from the initial clone. Different approaches are being used to circumvent this problem. Chemotherapuetic agents are used in combination either with other cytotoxic drugs or with an agent that will enhance drug uptake, inhibit drug efflux thereby increasing cytotoxicity. Other approaches are also under investigation that involve tumour

killing by indirect means rather than by direct killing e.g inhibition of invasion and metastasis. Induction of differentiation and suppression of malignancy is a more novel approach that is target specific, non-cytotoxic, and applies the biological rules to modify the abnormal cell behaviour.

H69 cell line was selected because of its potential stem cell or precursor cell nature and therefore greater potential to alter the phenotype using phenotypic modulators. After the study of spontaneous phenotypic changes in H69 cell line in the previous chapter it was decided to examine whether similar changes could be induced by various chemical inducers known to induce phenotypic changes in other systems. It may be possible to induce NSCLC-like phenotype in H69 cells following treatment with these agents. Some phenotypic alteration has been shown in H69 cells following a 72 hour treatment with phenotypic modifiers [Murray, 1989]. The present study was aimed at investigating the effects on the H69 cell phenotype of prolonged treatment with the phenotypic modulators as has been seen in other systems [Chun *et al.*, 1986; Toscani *et al.*, 1988].

The activities of chemical inducers used in this study (HMBA, Na-But, dbcAMP and Dex) have already been described in Chapter one. They have been used either alone or in various possible combinations in this study. Following drug treatment cell cultures were examined for any shift in the SCLC phenotype. Specific objectives were: 1) To investigate the effects of a more prolonged (7 days) treatment on H69 cells morphology and growth in culture, clonogenicity in soft agar, and expression of NE-cell markers by both biochemical and immunocytochemical methods. 2) To study the effects of chemical agents on invasion of H69 cells *in vitro*, using chick heart assay (Mareel *et al.*, 1979). 3) In parallel studies, to investigate the effects of 14 days treatment on growth, histology, invasion and NE-cell marker expression by immunohistochemical staining of H69 xenografts grown in athymic nude mice.

## 3B.2 MATERIALS AND METHODS

### 3B.2.1 Cell Lines

A classic SCLC cell line (NCI-H69) was used in this study. The cell line was grown and maintained in culture as described in the previous chapter.

### 3B.2.2 Chemical Inducers

The chemical agents (see Chapter one) used as phenotypic modulators included $N^6$-2-O-dibutyryl adenosine 3':5'-cyclic monophosphate, sodium salt (dbcAMP) [Sigma], N,N'-Hexamethylene bisactamide (HMBA) [Sigma], N-butyric acid, sodium salt (Na-But) [Sigma], and dexamethasone sodium phosphate (Dex) [Decadron, Merk, Sharp & Dohme Ltd]. Dosage is shown in Table 3B.1. All drugs were dissolved in culture medium.

### 3B.2.3 *In Vitro* Assay

Cells were seeded in 24 well plates at a final cell concentration of $10^5$ cells/ml/well. Exponentially growing cells were exposed to the phenotypic inducers i.e. 1 mM cAMP, 0.25 $\mu$M Dex, 5 mM HMBA, and 1 mM Na-but, individually, in culture medium, for 7 days. Cells were fed on alternate days with growth medium containing drug (test) or drug vehicle (control). The test group was further divided into two parts: 1) cells received drug treatment throughout the experiment, 2) cells received drug treatment for 7 days only, and after drug removal cells were either allowed to recover in drug free medium for further 9 days or processed immediately for study of growth, morphology and marker expression. Cell number was determined by electronic particle counter, following trypsinization of at least 3 wells. Culture morphologies of control and treated cells were also examined and compared at the end of experiment. Cell viability was tested by trypan blue exclusion.

### 3B.2.4 *In Vivo* Assay

Cells were harvested from exponentially growing cultures, washed and resuspended in PBS at $2x10^7$ cells/ml. An aliquot of 0.5 ml cell suspension was injected subcutaneously into the flanks of six to eight weeks old male or female nude mice. For passage a 2x2x1 mm tumour piece was transplanted subcutaneously into the flank of each animal. When the tumours reached a size of approximately 4x4 mm in two perpendicular diameters, animals were assigned randomly into control

**Table 3B.1:- Dosage of phenotypic inducers *in vitro* and *in vivo*.**

| DRUG | DOSAGE | |
|---|---|---|
| | *In Vitro* | *In Vivo* |
| HMBA | 5.0 mM | 1.5 mg/gm body wt. |
| Na-But | 1.0 mM | 2ug/gm body wt. |
| dbcAMP | 1.0 mM | 2ug/gm body wt. |
| Dex | 0.25 uM | 2ug/gm body wt. |

HMBA: hexamethylene bisacetamide, Na-But: sodium butyrate, dbcAMP: dibutyryl cyclic adenosine monophosphate, sodium salt, Dex: dexamethasone, sodium phosphate.

and treated groups, 4-6 animals per group. Each of the test animals received a daily i.p injection of drug [Table 3B.1] for two weeks, at dose adjusted to the body weight at the time of injection. The control animals received an equal volume of drug vehicle. Animals were examined twice weekly, tumour volumes were measured for each animal, and total body weight recorded, according to General Methods

### 3B.2.5 Other Methods

The details of primary antibodies used in this chapter for immunohistochemical methods have been shown in table 3A.2 (see above). Other methods employed in this Chapter have been described in General Methods.

## 3B.3 RESULTS

### 3B.3.1 Growth *In Vitro*.

Cells treated with 1 mM sodium butyrate showed suppression of growth, but the cell number did not decrease when grown in the continuous presence of drug. When drug was removed cells reentered exponential growth after 3-4 days with a doubling time equal to that of control cells [Table 3B.2]. However treated cells did not reach the concentration of controls in plateau by day 19 [Figure 3B.1].

When cells were treated with 5 mM HMBA, growth suppression was noticed within 24 hours of drug addition compared with control. Treated cells showed a different growth pattern [Figure 3B.2]. There was no complete growth suppression as observed with sodium butyrate, rather cells continued to proliferate, though at a very low rate with a prolonged doubling time [Table 3B.2]. When drug was removed after 7 days exposure, cells showed a lag of 3 days before resuming exponential growth, when the normal growth rate was restored with a doubling time of around 40 hours. However, cell number did not reach control levels even after 9 days in drug free conditions.

The growth pattern of cells treated with dbcAMP was similar to that seen in sodium butyrate treated cells. When cells were exposed to 1 mM dbcAMP growth was suppressed within 24 hours [Figure 3B.3] with only a minor rise in cell number. When drug was removed after 7 days exposure, cells reentered exponential growth

**Table 3B.2:- Effects of phenotypic inducers on growth properties of H69 cells *in vitro*.**

| INDUCER | DOUBLING TIME (days) | | SATURATION DENSITY ($X10^{-6}$ cells/ml) | | COLONY NUMBER | [x]CLONING EFFICIENCY |
|---|---|---|---|---|---|---|
| | A | B | A | B | Mean ± SEM | (%) |
| Control | 2.2 | 2.2 | 12.4 ± 0.6 | 12.4 ± 0.6 | 494 ± 41 | 100 |
| HMBA | G.S | 1.7 | [*]1.3 ± 0.04 | [*]5.6 ± 0.3 | [*]84 ± 5 | 17 |
| Na-But | G.S | 1.7 | [*]0.2 ± 0.02 | [*]4.6 ± 0.07 | [*]83 ± 13 | 17 |
| dbcAMP | G.S | 1.8 | [*]0.3 ± 0.02 | [*]5.4 ± 0.2 | [**]210 ± 35 | 43 |
| Dex | 2.1 | 2.5 | [*]11.6 ± 2.5 | 12.3 ± 1.5 | 613 ± 43 | 124 |

A (in drug), B (after drug removal). Data are from at least 2 (growth curves) or 3 (cloning) independent experiments. G.S: Growth suppression. [x]% of control, where control is taken as 100%. Other abbreviations as above [see Table 3.4].
[*]$P<0.0001$, [**]$P<0.001$ (analysis of variance and Bonferoni adjustment).

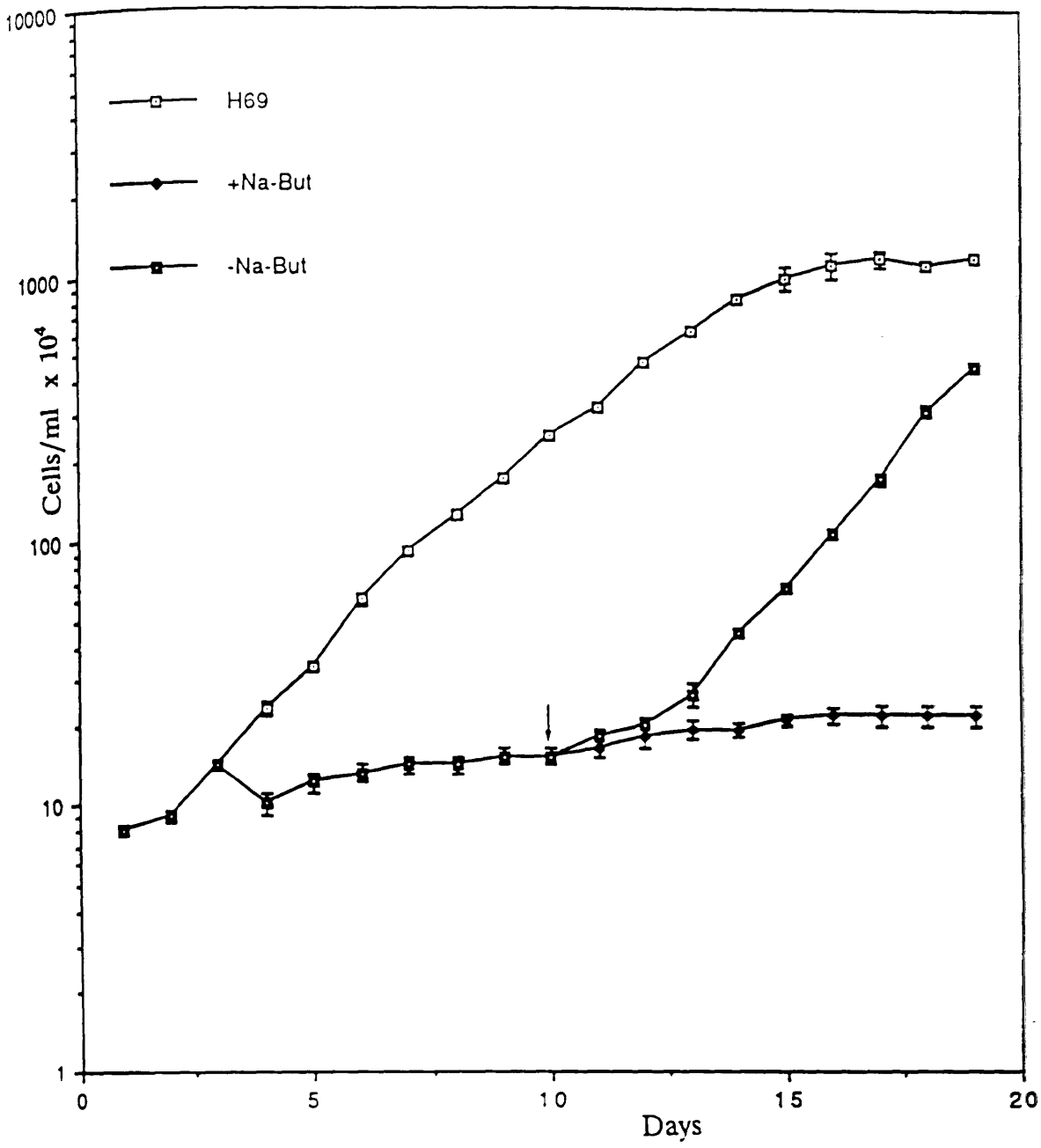



**Figure 3B.1:- Effects of sodium butyrate on growth of H69 cell in vitro.**

The cells were seeded in 24 well plates at $1x10^5$ cells/ml on day 0. Exponentially growing cells (day 3) were treated with 1.0 mM sodium butyrate, continuously for up to 19 days (+Na-But) or allowed to recover in absence of drug after 1 week drug exposure (-Na-But). The drug treated cells showed growth inhibition, and following drug removal cells were able to reenter exponential growth again. Results are from one experiment, and similar results were obtained in a second experiment. Each point represents mean ± SEM (bars). Arrow, time at which the drug was removed from culture.

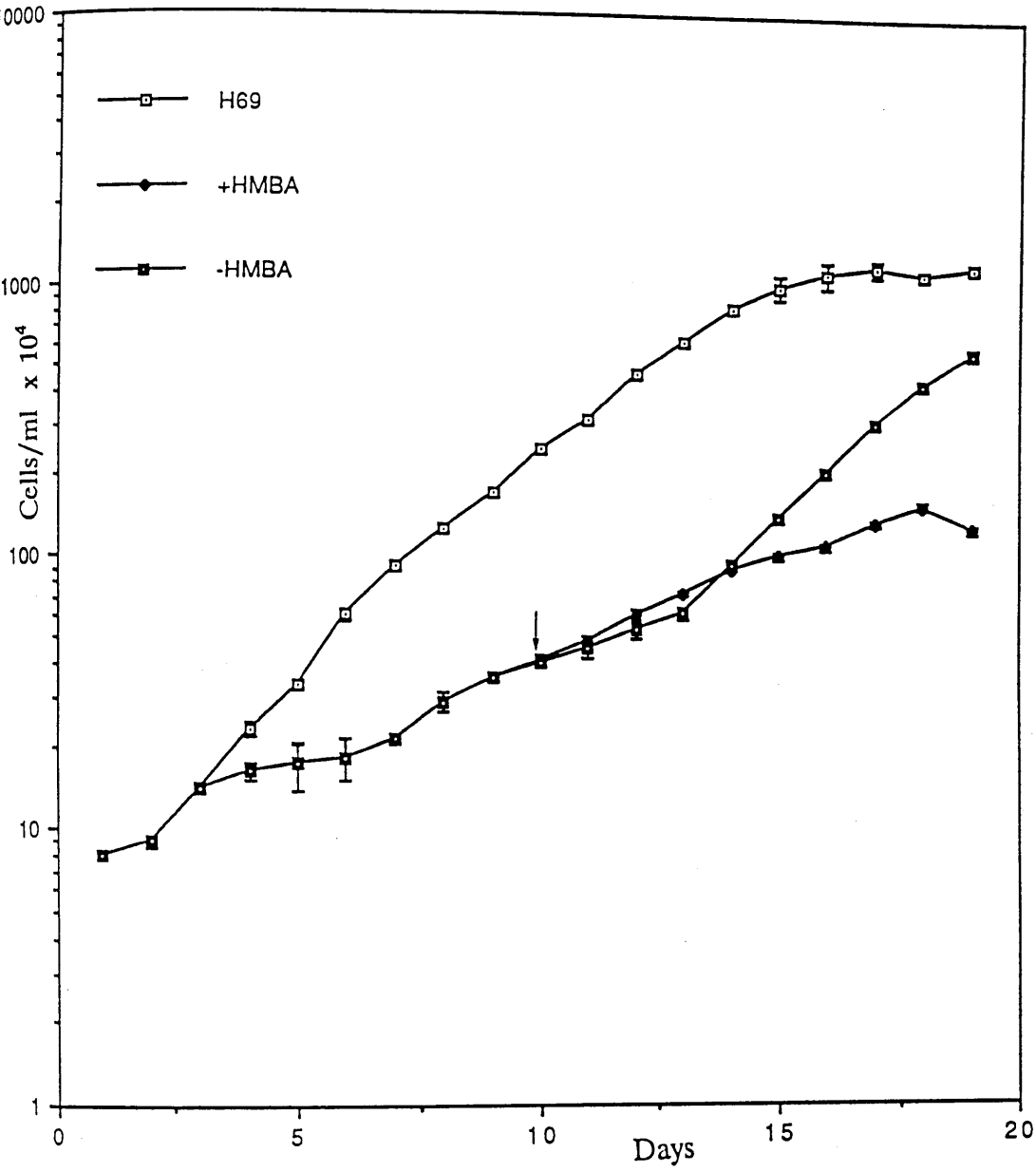



**Figure 3B.2:- Effects of HMBA on growth of H69 cells in vitro.**

As [Figure 3B.1] except that the inducer used was HMBA (5.0 mM).

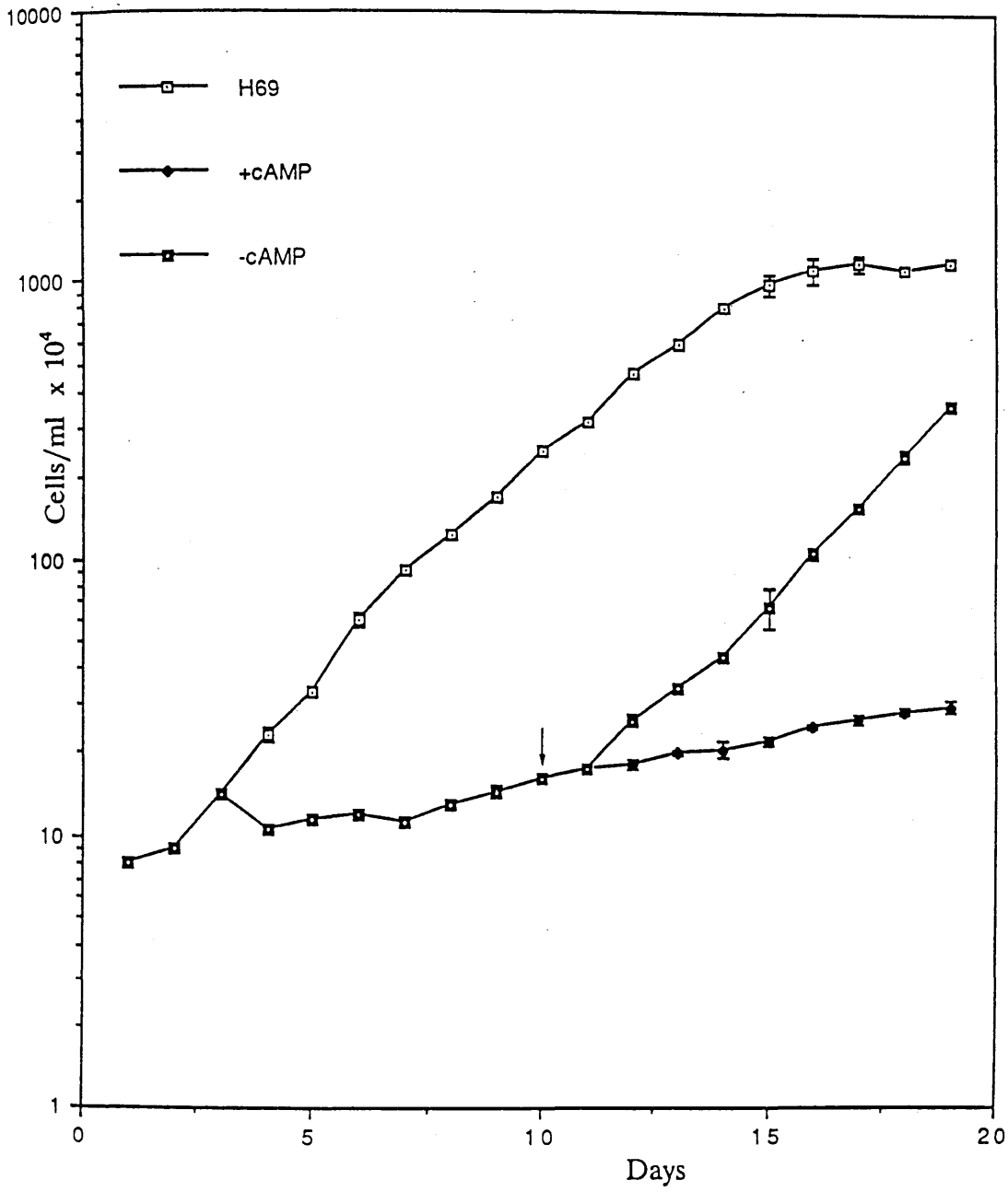



**Figure 3B.3:- Effects of dibutyryl cyclic AMP on growth of H69 cells *in vitro*.**

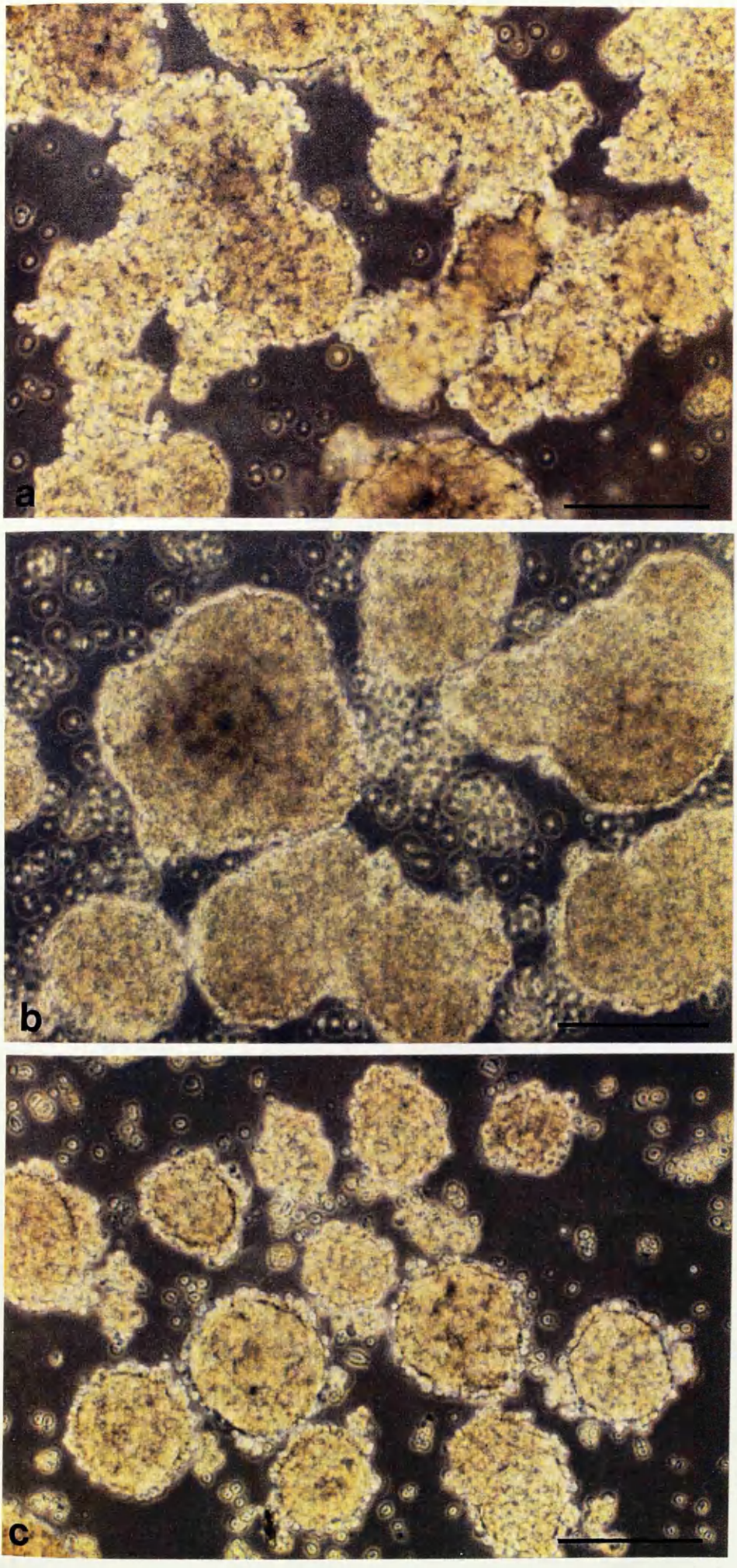
As (Figure 3B.1) except that inducer used was dibutyryl cyclic AMP (1.0 mM).

**Plate 3B.1:- Effects of phenotypic inducers on morphology of cells in culture.**

(a) H69 cells seeded in 25 $cm^2$ flasks ($10^5$/ml), grew as irregular cellular aggregate Aggregates were collected together for photographs on day 10.
(x 20 objective, phase-contrast, bar = 100 $\mu$m)

(b) H69 cells treated with sodium butyrate (1.0 mM) for 7 days in log phase growth formed larger aggregates, than the control, and with a more round morphology. However, the number of aggregates was reduced compared to cont In addition there were more single cells in the medium and attached to the substr compared to the untreated control.

(c) H69 cells treated with HMBA (5.0 mM) as in (b). These grew as small aggregates than the control with more rounded outline. However, the number aggregates was reduced compared to control. Single cells were found in the medi but to a lesser extent than following sodium butyrate treatment.


a
b
c

Dexamethasone treated cells showed no significant morphological changes compared to control.

*Cytology*

There were no differences in the cytological appearances of treated and untreated cells, in Giemsa stained cytospin preparations.

### 3B.3.4 Neuroendocrine Marker Expression

*Effects on Bombesin like immunoreactivity (BLI).*

Table 3B.3 shows the effect of various phenotypic modulators on the expression of BLI of H69 cells, used either alone or in combination. Both cell associated and secreted levels were examined from each replicate sample, using RIA, and activity was expressed as pg/mg total cellular protein. The control showed slightly less cell associated than secreted BLI, but not significant. There was no significant effect of drug treatment on the cellular levels of BLI, used either alone or in different combinations, except an additive effect, following treatment with dbcAMP and Na-But in combination. However, there was a slight increase in the levels of secreted BLI, following treatment with single agent (HMBA) or a combinations of two agents (dbcAMP+HMBA; HMBA+Na-But).

*Effects on DDC Expression*

Table 3B.4 shows the effects of various drugs alone or in combination on expression of DDC in H69 cells. There was no significant effect of dbcAMP or dex, either alone or in combination. HMBA and Na-But alone and in different combinations (dbcAMP + HMBA; dbcAMP + Na-But; Dex + HMBA; Dex + Na-But; HMBA + Na-But) decreased significantly the DDC activity of the treated cells (by approximately 50%).

*Effects on CKBB Expression*

H69 cells expressed creatine kinase, predominantly, CKBB isoenzyme, a very small fraction of CKMM, but undetectable levels of CKMB [see above, Figure 3A.4] Treatment of cells with various phenotypic modulators did not have any significant effect on levels of either total enzyme activity or alteration of its relative isoenzyme expression [Table 3B.5].

**Table 3B.3:- Effects of phenotypic inducers on the expression of bombesin like immuoreactivity (BLI).**

| DRUG | BOMBESIN LIKE IMMUNOREACTIVITY (pg/ug) | | | |
|---|---|---|---|---|
| | CELLULAR | | SECRETED | |
| | Mean ± SEM | (%) | Mean ± SEM | (%) |
| Control | 0.48 ± 0.04 | 100 | 0.67 ± 0.08 | 100 |
| dbcAMP | 0.47 ± 0.01 | 99 | 0.82 ± 0.10 | 122 |
| Dex | 0.46 ± 0.02 | 96 | 0.74 ± 0.08 | 110 |
| HMBA | 0.44 ± 0.02 | 91 | 1.12 ± 0.30 | 167 |
| Na-But | 0.48 ± 0.03 | 101 | 0.49 ± 0.09 | 73 |
| dbcAMP+Dex | 0.45 ± 0.02 | 94 | 0.61 ± 0.08 | 91 |
| dbcAMP+HMBA | 0.42 ± 0.03 | 87 | 1.26 ± 0.22 | 188 |
| dbcAMP+Na-But | 0.52 ± 0.06 | 109 | 1.43 ± 0.14 | *213 |
| Dex + HMBA | 0.41 ± 0.01 | 86 | 0.688 ± 0.10 | 102 |
| Dex + Na-But | 0.50 ± 0.04 | 105 | 0.61 ± 0.07 | 91 |
| HMBA+Na-But | 0.47 ± 0.04 | 98 | 1.03 ± 0.14 | 153 |

$10^4$ cells/ml were seeded in 75 $cm^2$ flasks, late log phase cells were treated continuously with drug for 7 days (fresh drug daily). Cell and spent media were assayed for BLI by RIA as in methods. BLI was expressed as pg/ug total cell protein. Results are at least from 6 replicates of at least 2 independent experiments. *$P<0.05$ (analysis of variance)

**Table 3B.4:- Effects of phenotypic inducers on DDC expression.**

| INDUCER | DDC ACTIVITY (uIU/min/mg) Mean ± SEM | % of control |
|---|---|---|
| Control | 2853 ± 355 | 100 |
| dbcAMP | 3389 ± 74 | 122 |
| Dex | 3608 ± 42 | 131 |
| HMBA | 1524 ± 204 | *54 |
| Na-But | 1827 ± 255 | *64 |
| dbcAMP + Dex | 2788 ± 215 | 99 |
| dbcAMP+ HMBA | 1543 ± 199 | *57 |
| dbcAMP+ Na-But | 1887 ± 97 | *69 |
| Dex + HMBA | 1684 ± 416 | *58 |
| Dex + Na-But | 1607 ± 275 | *56 |
| HMBA + Na-But | 1408 ± 108 | *50 |

Cells were seeded in 75 $cm^2$ flasks, exposed to drug in late log phase for 7 days,fresh drug was added daily. Cellular DDC activity was determined and expressed as uIU/min/mg total cellular protein. Values are from at least three independent experiments.

*$P<0.05$ (analysis of variance).

Table 3B.5:- Effects of phenotypic inducers on creatine kinase (CK) activity.

| INDUCER | TOTAL CK ACTIVITY (IU/mg) | % OF CONTROL | ISOENZYMES ANALYSIS CKBB | CKMB | CKMM |
|---|---|---|---|---|---|
| Control | 3.12 ± 0.36 | 100 | 95 | 0 | 5 |
| dbcAMP | 3.88 ± 0.73 | 124 | 96 | 0 | 4 |
| Dex | 3.20 ± 0.56 | 103 | 95 | 0 | 5 |
| HMBA | 2.78 ± 0.62 | 89 | 94 | 0 | 6 |
| Na-But | 3.54 ± 0.94 | 113 | 96 | 0 | 4 |
| dbcAMP + Dex | 3.38 ± 0.60 | 108 | 94 | 0 | 6 |
| dbcAMP + HMBA | 3.50 ± 0,26 | 112 | 94 | 0 | 6 |
| dbcAMP + Na-But | 3.21 ± 0.63 | 103 | 95 | 0 | 5 |
| Dex + HMBA | 3.14 ± 0.70 | 101 | 90 | 0 | 10 |
| Dex + Na-But | 2.93 ± 0.89 | 94 | 96 | 0 | 4 |
| HMBA + Na-But | 3.64 ± 0.68 | 117 | 95 | 0 | 5 |

Cells were grown as for DDC (see above Table 3.15). The results are Mean ± SEM of 3 independent experiments. Results are also shown as percentage of control, where the control value is taken as 100.

### 3B.3.5 Immunohistochemical Marker Expression

Expression of immunohistochemical markers by the H69 cell line was shown in the previous chapter. There was no detectable alteration in the expression of NSE; BLI; and chromogranin-A, following drug treatment either alone or in combinations.

### 3B.3.6 Invasiveness *In Vitro*

When combined in confronting culture with chick heart the H69 control cells showed invasion within the first 48 hours [Plate 3B.2 a], while invasion was found only by day 4 in HMBA or Na-but treated H69 cells. Following a 72 hours pre-treatment with HMBA (2.5 mM) there was a delay of approximately 4 days in invasion compared to control cells [Plate 3B.2 b]. Invasion was also delayed for the similar period of time in treated cells following a 14 days recovery period in drug free medium before co-culture [Plate 3B.2 c]. Cells treated with 1 mM sodium butyrate for 72 hours before confrontation did not invade the fragment for at least 5 days, and significant invasion was not observed until day 7 [Plate 3B.2 d]. Viability determined by dye exclusion, was found to be over 80-90% in treated non-invasive cells in co-culture assay.

### 3B.3.7 Growth of Xenografts

There was 100% tumour take following transplantation of 2x2x1 mm tumour pieces subcutaneously, after a latency period of around 17 days [Figure 3B.5]. Tumours grew with a doubling time of around 5 days. They attained an average maximum volume of approximately 2 $cm^3$ in a period of around 5 weeks [Table 3B.6]. Following HMBA treatment, there was a growth suppression compared to untreated controls [Figure 3B.5, Table 3B.6]. HMBA given i.p daily for 14 days increased the tumour doubling time 3-fold and reduced the final tumour volume 8-fold. Na-But [Figure 3B.5, Table 3B.6] increased the tumour doubling time by 5-fold, and reduced the final tumour volume by 13-fold. Treatment for 14 days with dbcAMP slightly increased the tumour doubling time (around 1-fold), and reduced the final tumour volume [Figure 3B.5, Table 3B.6]. The effects were less than those of HMBA or Na-But. There was no effect of dexamethasone treatment on tumour growth [Figure 3B.5, Table 3B.6].

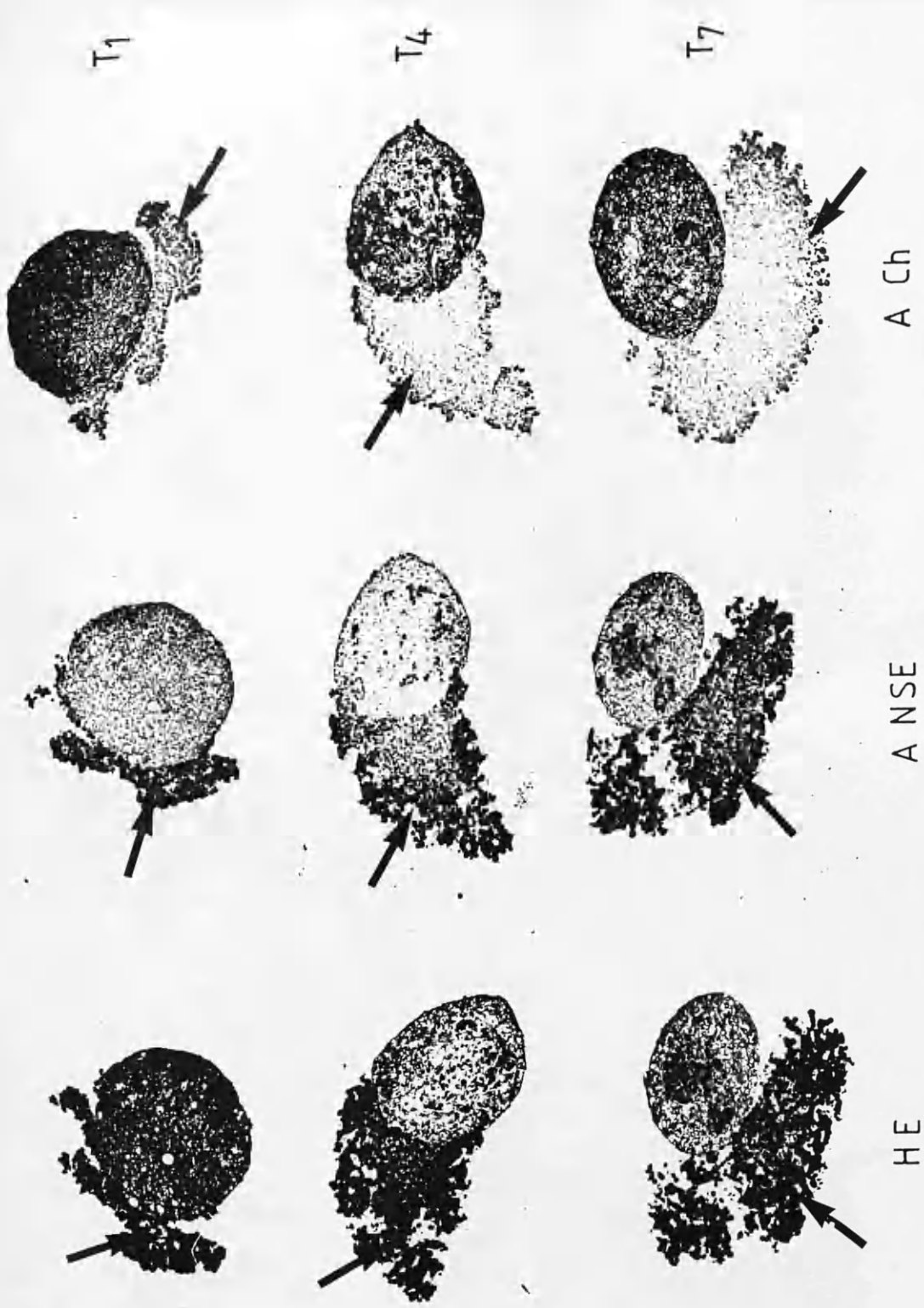
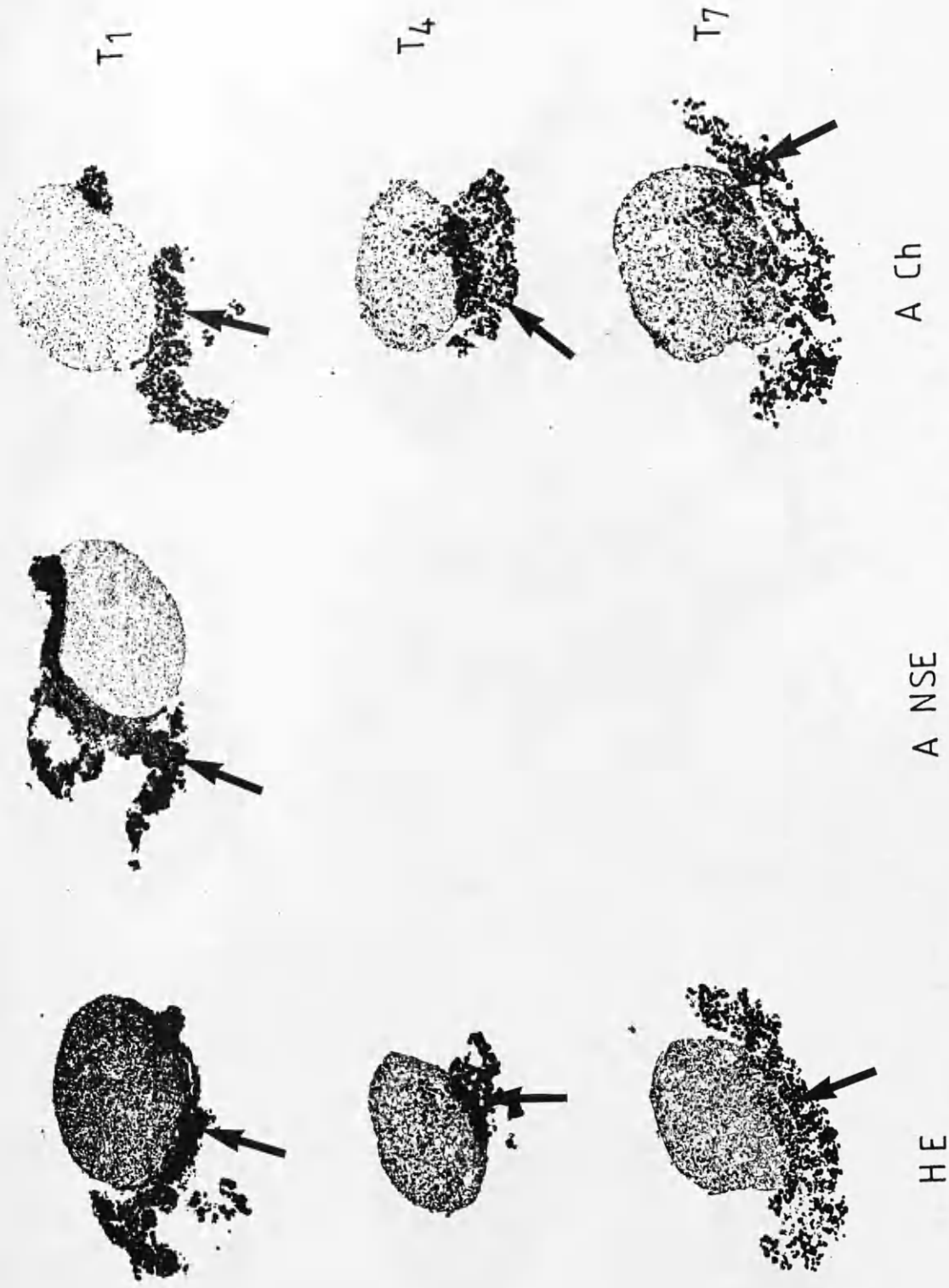
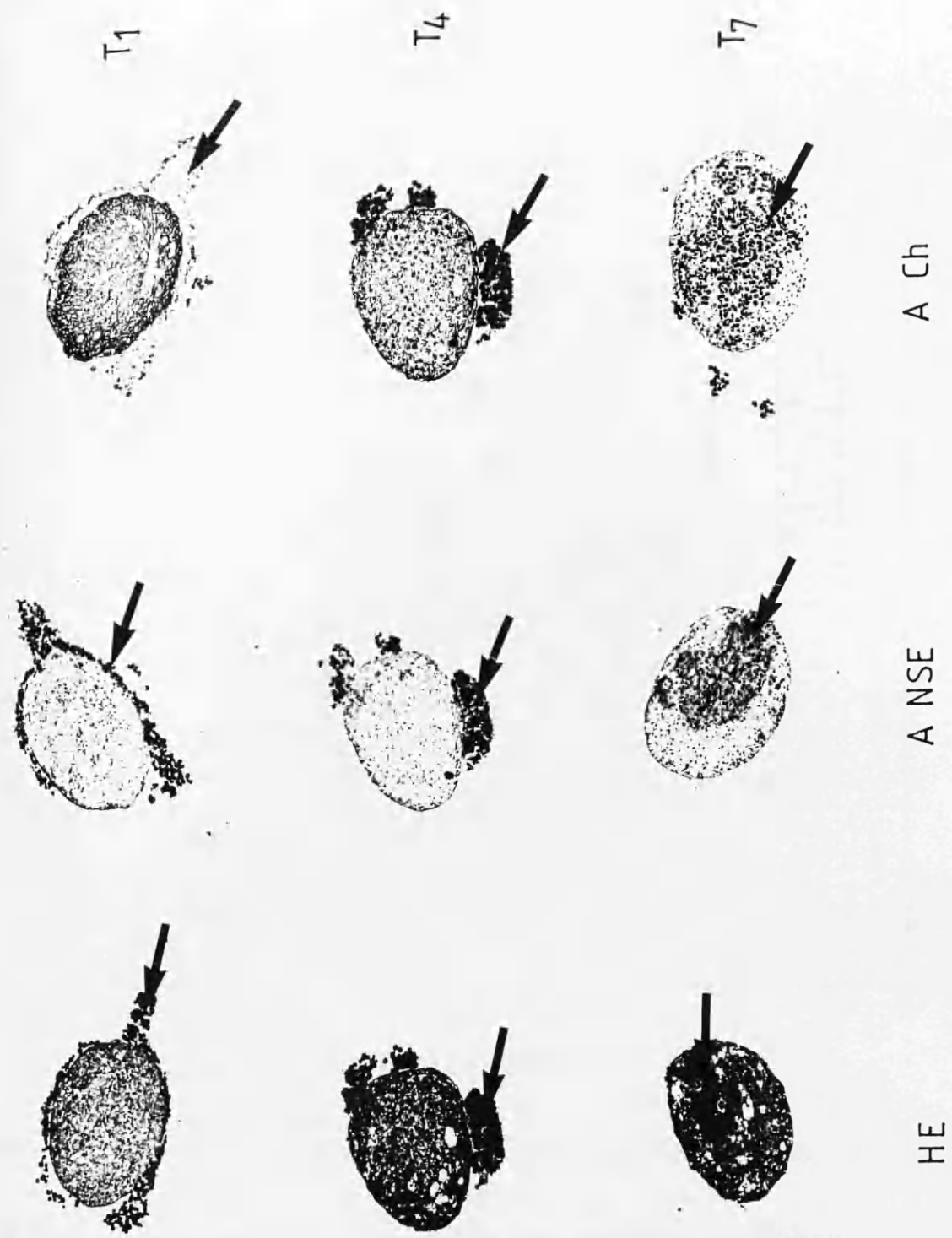



Plate 3B.2:- **Effects of phenotypic modulators on invasion of H69 cells *in vitro*.**
(a). H69 cells co-cultured with embryonic chick heart show invasion within 48 hours. Tumour cells (arrow) are identified in the centre of the heart fragment, by haematoxylin & eosin (HE) staining, and confirmed by immunoperoxidase staining using anti-neuron specific enolase (ANSE), and anti-chick (ACh) antibodies. Samples at day 1 (T1), 4 (T4) and 7 (T7), after co-culture are shown (x 250, H&E, and IP).




(b). Tumour cells (arrow) treated with HMBA (2.5 mM) for 3 days, then co-cultured with pre-cultured heart fragment show invasion by day 4 (T4) compared to 48 hours for untreated H69 cells (see above, a). Cells remained alive outside the fragment during this period but did not invade. Invasion after day 4 was progressive. By day 7 (T7) tumour cells are seen throughout the heart fragment, but complete replacement was not seen (x 250, H&E, IP).

(c) As (b), except that tumour cells (arrow) were allowed to recover for 14 days in drug free medium, before co-culture. Invasion is observed at day 4 (T4), and more extensive at day 7 (T7) (x 250, H&E, IP).




(d) H69 cells (arrow) treated with sodium butyrate (1.0 mM) for 3 days, and co-cultured with heart fragment. Evidence of invasion is not identified by day 4 (T4). Cell are seen in the centre of the heart fragment at day 7 (T7). Cells were found alive around the heart fragment before that time (x 250, H&E, IP).

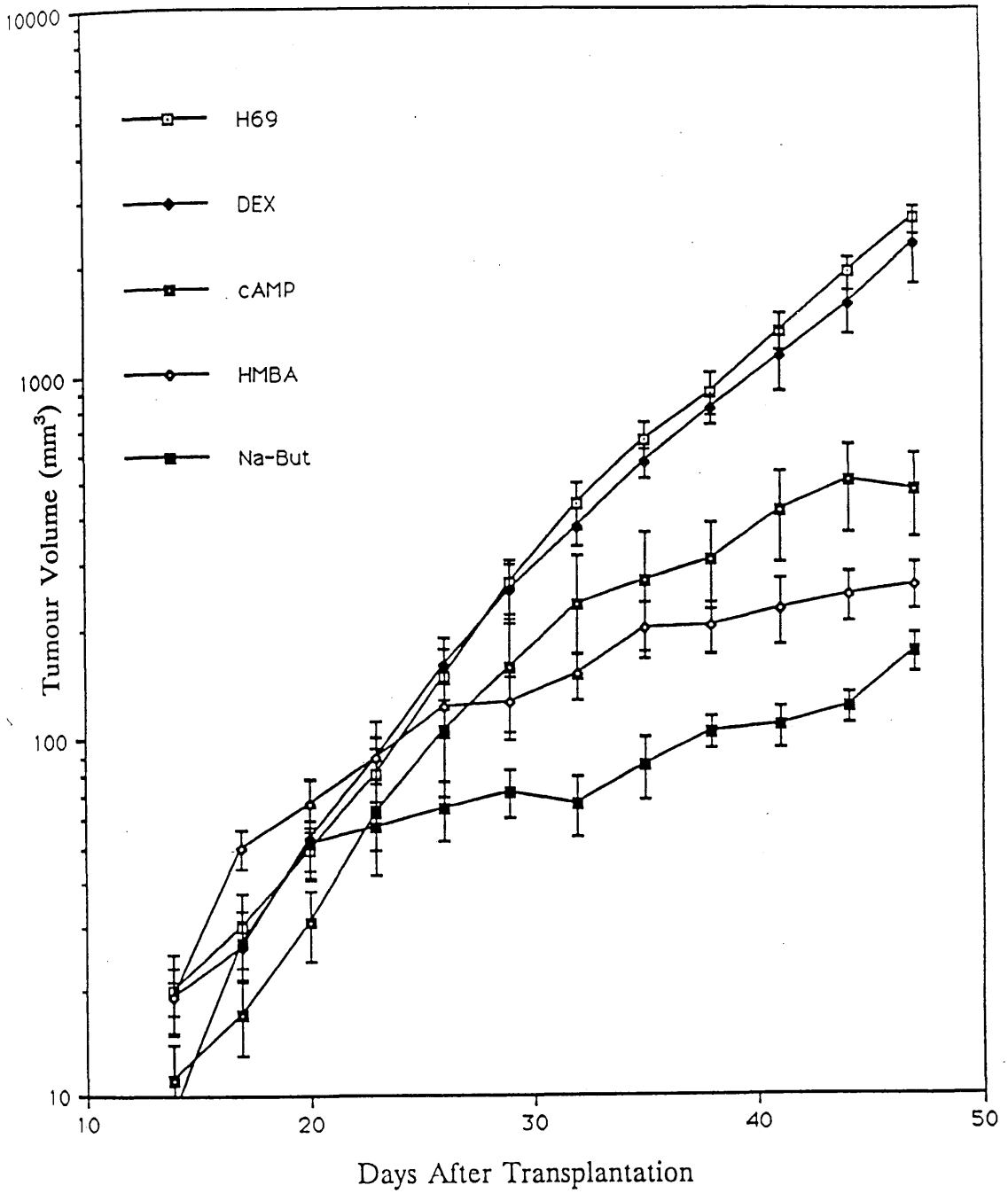



**Figure 3B.5:- Effects of phenotypic inducers on growth of H69 xenograft.**

H69 xenografts were produced in athymic nude mice following s.c transplantation of 2x2x1 mm tumour piece in each animal. Animal bearing tumours of approximately same size were randomly assigned to test and control groups. Each of the test animals received a daily i.p dose [see Table 3.2] of the phenotypic inducer for 14 days, while controls received same dose of drug vehicle. Tumour size was measured twice a week in maximum and minimum cross diameters, and tumour volume was determined as described in General Methods. Each point represents mean ± SEM (bars) from at least 7 replicate observations, from at least two separate experiments.

**Table 3B.6:- Effects of phenotypic inducers on the growth of H69 xenografts in nude mice.**

| INDUCER (Drug) | Tumour Bearing time (days) | Tumour doubling time (days) | Duration of treatment (days) | Recovery period (days) | Maximum tumour volume ($cm^3$) |
|---|---|---|---|---|---|
| Control | 30 | 4.6 ± 0.1 | 14 | 16 | 2.3 ± 0.3 |
| HMBA | 30 | *13.8 ± 1.1 | 14 | 16 | *0.3 ± 0.1 |
| Na-But | 30 | *21.1 ± 1.5 | 14 | 16 | *0.2 ± 0.0 |
| dbcAMP | 30 | 6.7 ± 0.6 | 14 | 16 | *0.5 ± 0.1 |
| Dex | 30 | 4.3 ± 0.2 | 14 | 16 | 2.7 ± 0.2 |

Approximately 2x2x1 mm tumour pieces were transplanted subcutaneously into flanks of nude mice, at day 0. Tumour take was 100% after a latent period of around 17 days. Treatment by daily i.p injection of drugs as shown in Table [3.2] was started when tumours reached a volume of $\geq 33$ $mm^3$. Infected or grossly necrotic tumours were not included in results. Data are Mean ± SEM of at least 8 replicate values from at least 2 separate observations.

*$P<0.0001$ (analysis of variance and Bonferoni adjustment).

### 3B.3.8 Tumour Morphology.

Grossly, control tumours of H69 grew with an irregular nodular outline, the overlying skin showing prominent vasculature, with frequent ulceration, and macroscopic evidence of tumour infiltration into the surrounding host tissues. The cut surface showed evidence of necrosis and cavitation. The tumour was firmly attached to both skin and deep tissues.

Animals treated daily with HMBA, Na-But, or dbcAMP [see above, Table 3B.1] for 14 days had smaller sized tumours, with well defined edges, smooth surface, and sparse blood vessels. Tumours were adherent to the skin, but attachment to the deep tissues was not firm. Cut section showed central areas of dead tissue with viable tissue around. Tumours were firm. Following treatment with dexamethasone tumour showed features similar to control, but with more necrotic tissues and central cavitation.

Histologically, the control tumours showed necrotic areas, with viable tumour tissues interposed, as small islands. The tumour appeared poorly differentiated with both cellular and nuclear pleomorphism and also showed high mitotic activity [Plate 3B.3 a]. Local invasion into surrounding host tissue was found in all cases.

Tumours treated with dbcAMP showed moderate mitotic activity, and trabecular/alveolar-like morphology and less pleomorphism; however, overall features were not significantly different from the parental tumour [Plate 3B.3 b]. Tumours treated with HMBA [Plate 3B.3 c] and sodium butyrate [Plate 3B.3 d] had similar features to dbcAMP treated tumours. Dexamethasone treated tumours showed more central necrosis compared to control, but similar histology. All treated tumours showed less local invasion. These results were confirmed by more than one pathologist.

### 3B.3.9 Expression of SCLC Markers in Tumours.

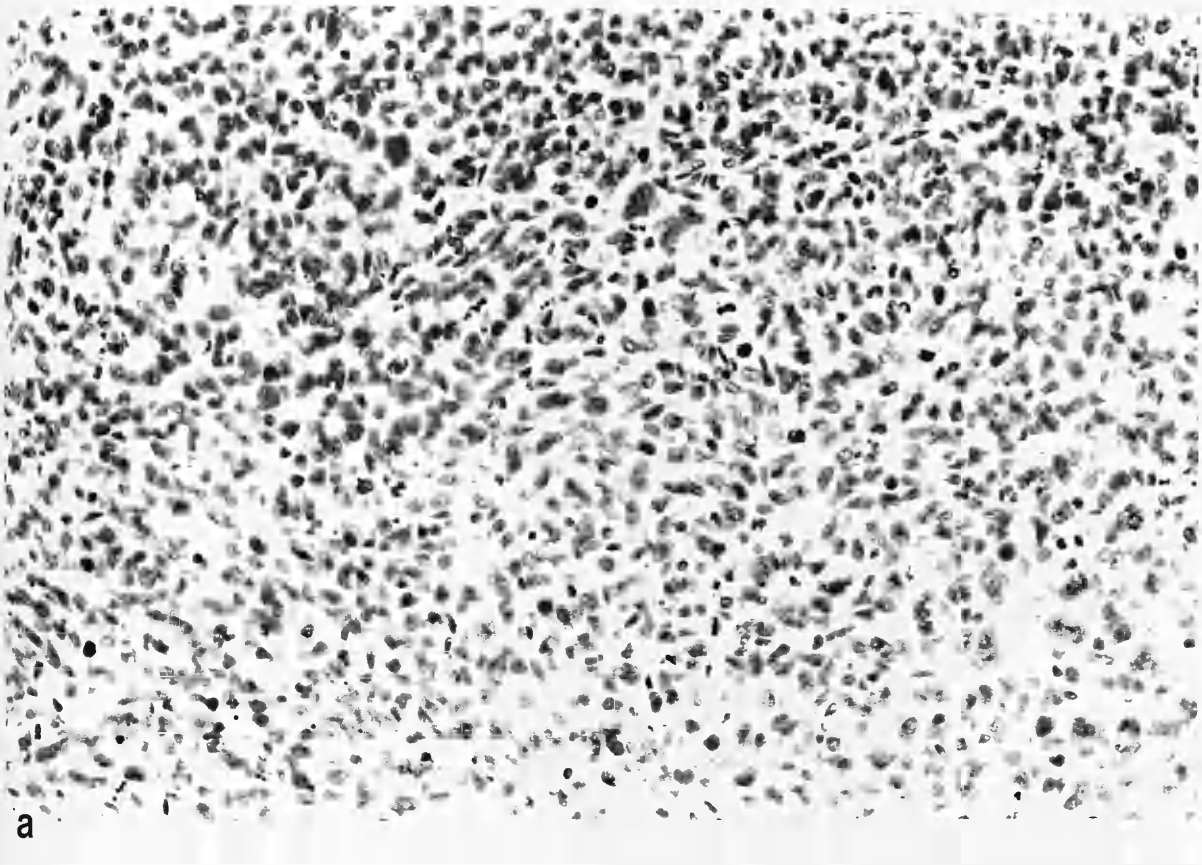
Tumours were processed for immunohistochemical staining as outlined above. Control tumour stained positive for all markers i.e. BLI, NSE, and chromogranin-A, however, staining was less intense in tumours compared to monolayer. There was no significant difference in the pattern of staining caused by treatment with any of the inducers, either in cell culture or in tumours.

**Plate 3B.3:- Effects of in vivo adminstration of inducing agents on tumour morphology.**

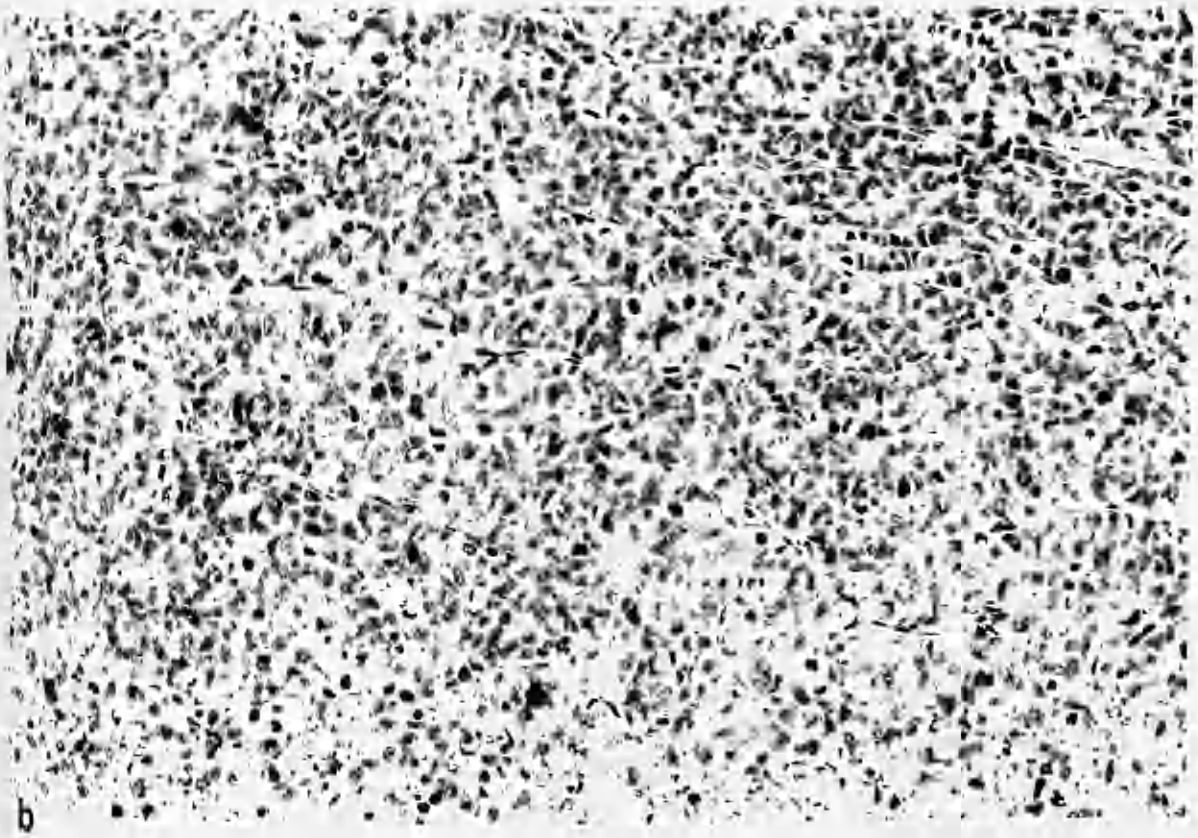
(a). H69 control tumour, shows undifferentiated pleomorphic tumour, with a high mitotic rate (x 247, H&E).

(b). H69 tumour treated with dibutyryl cyclic AMP (2 $\mu$g/gm body wt) for 14 days showing a suggestion of alveolar/trabecular morphology. Subjective mitotic figures are less frequent than in control (x 247, H&E).

\continued



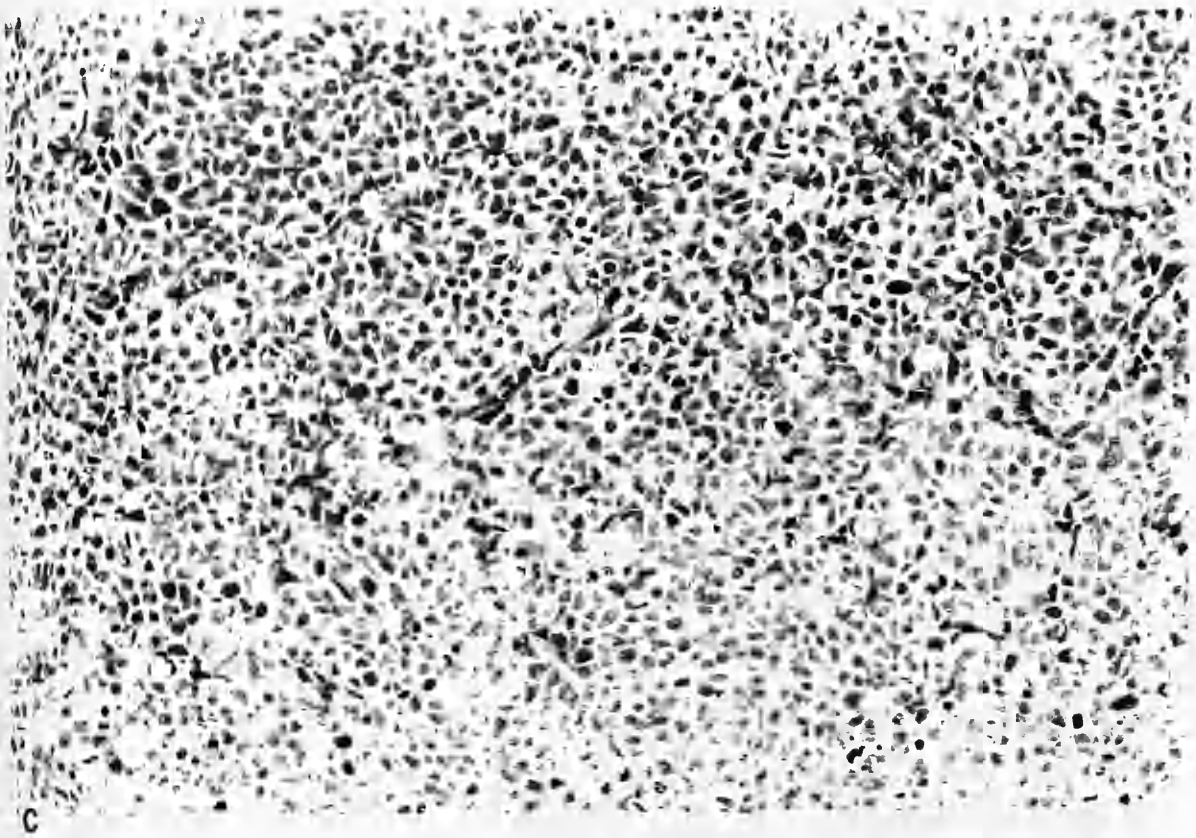
a



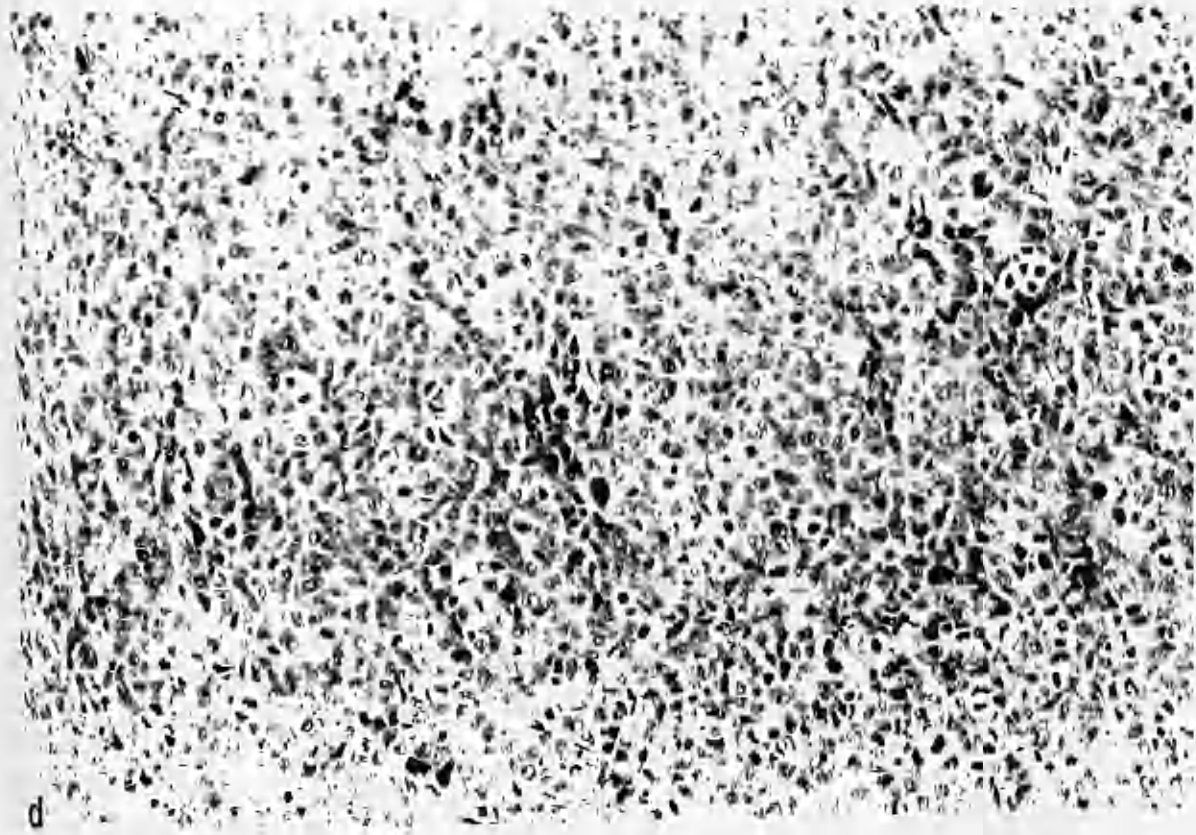
b

(c). H69 tumour treated *in vivo* with HMBA (1.5 mg/gm body wt) for 14 days shows features similar to (b), but the changes are less prominent compared to those induced by dibutyryl cyclic AMP (x 247, H&E).

(d). H69 tumours treated with sodium butyrate (2 $\mu$g/gm body wt) for 14 days again shows similar features as above (b&c), with less mitotic activity (x 247, H&E).



c



d

## 3B.4 DISCUSSION

While the phenotypic variation observed in the previous chapter may be due to genetic selection, similar behaviour of the derivative lines suggested that this may not be the case. This suggested that the expression of the phenotype may progress spontaneously to a more adherent NSCLC-type under regulatory control. To investigate this, SCLC H69 cells were exposed to various phenotypic modulators, known to have activity in other systems [Egorin *et al.*, 1988; Philippe *et al.*, 1987; Guiffre *et al.*, 1988], and any change in SCLC phenotype was measured. While it was relatively easier in other systems to show whether the effects were differentiation associated or otherwise, it is not straightforward in SCLC, because of the lack of appropriate markers of differentiation. SCLC may represent an intermediate progenitor tumour cell population, characterized by NE-cell markers expression, and sensitivity to cytotoxic agents. As the cell population progresses towards a more differentiated phenotype, they may change morphology, stop expressing NE-cell markers, become more resistant to therapy, and grow more slowly and start expressing new lineage specific markers. Previously, effects of the agents had been demonstrated in SCLC cell lines [Murray, 1989], where an alteration in marker expression was found following a 72 hours exposure. The changes in the phenotypic expression were not consistent among different SCLC cell lines, but were consistent within one cell line. The objectives of this study were to investigate the effects of prolonged treatment with the phenotypic inducers, within one cell line, alone and in different combinations, and both *in vitro* and *in vivo*. A period of 7 days (*in vitro*) or 14 days (*in vivo*) was selected for the induction of phenotypic changes in the present study, because similar exposure times used in other studies have shown significant effects [Chun *et al.*, 1986; Toscani *et al.*, 1988].

Now the question arises, is the phenotypic shift in SCLC differentiation associated? H69 cells treated with the phenotypic inducers (HMBA, Na-But, and dbcAMP) showed suppression of malignancy associated properties and expression of differentiated phenotype, both *in vitro* and *in vivo*. The growth of cells in culture was stopped as long as cells remained in drug containing medium, however, following drug removal cells were able to regain exponential growth, suggesting a cytostatic effect. Treated cells formed only fewer and small rounded colonies, compared to more numerous and large irregular colonies of the control cells. This

may imply increased cellular homogeneity in treated colonies possibly by effects of the inducer on the stem cells. It may be a differentiation associated effect; as tumour cells differentiate, they start receiving normal growth regulatory signals that prevent cellular diversification. Alternatively, phenotypic modulators could have prevented tumour cell diversification by inhibiting cell proliferation and thereby suppressing clonal expansion. It would be interesting to investigate this further by a comparative study of treated and untreated H69 colonies for cellular heterogeneity. Single viable cells in the treated cultures suggested that the effects were non-cytotoxic, and cytostatic. Similarly, the H69 xenografts treated with the inducers showed significant growth retardation. This *in vitro* and *in vivo* correlation suggests that the agents may have significant therapeutic potential.

The molecular mechanisms of growth arrest by these agents are only partly understood in other systems, where they appear to control the expression of specific genes, including both proto-oncogenes and oncogenes [Sporn *et al.*, 1985]. They have been shown to prevent the progression of a premalignant cell to a malignant cell, or in certain cases they could arrest or reverse the process of malignant transformation [Sporn *et al.*, 1985]. Polar planar compounds (e.g. HMBA) can induce differentiation of malignant cells, and the differentiated cells lose their ability to proliferate, to clone in suspension, and to propagate when transplanted into animals [Spermulli & Dexter, 1984]. HMBA induces a transient increase in intracellular level of cyclic adenosine 3':5'-monophosphate, followed by a prolongation of G1 phase, during differentiation in murine erythroleukemia cells [Gazitt *et al.*, 1978]. In the present report, HMBA had significant phenotypic effects on H69 cells *in vitro*. Further studies on cell kinetics are required to establish whether effects of HMBA on H69 cells are due to arrest in G1, by above mechanisms. This may involve investigating the effects of modulating agents by growth curve analysis, and analysis of cellular DNA synthesis, and growth associated genes (e.g c-myc, c-fos).

Sodium butyrate has been shown to cause an inhibition of DNA synthesis by arresting the treated cells in the G1 stage of the cell cycle [Wintersberger *et al.*, 1983]. It also modulates the expression of a variety of genes [Andrews & Adamson, 1987], and induces terminal differentiation in other systems [Philippe *et al.*, 1987]. It appears to be an active process which either directly or indirectly represses the expression of some genes such as those involved in cell proliferation (e.g c-myc)

while inducing the expression of others such as those involved in cell differentiation e.g. c-myc, c-fos [Toscani *et al.*, 1988]. H69 cells treated with Na-But showed complete growth arrest. It had the maximum cytostatic effect among the inducers used in this study. Na-But in the present study may have induced these phenotypic changes in H69 cells, by expression or suppression of specific genes related to growth and differentiation. Future studies may be carried out to find an answer to these possibilites by looking at the effects of, myb or fos transfection on H69 cell phenotype and also by antagonizing their effects by antisense oligonucleotides.

Dibutyryl cyclic AMP can exert its effects due to both released butyrate and cAMP. The activities of many genes are regulated by binding of ligands to cell surface receptor proteins. Transmission of the signal from the plasma membrane to the nucleus, where regulation of transcription occurs, is mediated by second messengers. Cyclic AMP acts in the signal transduction pathway for a number of different external stimuli [Alberts *et al.*, 1889]. Cyclic AMP exerts its effects in animal cells by activating an enzyme called cAMP dependent protein kinase (A-Kinase). A-Kinase catalyses the transfer of the terminal phosphate group from ATP to specific serine or threonine residues of selected proteins in the target cell, resulting in activation or inactivation of a number of different enzymes. H69 cells treated with dbcAMP exhibited a growth suppression throughout drug exposure, similar to sodium butyrate. However, following drug removal, cells were able to regrow exponentially, implying reversibility. So cyclic AMP in these studies may have induced more differentiation related effects by the signal transduction mechanisms and this requires more investigations to elucidate the effects of cyclic AMP further. It may be that the effects of cyclic AMP observed in the present study were due to cleaved butyrate as seen above. Therefore, it would be interesting to analyse these finding further by specifically blocking the effects of one of the compounds at a time.

Production of neuroendocrine markers in SCLC may indicate an undifferentiated, intermediate cell phenotype in the spectrum of lung cancer progression or a tumour differentiated towards a more neuroendocrine phenotype, after originating from a stem cell compartment, under the influence of different microenvironmental factors. Some of these may be cell derived autocrine growth factors. Bombesin has been proposed as an autocrine growth factor in SCLC. The action of bombesin is believed to be mediated via a membrane receptor [Moody *et*

*al.*, 1985], which activates a post-receptor signal transduction mechanism resulting in mitogenic response. Other studies *in vitro* have demonstrated a role of bombesin as an autocrine growth factor in SCLC [Cuttitta *et al.*, 1985; Woll & Rozengurt, 1988]. In the present study, there was no significant change in cellular BLI, however, a slight increase in secreted BLI was found after treatment with dbcAMP alone, and also in combination with HMBA or Na-But. As the increase in released BLI exceeded the fall in cellular BLI a total increase rather than simply secretion is implied. Preliminary data from the present study have suggested that bombesin may not have an autocrine growth effect on H69 cells, in contrast to the above hypothesis. Further studies could be carried out, to determine how the phenotypic inducers could modulate the cellular and secreted BLI in H69 cells, perhaps by labelling, and also by investigating the effects of inducers on BLI inhibitors. It could be that the increase in secreted BLI was due to inhibition of BLI inhibitors.

Treatment of H69 cells with HMBA either alone or in combination with other inducers significantly reduced DDC. Similarly, Na-But either alone or in combinations with other inducers also reduced DDC levels significantly, and the maximum effect was found in combination with HMBA. Cyclic AMP in combination with HMBA or Na-But decreased DDC expression, suggesting an additive cytostatic effect. These data are consistent with previous observations [Murray, 1989], where a 50% reduction in DDC was found following a 72 hours treatment with HMBA. More investigations are required to delineate the role of DDC in growth of SCLC. DDC has no direct role in cell growth, however it may alter cell growth by catacholamine regulation. The effects of catacholamine agonists (e.g noradrenaline) and antagonists (e.g phentolamine, propranalol) may be investigated in future studies. Selection of appropriate models from lung cancers, with relatively lower and higher levels of marker expression may show whether NE-cell markers have a definite role to play in the growth regulation of human lung cancer.

Morphological changes have been observed following treatment of H69 cells *in vitro* with HMBA and Na-But. HMBA treated H69 cells formed small regular aggregates in culture, while Na-But treated cells showed regular, but, larger sized aggregates, compared to the irregular type aggregates of the parental line. More cells were found sticking to the substrate, similar to the cells found spontaneously in H69 culture adhering to the substrate as described in the previous chapter,

implying again, a cytostatic rather than cytotoxic event. The changes could be due to alterations in cell surface molecules, either directly or via specific gene expression (e.g. glycosyl transferase activity, inhibition of proto-oncogenes). The modification in cell surface glycoproteins and proteoglycans by chemical agents like retinoids have been shown to induce cytostasis [Lotan *et al.*, 1978]. Cell surface molecules may be altered in several different ways, following treatment with inducers. They may be deleted, truncated, or altered by sialation of carbohydrate moieties of cell membrane proteins. This could modify the receptors, cell adhesion molecules or matrix interactions. Alteration in aggregate size and substrate attachment implies an alteration in cell-cell adhesion and cell-matrix adhesion molecules. Morphological change may also be due to cytoskeletal alteration due to phosphorylation or dephosphorylation of vinculin and tubulin. Changes have also been found in H69 tumours grown in nude mice. These changes, though not very prominent, may be significant. A trabecular or alveolar type pattern emerged in treated tumours, suggesting a shift towards a more differentiated phenotype. It would be interesting to see the effects of cytotoxic agents on these induced tumours *in vivo*. More differentiated cells may become resistant to cytotoxic drugs. It has been shown [Murray, 1989] that induction of SCLC cell lines with chemical agents (HMBA, Na-But, dbcAMP, and Dex) for 72 hours did not have any significant effects on sensitivity of cells to cytotoxic drugs (i.e adriamycin, vincristine, and VP-16). It may be that 72 hours time was not enough to exert phenotypic effects that could have altered the drug sensitivity. Therefore, it may be appropriate to test sensitivity after 7 days pretreatment with the inducers.

Pretreatment of H69 cells with HMBA or Na-But delayed invasion without affecting cell viability. The effects could be due to growth retardation as a result of cytostasis, although Storme *et al.* [1985] found inhibition of cell proliferation with 5-fluorouracil did not inhibit invasion in this assay. More important may be the possible cell surface changes. Changes in matrix receptor could result in an altered interaction between the cell and matrix components. Carbohydrates exposed at cell surface might be implicated in invasion of tumour cells [Smets *et al.*, 1984]. However, both treated and untreated cells were able to attach to the heart fragment, and as invasion was delayed in treated cells compared to the control, this may implicate cytoskeletal interaction and cell motility rather than adhesiveness. The effects were reversible, once the drug was removed. This suggests a regulatory

phenotypic effect, rather than an irreversible change in gene expression, and might be associated with terminal differentiation.

In treated animals, the tumour cells were found in subcutaneous fat only, whereas in untreated control tumours invasion was found in subcutaneous fat, skin, and deep into the muscles, with the evidence of the destruction of the invaded host tissues. Tumours treated with inducers were not processed for evidence of metastatic growth, in this study, because of the large number of samples and the shortage of time. However, this remains an interesting avenue of investigation that may well be worth pursuing in future studies. H69 is metastatic, and it may be that treated tumours could be induced to a non-metastatic phenotype, as there was only minimal invasion in treated tumours. An anti-metastatic phenotypic effect of a chemical agent may have significant therapeutic implications.

Therefore, it can be concluded from these data that the effects of phenotypic inducers on H69 cells are cytostatic, non-cytotoxic, and related to a more differentiated phenotype (morphologically, anti-invasive, anti-clonogenic, anti-tumorigenic), compared to the malignant phenotype of the H69 cells, both *in vitro* and *in vivo*.

The clonal nature of H69 cells implies that the malignancy begins with a single aneuploid cell whose progeny proliferate to produce clinical disease. It follows that in order to achieve clinical cure of malignant disease, the last aneuploid malignant cell must be eradicated. Reversibility and lack of any significant loss of viability of cells treated with the inducers in this study suggests that the effects were cytostatic and not cytotoxic. This could lead to the possible combination of cytotoxic and cytostatic agents in a coordinated schedule, as has been proposed by Lotan and Nicolson [1988], and has also been shown in a human colonic carcinoma cell line by combination of 5-Fluorouracil with N-methylformamide [Zupi *et al.*, 1988], suggesting a possible role of this potential therapeutic approach in future. In contrast to the conventional cytotoxic therapy, whose objective is tumour cell destruction, the rationale for using cytotoxic therapy alternating with cytostatic therapy in the clinic would be to kill tumour cells using cytotoxic agents while to prevent the diversification and heterogeneity of surviving tumour cells. The cytostatic agents could be used to suppress tumour cell diversification during therapeutic recovery periods between cycles of cytotoxic therapy. This therapeutic regimen can hopefully improve the outcome of cancer treatment in future.

# CHAPTER FOUR

## PHENOTYPIC REGULATION IN HUMAN NON-SCLC

This Chapter consists of three parts. The first part deals with the effects of stromal interaction on the tumour cell phenotype, and its relationship to chemosensitivity, the second part describes the possibility of induction of a differentiation associated phenotype in malignant cells, by allowing them to grow in a histotypic tissue culture model system, while the third part compares the phenotypic changes in histotypic culture with *in vivo* growth characteristics of the tumour cells.

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## 4.1 INTRODUCTION

The role of substratum in growth and differentiation of cells has been described [Kleinman *et al.*, 1981]. Extracellular matrix (ECM) has been reported to be an important substratum for cultured cells. Normal epithelial cells generally attach, grow and maintain differentiated functions, when components of the basement membrane or stroma are used as the substratum instead of plastic [Emerman and Pitelka, 1977], although, the effects of an ECM on growth and differentiation appear to be different in different cell types [Gospodarowicz *et al.*, 1984], and it is still unclear which ECM components are related to such effects. Morphogenesis or three-dimensional development of epithelial tissue is brought about by its close association with the mesenchyme *in vivo*. This process can be partially simulated *in vitro* by growing epithelial cells cultures on type 1 collagen with the formation of multicellular structures [Emermann *et al.*, 1979]. A specific role of collagen in the maintenance of the functional state of membrane structures associated with specific functions has been suggested for hepatocytes [Michalopoulos and Pitot, 1975], mammary cells [Emerman *et al.*, 1979], and thyroid epithelium [Chambard *et al.*, 1981; 1982].

It has been shown that normal respiratory epithelium expresses differentiated functions when grown in the proper micro-environment [Wu and Wu, 1984; Wu *et al.*, 1985]. An alveolar cell carcinoma cell line A549 has been shown to produce the differentiated phenotype when grown on top of a filter near the air-liquid interphase in culture [Speirs & Freshney, 1990].

The work presented in this Chapter was an effort to investigate whether a cell line derived from a human lung carcinoma could be manipulated to express a differentiated phenotype when grown in optimal culture conditions.

The WIL cell line was selected among the different NSCLC cell lines available. It showed an intermediate response to the cytotoxic agents. It had the property of dome formation in culture implying a capacity to differentiate. It was originally derived from lung adenocarcinoma, and was mucin positive. Histologically, WIL has also been described as both a squamous and an adenocarcinoma, and may in fact be a stem cell which could become either with the appropriate inducer and/or micro-environment. Manipulation of its phenotype may influence its

malignancy and/or differentiation associated properties. Hence, if WIL can be manipulated from a stem cell-like phenotype to a more mature normal or differentiated phenotype, then it might be a good model for the study of relationship between malignancy and differentiation and between differentiation and chemosensitivity. Previously, it has been shown that WIL cells treated with dexamethasone exhibited reduced malignancy associated properties [McLean *et al.*, 1986]

Assuming that tumour cells are primitive progenitor-like cells, which exhibit a more fetal type (quasi-fetal) phenotype, it was appropriate therefore to use a stromal cell line of fetal origin. The cell lines EWLU and LF113 were selected as they were derived from normal human fetal lung, and grew in culture with typical fibroblastic morphology.

Malignant tumours with a high proliferative rate are generally more sensitive to the effects of cytotoxic agents, while differentiated tumours or normal tissues are resistant. The second aim of the study was to assess the effects of phenotypic alterations on sensitivity of tumour cells to cytotoxic drugs. The specific aim was to compare the growth of tumour cells plated on plastic with and without stroma, on a filter well system, and on a collagen gel matrix and in the presence and absence of chemical inducers.

A third aim was to investigate whether a differentiated phenotype could be induced in the WIL cell line if a histotypic tissue culture environment was provided. The purpose was to develop a histotypic model, one which should represent *in vivo* conditions as closely as possible, and then to stimulate the tumour cells growing in such an environment with agents known to cause phenotypic changes in other systems.

Various chemical substances have been shown to induce differentiation associated functions in cells of respiratory epithelium. The agents used in the present study included: retinoic acid, TGF-$\beta$, dexamethasone, HMBA, Na-But, and dibutyryl cyclic AMP (see Chapter one).

A number of different parameters were selected for the determination of a shift in tumour cell phenotype by stromal influences. These included morphological changes, effects on tumour cell growth, alterations in mucin secretion, and changes in expression of cytokeratins, vimentin, epithelial related membrane proteins (human

milk fat globulins 1 & 2, HMFG 1 & 2) epithelial membrane antigen (EMA), and the oncofetal proteins, carcinoembryonic antigen (CEA), and sensitivity of cells to the cytotoxic drugs.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Cell Lines

A panel of non-small cell lung carcinoma (NSCLC) cell lines was used [Table 4.1], for cytotoxicity assays. All cell lines grew as adherent monolayer, in F10:DMEM.

**Table 4.1:- Cell lines.**

| Cell line | Origin | Lineage | Pathology | Source |
|---|---|---|---|---|
| WIL | human lung | epithelial | adeno ca | Haddow, Sutton |
| [1]WIL+b | human lung | epithelial | adeno ca | Medical Oncol |
| A549 | human lung | alveolar | alv cell ca | ATCC, MD |
| SKMES | human lung | epithelial | sq cell ca | ATCC, MD |
| NCI-H125 | human lung | epithelial | adeno ca | NCI, MD |
| [2]MOG-EWLU | human lung | fibroblast | normal | Med Oncol |
| [2]MOG-LFII3 | human lung | fibroblast | normal | Med Oncol |
| MOG-MLC | mouse lung | lung | normal | Med Oncol |

[1]The cell line derived from WIL xenograft. [2]Fetal. ATCC: American type culture collection

### 4.2.2 Cytotoxic Drugs

Vincristine was obtained from Sigma Co Ltd, and adriamycin (doxorubicin) was obtained from Farmitalia Co Ltd. The drugs were dissolved in PBS and diluted in culture medium in final concentration.

### 4.2.3 Chemical Inducers

Retinoic acid, sodium n-butyrate, HMBA, cyclic AMP were obtained from Sigma Co Ltd, and TGF-$\beta$ was kindly provided by Dr David Kerr. Retinoic acid was dissolved in ethanol, other chemicals were dissolved in complete culture medium. The doses used in this study are shown in table 4.4 (see below).

### 4.2.4 Tumour-Stromal Interaction

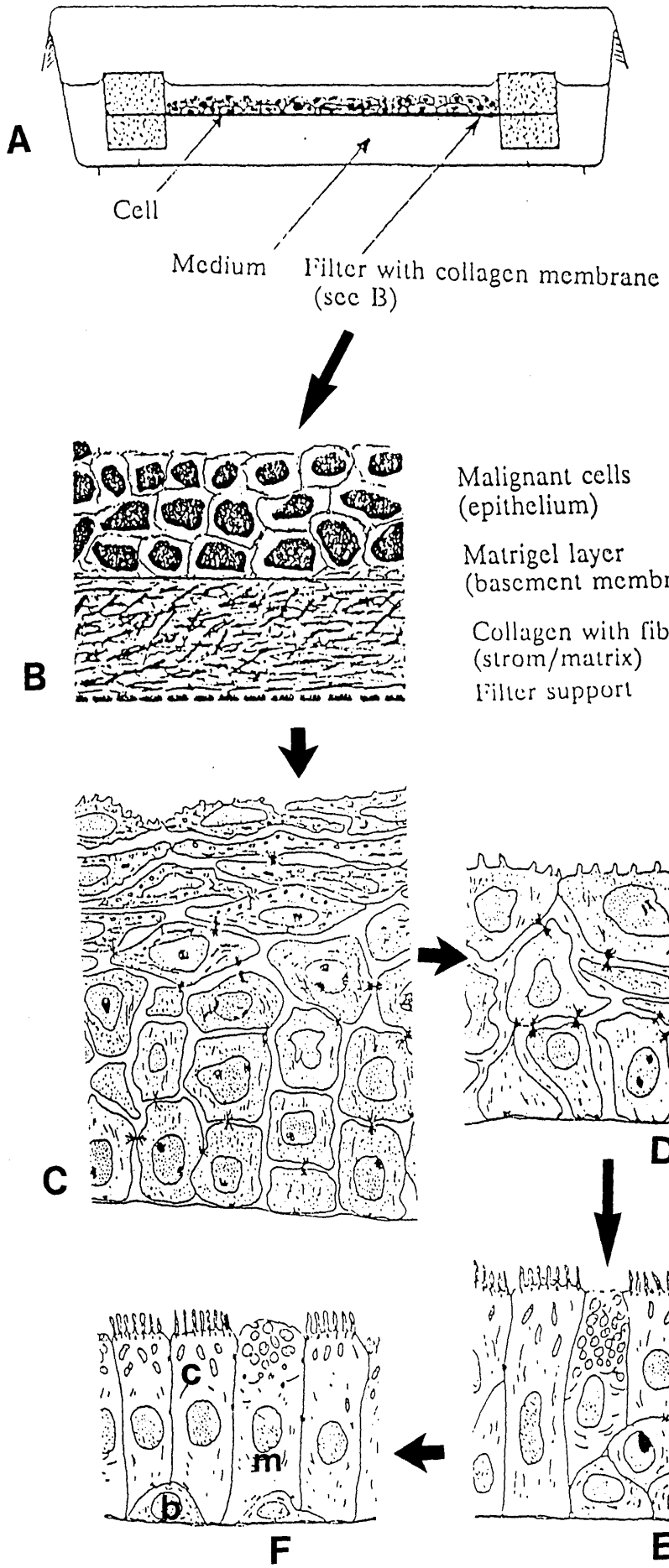
The tumour cells were co-cultured with fibroblasts in conventional monolayer for different time periods, after which they were examined using phase-contrast microscopy of living cultures, or light microscopy of Giemsa stained preparations, immunohistochemical staining for expression of different markers, and histochemical detection of mucin synthesis. Cells were also treated with drugs in co-culture to observe any effects of stromal interaction on tumour cell sensitivity to cytotoxic drugs by clonogenic assay.

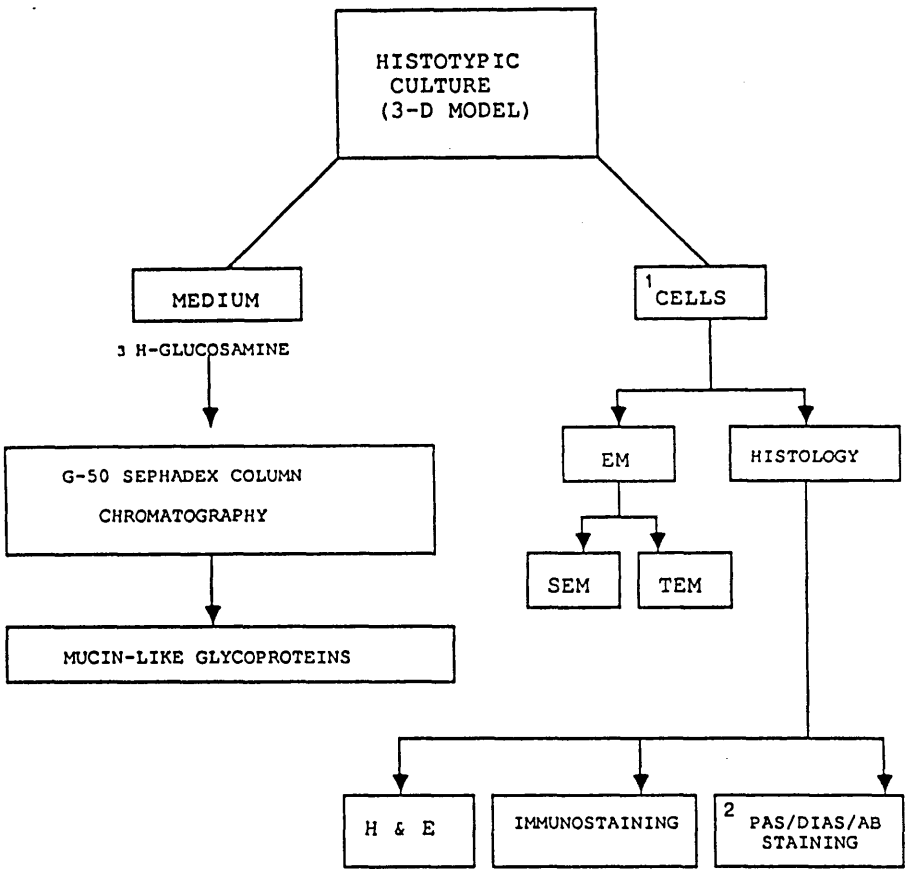
### 4.2.5 Development of Histotypic Tissue Culture Model

A three dimensional complete histotypic tissue culture model was constructed for induction of differentiated phenotype in malignant (WIL) cells [Figure 4.1]. It consisted of a polycarbonate filter [8 $\mu$, Nuclepore] suspended in a filter unit, which was used as a basic supporting structure, and secondly to keep cultures at an air-liquid inter-phase (respiratory epithelium) for maximum inductive effects. A complete fibroblast layer was grown on the filter, then a layer of collagen gel containing fetal lung fibroblasts was laid on the top of fibroblast layer, representing stroma in an *in vivo* environment. Then a thin layer of matrigel (collagen type IV + laminin) represented the basement membrane in natural conditions. This was called a 3-D histotypic tissue culture model. Malignant cells were cultured in this inductive environment in presence of a known inducer of phenotypic changes. Culture medium was saved from filter well cultures and analyzed for mucin-like glycoproteins, the cells were processed for histology, immunohistochemistry and electron microscopy as shown in figure 4.2. Mucin was identified using the PAS/diastase-alcian blue stain. Cells were counterstained with haematoxylin for 1 min. Stained slides were then examined by light microscopy using a x 100 objective (oil immersion). Blue (acid mucins) and red (neutral mucins) granules were seen.

**Figure 4.1:- Proposed model for induction of differentiated phenotype in NSCLC *in vitro*.**

A schematic representation of sequences of steps leading towards a differentiated respiratory epithelium, in a three dimensional histotypic tissue culture model. The model provides optimum inductive microenvironment. Filter Well Assembly with malignant cells (A). Malignant cells (WIL) in histotypic tissue culture model grown at high cell density (B). An initial stage of polarity and orientation (C & D). Differentiated cells [E]. Normal epithelium (F), with basal (b), mucous (m), and ciliated columnar (c) cells.


A
Cell
Medium
Filter with collagen membrane
(see B)
B
Malignant cells
(epithelium)
Matrigel layer
(basement membrane)
Collagen with fibroblasts
(strom/matrix)
Filter support
C
D
E
F
c
m
b




1 Cells in complete histotypic culture (filter + Fibroblasts + collagen gel + mat rigel).

2 Periodic acid-Schiff/diastase/alcian blue.

**Figure 4.2:- Schematic diagram of experimental strategies.**

### 4.2.6 Biochemical Analysis of Mucin-like Glycoproteins.

Cells growing in histotypic culture were incubated with 10 $\mu$Ci/ml $^3$H-glucosamine (29 Ci/mmol, NEN) in 2 ml of medium/well. Following incubation with precursor, medium was collected by simple pipetting followed by an additional wash with 1 ml of the fresh medium. The spent medium was centrifuged to remove cell debris, and the supernatant was lyophilized and then stored at -20 °C. The lyophilized material was dissolved in PBS containing 2% v/v 2-mercaptoethanol and applied to a Sepharose G-50 column (Nick Columns, Pharmacia, 2.5 cm x 10 mm) and eluted with the same buffer. The excluded fractions which contained high molecular weight glycoproteins were pooled and subjected to enzymatic digestion. Hyaluronidase treatment was performed at 37 °C for 16 hours with 10U/ml hyaluronidase (streptomyces, Miles). Fractions of 75 $\mu$l were collected, mixed with 5 ml Ecoscint and radioactivity of the sample counted, in a scintillation counter (Packard). A sum of 5 void volume peak fractions was used as the amount of hyaluronic acid free mucin-like glycoprotein present in the sample. Results were expressed as percentage of total counts per sample.

## 4.3 RESULTS

### 4.3.1 TUMOUR-STROMAL INTERACTION IN CONVENTIONAL MONOLAYER

#### 4.3.1.1 Tumour Cell Morphology

WIL grew in culture as a monolayer of two cell populations [Plate 4.1 a] a rounded small cell population and a large epitheloid cell population. The growth of cells in 24 well plates showed characteristic phases [Figure 4.3], a lag of around 2 days, exponential phase of approximately 5 days, and a plateau after around 8 days of subculture. The fibroblasts alone grew in culture with characteristic morphology [Plate 4.1 b].

WIL cells growing in 50:50 (WIL:EWLU) co-culture showed morphological changes after a week, becoming more like cells of primary cultures from WIL xenografts or mouse lung growing in culture. Tumour cells grown on top of a

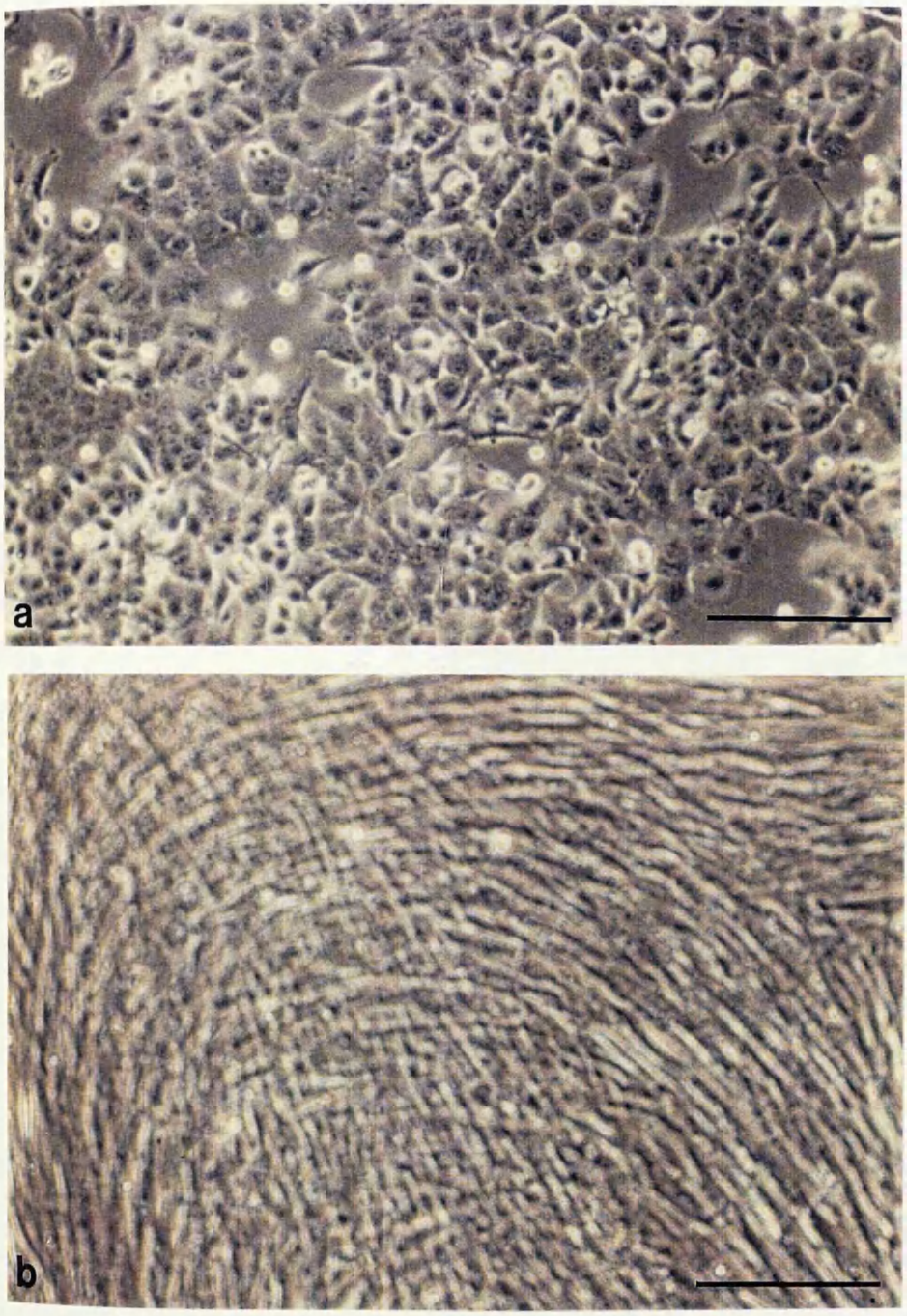



**Plate 4.1:- Morphology of tumour and stromal cells in culture.**

Tumour (WIL) cells showing epithelial morphology (a), and stromal (EWLU) cells showing typical fibroblastic morphology (b). One week after subculture on the plastic substrate (x 10 objective, phase-contrast, bar = 200 $\mu$m) .

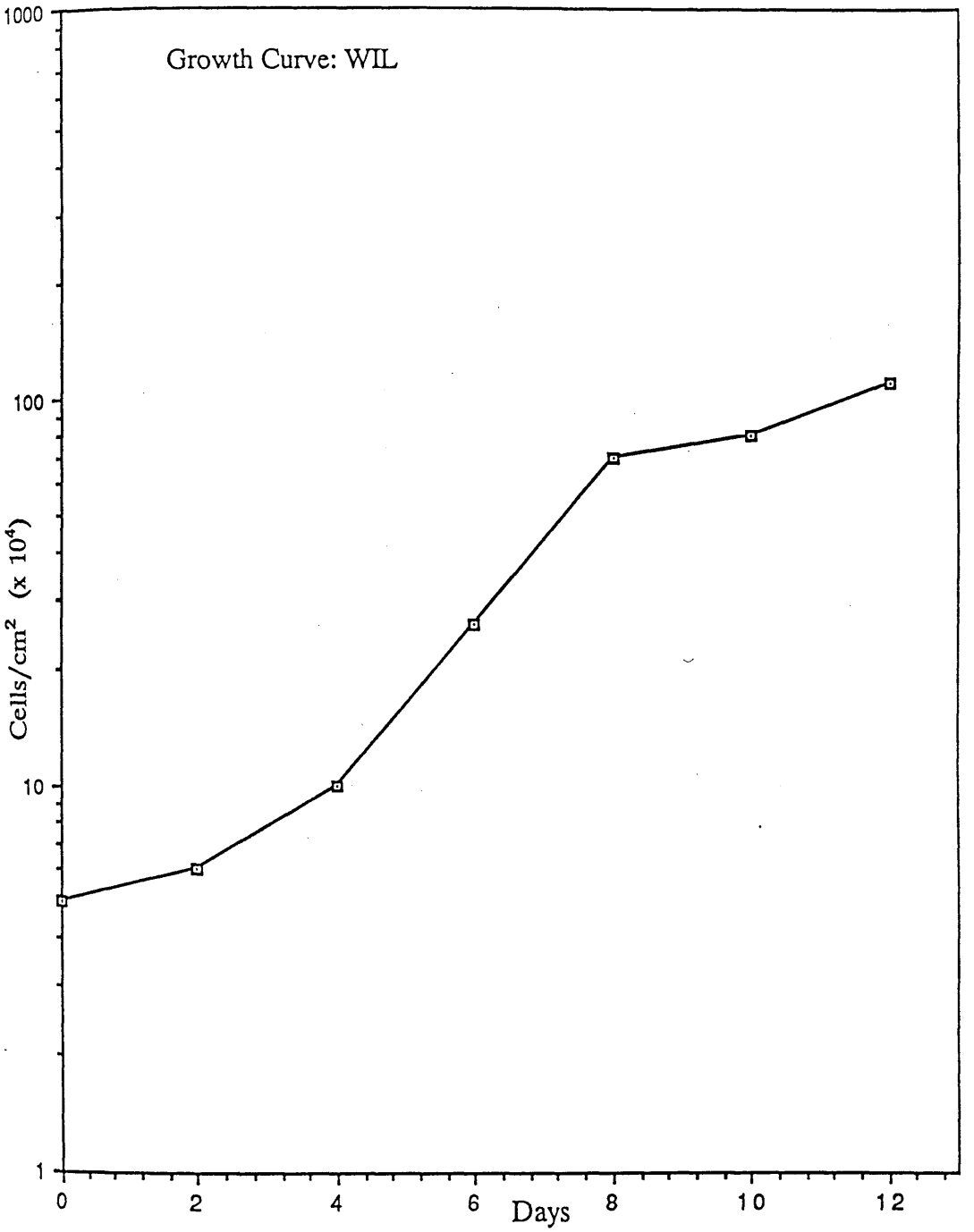



**Figure 4.3:- Growth curve of WIL cell line in vitro**

A semi-logarithemic plot for WIL cells grown in 24-well plates. The cells were seeded at $5x10^4$ cells/$cm^2$ on day 0. They were counted by trypsinization, of at least triplicate wells on alternate days and fed. Each data point represents mean value.

confluent dead fibroblast layer did not show any morphological change, implying that the effects were not just physical, but needed viable cell interaction. After 3 weeks circular ridges appeared which grew as a complex pattern [Plate 4.2] not found in pure cultures.

The histology of cells grown on the filter well did not show any gross change in co-culture compared to the cell lines alone.

#### 4.3.1.2 Marker Expression

There was no significant change in the expression of intermediate filaments or membrane proteins as identified by immunohistochemical staining techniques, between co-cultures and tumour cells alone [Table 4.2].

#### 4.3.1.3 Colony Forming Efficiency

There was a significant increase in the cloning efficiency of WIL cells following interaction with fibroblasts (297 ± 16), compared to WIL cells cloned alone (183 ± 18), the difference was consistent in 8 different observations (Mean ± SEM). Statistical analysis ($P < 0.001$) was carried out by analysis of variance and adjustment [see Figure 4.4].

#### 4.3.1.4 Expression of Mucin-Like Glycoproteins

The histochemical staining showed that there were no significant differences in the expression of mucin-like substances by the tumour cells grown either alone or with fibroblasts.

#### 4.3.1.5 Sensitivity of Tumour Cells to Cytotoxic Drugs.

The effects of vincristine and adriamycin on sensitivity of tumour cells alone or in co-culture are shown in table 4.3. The results are shown as $ID_{50}$ values obtained from dose response curves of the WIL cells to adriamycin [Figure 4.4 a] and vincristine [Figure 4.4 b]. There were no significant differences in the sensitivity of tumour cells by increasing duration of interaction (3 days, 1 week, 3 weeks) duration of drug exposure (24 h, 48 h, and 72 h), or by varying the cell ratios (WIL:EWLU, 30:70; 50:50; 80:20). The results are shown from one representative

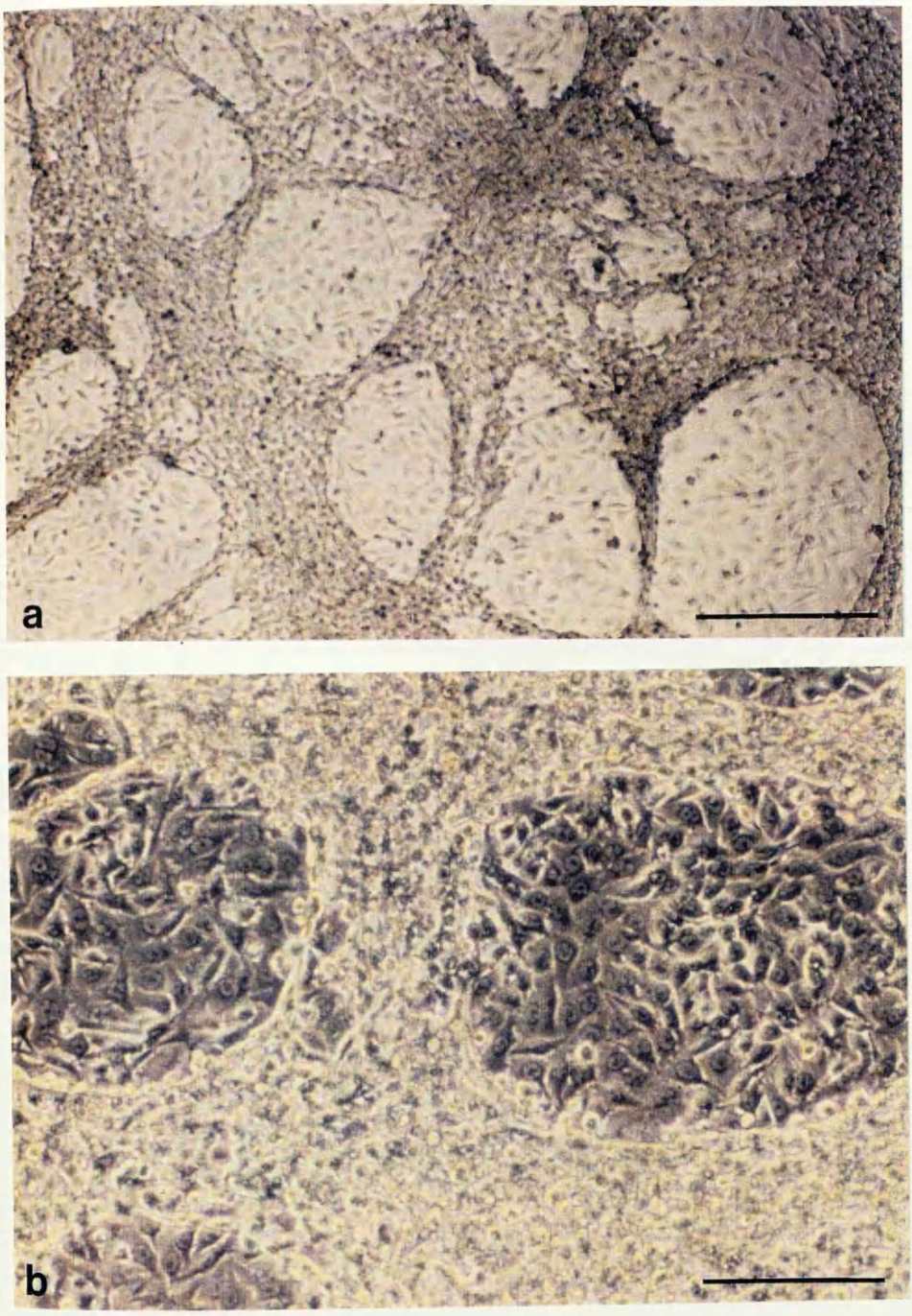



**Plate 4.2:- Morphology of tumour-stromal co-culture.**

WIL and EWLU cells plated at $1x10^5$ cell/$cm^2$ in 50:50 ratio showed formation of circular patterns after 3 weeks. (a). (x 10 objective, phase-contrast, bar = 200 $\mu$m). (b). (x 20 objective, phase contrast, bar = 100 $\mu$m).

**Table 4.2:- Effects of microenvironment on malignant cell phenotype.**

| Phenotypic Marker | Monolayer Culture | Histotypic Culture | Xenograft (Vivo) |
|---|---|---|---|
| Morphology | smooth surface | microvilli connections | adeno-squamous differentiation |
| Mucin | + | + + | + + |
| CEA | + + | + | + |
| HCG | + + | + | + |
| CAM5.2 | + + + | + + + | + + + |
| Polykeratin | + | + | + |
| Prekeratin | + | + | + |
| EMA | + | + | + |
| Vimentin | + + + | + + | + + |

CEA: Carcinoembryonic antigen. HCG: Human chronic gonadotrophin. EMA: Epithelial membrane antigen.

**Table 4.3:- Sensitivity of WIL cells to cytotoxic drugs with or without stromal interaction by clonogenic survival assay.**

| Cell line | [a]$ID_{50}$ | |
|---|---|---|
| | Vincristine ($\times 10^{-9}$ M) | Adriamycin ($\times 10^{-11}$ M) |
| WIL | 4.4 ± 0.3 | 7.5 ± 0.1 |
| WIL + EWLU | 4.0 ± 0.4 | 6.5 ± 0.9 |

[a]The $ID_{50}$ was defined as the drug concentration required to inhibit cell growth to 50% of that in untreated control. Values are arithmetic mean ± SEM from at least 3 experiments, carried out at different occasions.

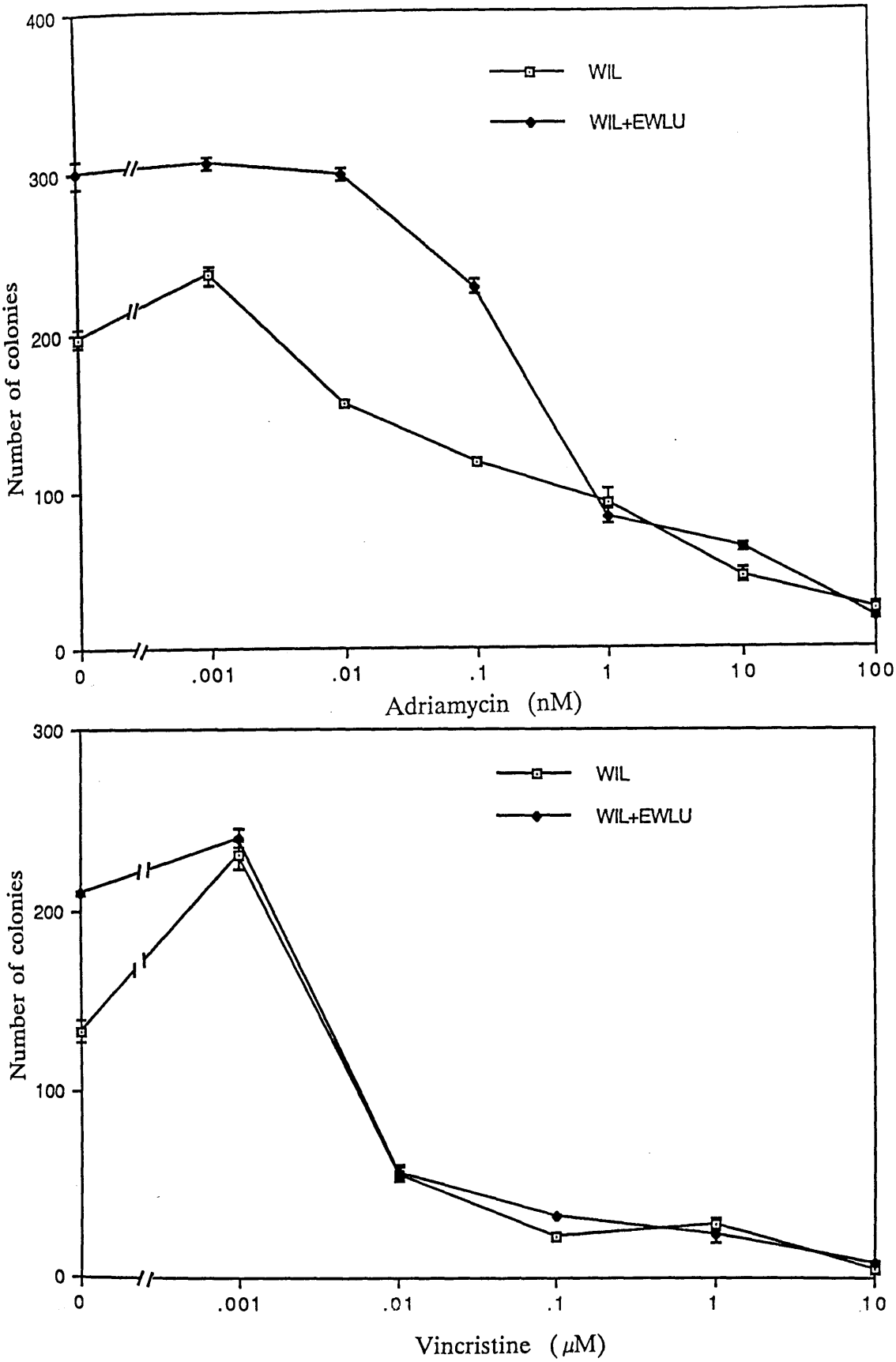



**Figure 4.4:- Chemosensitivity of WIL cells to adriamycin and vincristine (24 hrs exposure) in presence or absence of fibroblasts.**

The chemosensitivity was determined using clonogenic survival assay as outlined in the text. Each point represents mean ± SEM (bars) from 3 replicate values of one representative experiment. Similar results were obtained from more than 3 other experiments.

set of data (50:50 ratio; 3 days interaction; and 24 hours drug exposure). A mitogenic effect was observed at lowest drug concentrations, in both tumour cells alone and following fibroblastic interaction.

## 4.3.2 INDUCTION OF PHENOTYPIC CHANGES IN MALIGNANT CELLS, GROWN IN A HISTOTYPIC TISSUE CULTURE MODEL

### 4.3.2.1 Effects on Morphology

Histology of WIL cells grown on three dimensional (3-D) model showed that the cells were aligned in a single cell layer on top of the gel, with some evidence of polarity, however there was little evidence of cell-cell interactions and findings on light microscopic observations did not suggest any morphological change. There was no resemblance of the sections of histotypic culture to the histology of WIL xenografts [Plate 4.3, see Plates 4.1 a & 4.6]. However, differences were obvious compared to monolayer morphology in culture, as the cells formed more than one layer.

There was no difference in the morphological features of the cells grown in the presence or absence of different inducers; however, substrate had some effects on tumour cell morphology. While cells grown on plastic showed a spread morphology, and covered the substrate completely, on a collagen substrate the cells did not form a complete monolayer; rather individual cells or isolated small colonies were found on the gel, with a reduction in the total cell number compared to plastic substrate.

Transmission electron microscopy of the control WIL cells grown in the histotypic model was typical of epithelial interaction, with development of adhesion specialisations [Plate 4.4 a], cytoskeletal elements and formation of surface microvilli [Plate 4.4 b]. Some cells showed evidence of mucin production [Plate 4.4 c]. Desmosomes, intermediate filaments, and tonofilaments were also found in cells grown on plastic, but microvilli and surface specialisations were not well developed, compared to cells grown in 3D model.

Scanning electron microscopy showed two types of cells; cells growing on

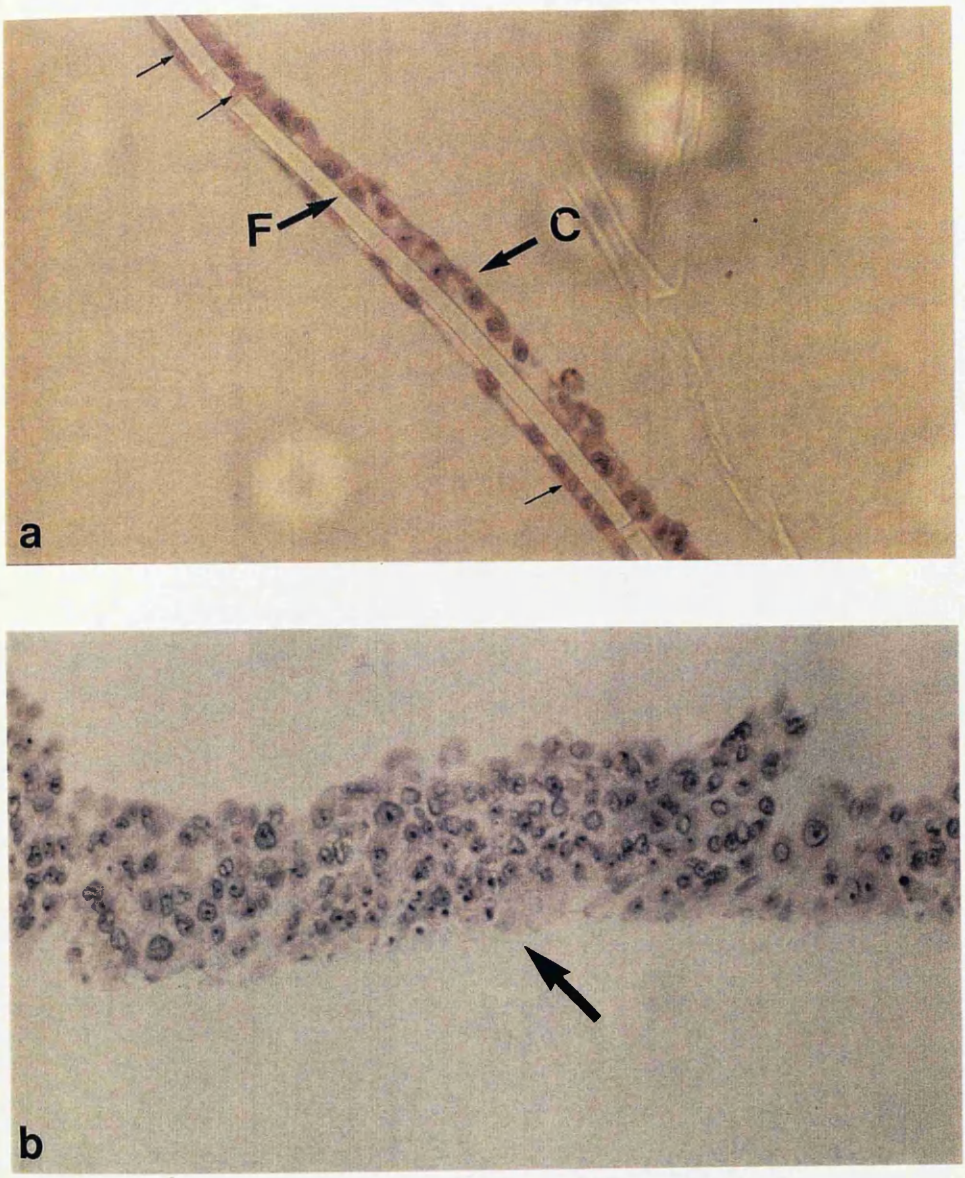



**Plate 4.3:- Morphology of cells grown on histotypic model**

(a). A single cell layer (C) is shown on top surface of Filter (F). The cells can also be seen crossing to the undersurface of the filter membrane (arrows).

(b). Culture from (a) 2 weeks later show approximately six cell layers. The filter membrane was separated from under surface (arrow) of the cell layer during histological processing (x 20, H&E, bar = 100 $\mu$m).

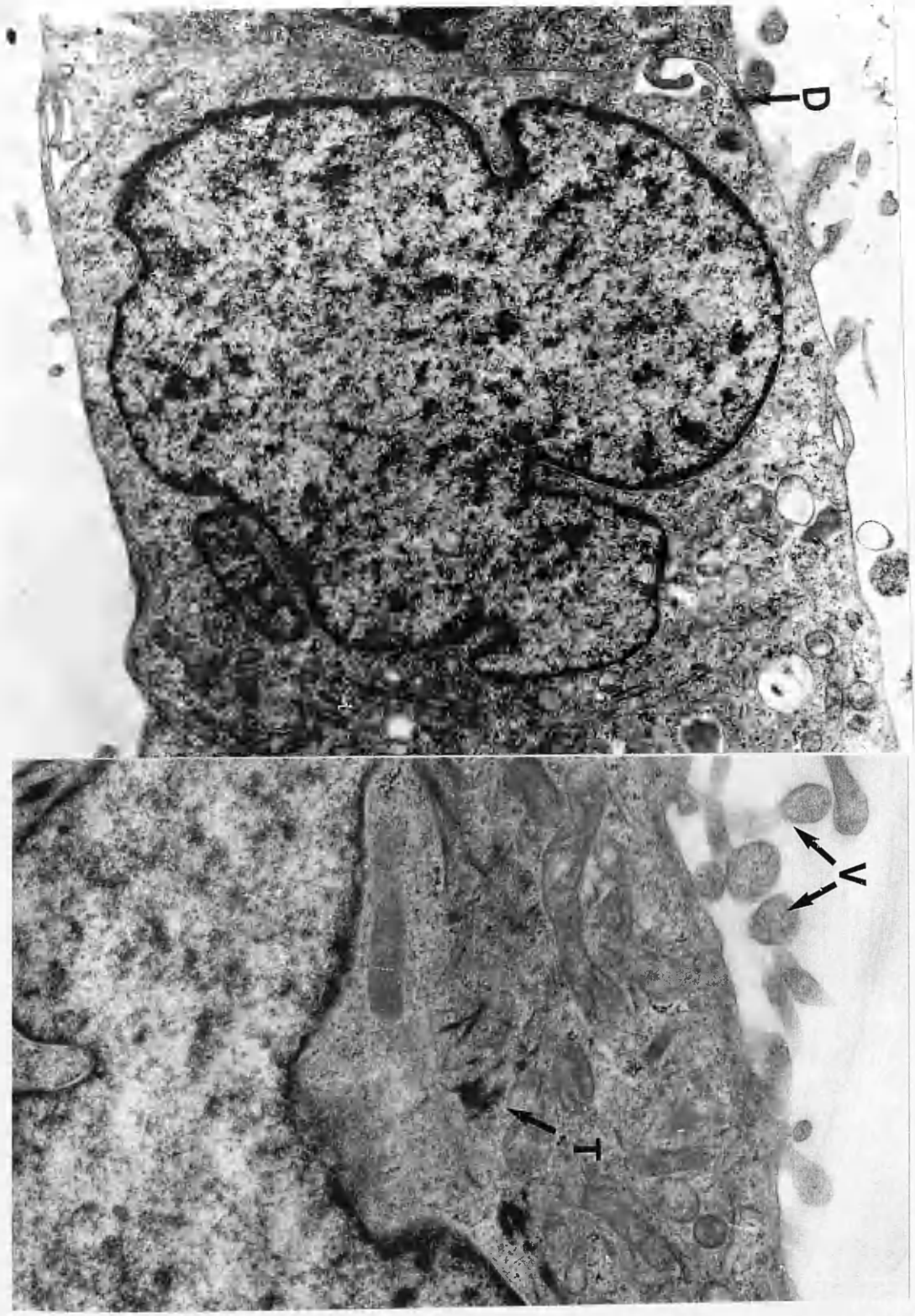
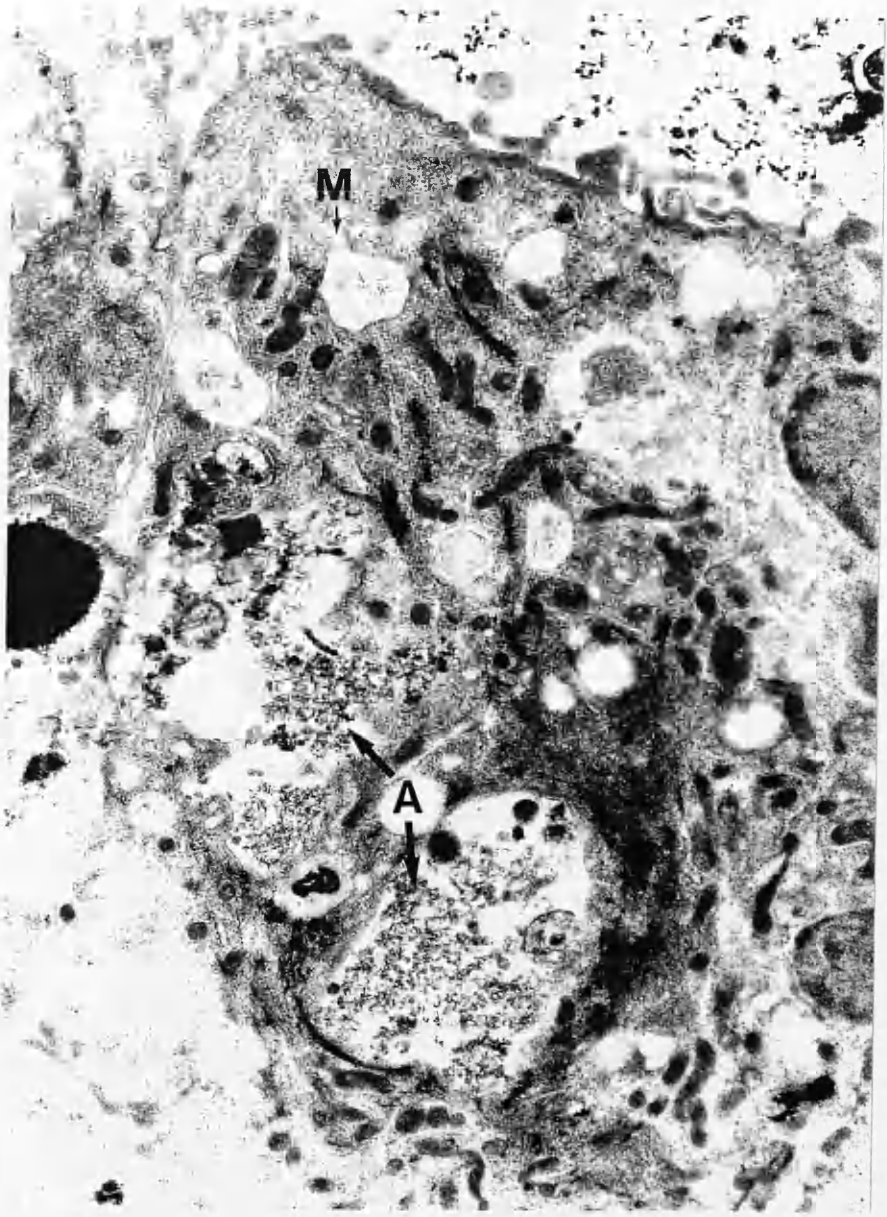



**Plate 4.4:- Transmission electronmicrographs of WIL cells.**

(a). The cells grown in histotypic culture show epithelial cell-cell relationships, and desmosome (D) (x 42500, Uranyl acetate and lead citrate stain). (b). Cell grown as (a) shows tonofilaments (T), and microvilli (V) (x 45000, Uranyl acetate and lead citrate stain).




(c). Cell grown as (a) shows apoptosis (A) and fluffy intracellular inclusions (? mucin) (M) (x 42500, Uranyl acetate and lead citrate).

plastic substrate showed more smooth surfaced cells [Plate 4.5 a], while cells grown on collagen substrate showed more microvilli, and developed cell-matrix [Plate 4.5 b] and cell-cell [Plate 4.5 c] connections.

There was no significant effect of phenotypic inducers on the morphology of the cells growing in the histotypic model by both transmission and scanning electron microscopy.

#### 4.3.2.2 Expression of Mucin-Like Glycoproteins.

WIL cells grown on a collagen substrate did not show any significant increase in the expression of mucin by histochemical staining. Positive staining was observed in 5-10% cells [see below, Plate 4.7], irrespective of the inducer used.

The results of biochemical analysis are shown in table 4.4. Growth of cells on a collagen substrate showed a peak for secreted mucin/GAGS in the conditioned medium, however, no difference was found following induction with different inducers. The peak was very low or absent in medium conditioned with WIL cells alone, grown on plastic substrate.

#### 4.3.2.3 Immunohistochemical Marker Expression

There was little difference in the expression of various antigenic markers expression on WIL cell grown in different micro-environments [Table 4.2].

### 4.3.3 EFFECTS OF *IN VIVO* GROWTH ON PHENOTYPE OF THE WIL

Histology of xenografts grown in athymic nude mice showed areas of mixed cellularity with both squamous and glandular differentiation [Plate 4.6]. Cell lines derived from xenografts (WIL+b) showed an increase in the expression of mucin-like glycoproteins both histochemically [Plate 4.7] and by biochemical analysis [Table 4.4]. The expression of mucin-like glycoproteins decreased with increasing passage number in culture. There was no difference in expression of intermediate filaments or membrane proteins, compared to the WIL cells grown as monolayer in plastic or in histotypic culture, however, a slight decrease in the expression of CEA and HCG was observed [Table 4.2].

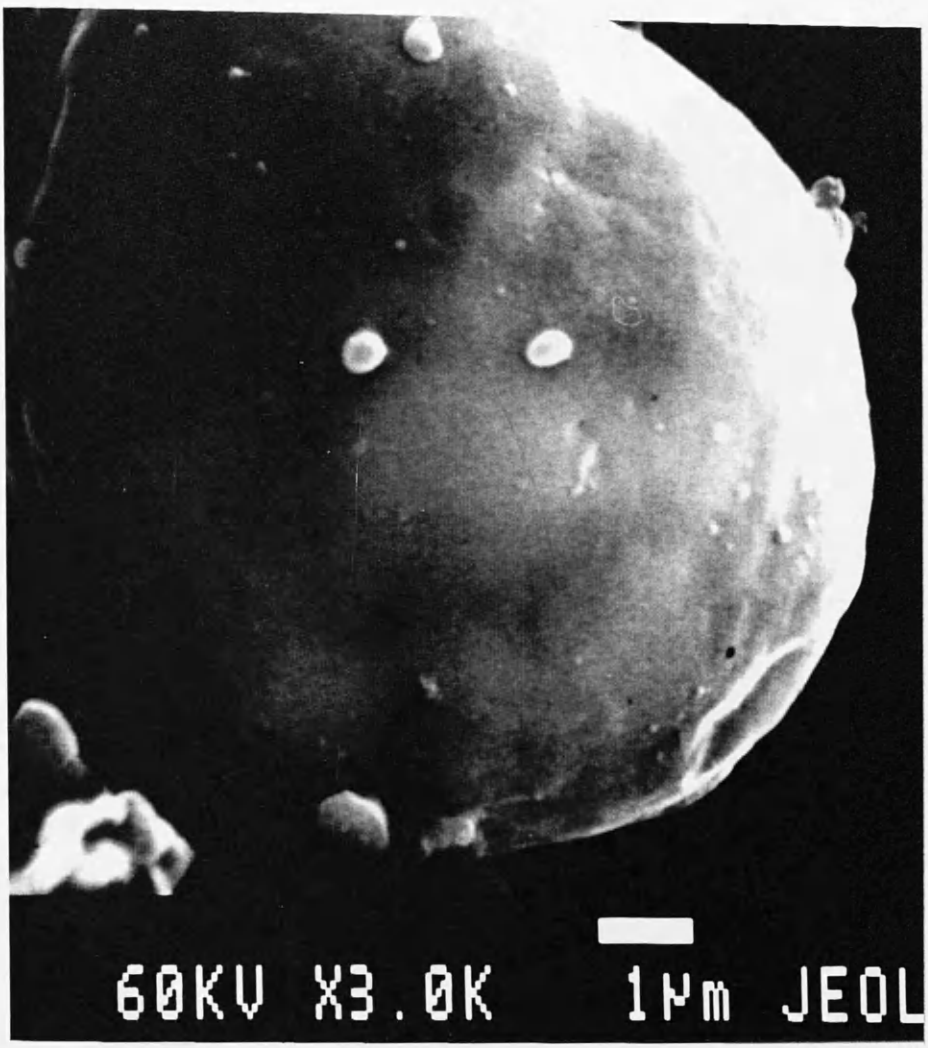
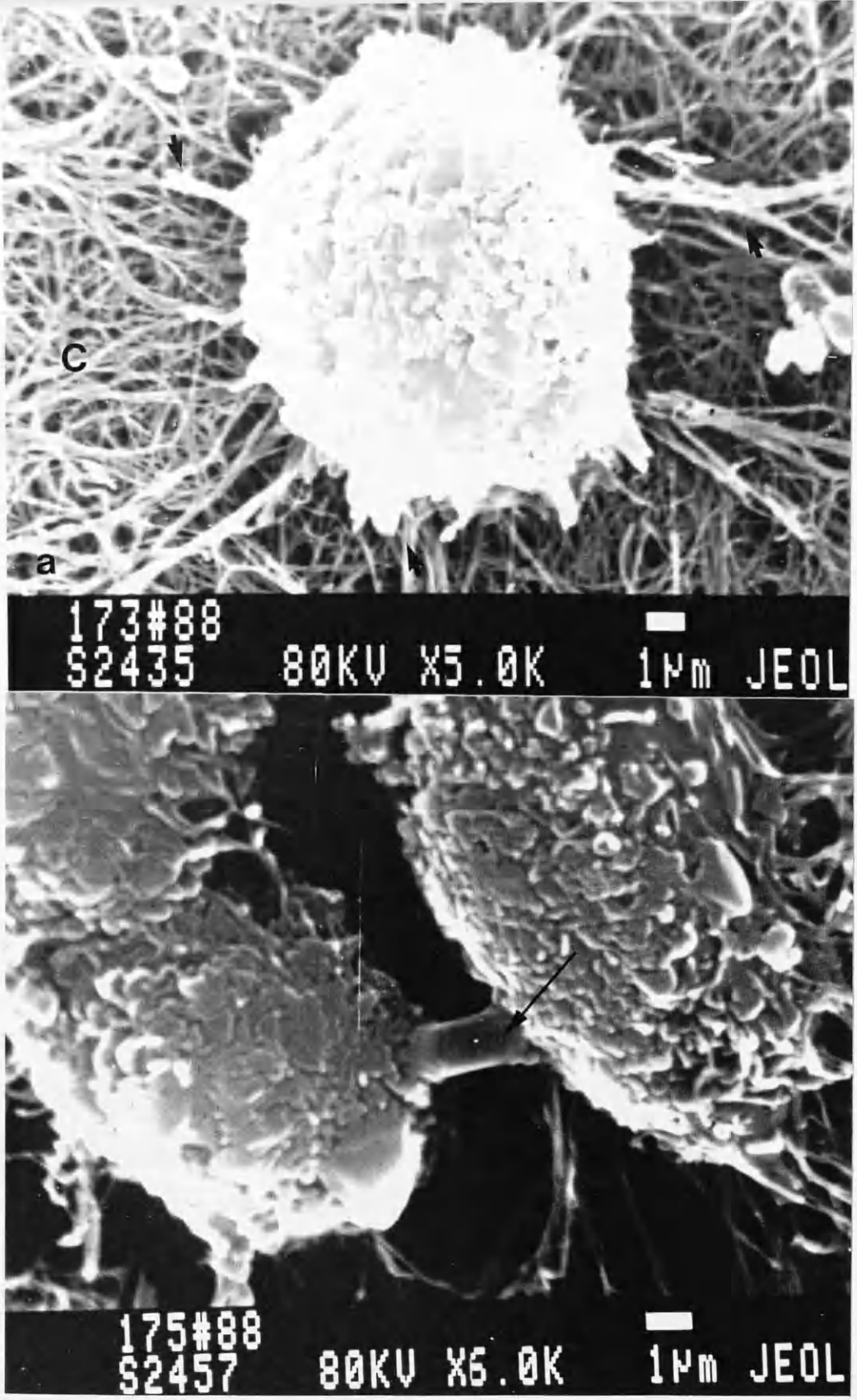



**Plate 4.5:- Scanning electronmicrographs of WIL cells.**

(a). WIL cell grown on plastic substrate for 10 days, shows smooth surface.




(b). The WIL cell grown on collagen substrate (C) in histotypic culture shows formation of surface microvilli and cell-matrix connections (arrows).

(c). The cells grown as (b) show cell-cell connection indicated by arrow (specimen was tilted for full view).

**Table 4.4:- Effects of phenotypic inducers on expression of mucin-like glycoproteins/GAGS in WIL cells.**

| CELL LINE/INDUCER | GAGS (% OF TOTAL CPM) |
|---|---|
| WIL (plastic) | 2.1 ± 0.1 |
| WIL (histotypic culture) | *6.3 ± 0.4 |
| + Cyclic AMP (1 mM) | *6.0 ± 0.3 |
| + Dexamethasone (25 μM) | *6.1 ± 0.2 |
| + HMBA (5 mM) | *6.3 ± 0.1 |
| + Na-But (1 mM) | *6.2 ± 0.4 |
| + Retinoic acid (0.5 μM) | *7.4 ± 0.1 |
| + TGF-β (1 ng/ml) | *6.3 ± 0.4 |
| WIL + b (WIL xenograft derived line) | *7.0 ± 0.2 |

WIL cells in histotypic culture were treated with chemical inducers, conditioned medium was assayed for mucin-like glycoproteins/GAGS as described in Methods. Cells grown in histotypic culture showed induction of mucin-like glycoproteins/GAGS compared to WIL cells grown on conventional plastic substrate. Results are mean ± SEM from at least 3 experiments.

*$P<0.01$ (Mann-Whitney test and Bonferoni adjustment).

plastic substrate showed more smooth surfaced cells [Plate 4.5 a], while cells grown on collagen substrate showed more microvilli, and developed cell-matrix [Plate 4.5 b] and cell-cell [Plate 4.5 c] connections.

There was no significant effect of phenotypic inducers on the morphology of the cells growing in the histotypic model by both transmission and scanning electron microscopy.

#### 4.3.2.2 Expression of Mucin-Like Glycoproteins.

WIL cells grown on a collagen substrate did not show any significant increase in the expression of mucin by histochemical staining. Positive staining was observed in 5-10% cells [see below, Plate 4.7], irrespective of the inducer used.

The results of biochemical analysis are shown in table 4.4. Growth of cells on a collagen substrate showed a peak for secreted mucin/GAGS in the conditioned medium, however, no difference was found following induction with different inducers. The peak was very low or absent in medium conditioned with WIL cells alone, grown on plastic substrate.

#### 4.3.2.3 Immunohistochemical Marker Expression

There was little difference in the expression of various antigenic markers expression on WIL cell grown in different micro-environments [Table 4.2].

### 4.3.3 EFFECTS OF *IN VIVO* GROWTH ON PHENOTYPE OF THE WIL

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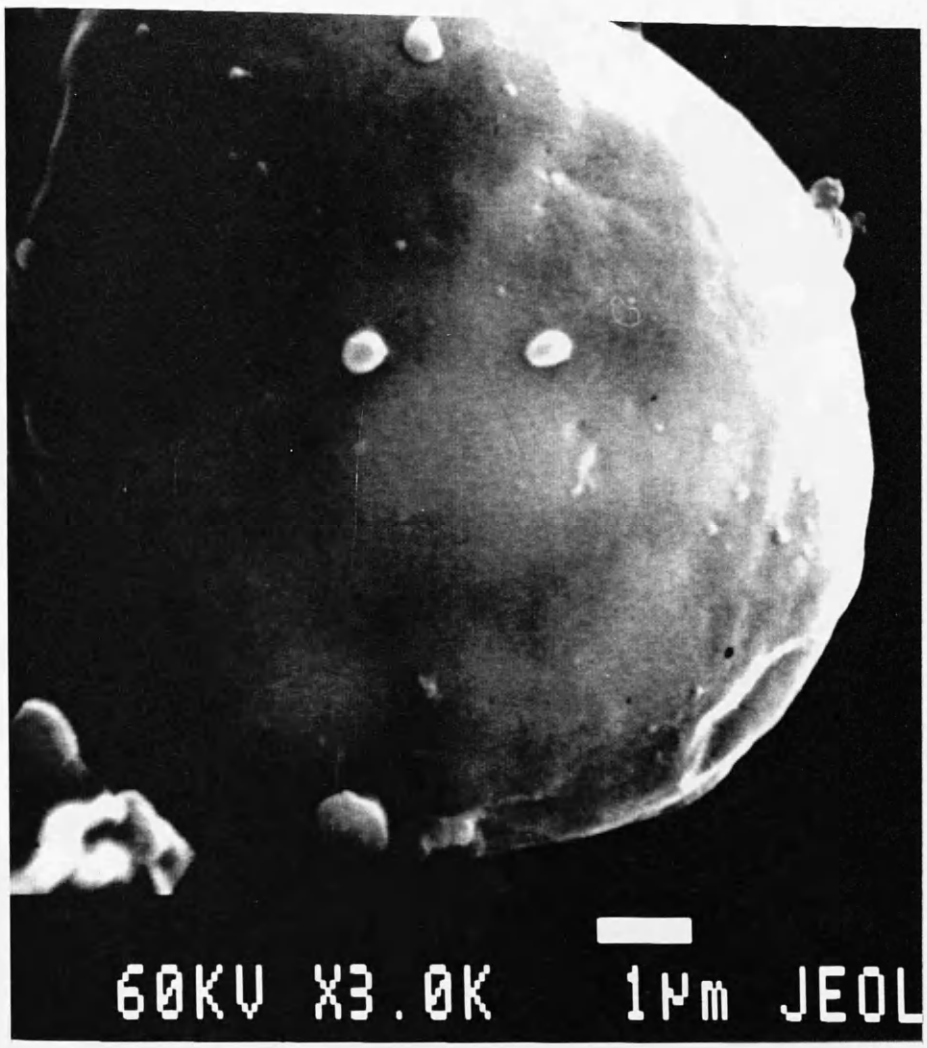
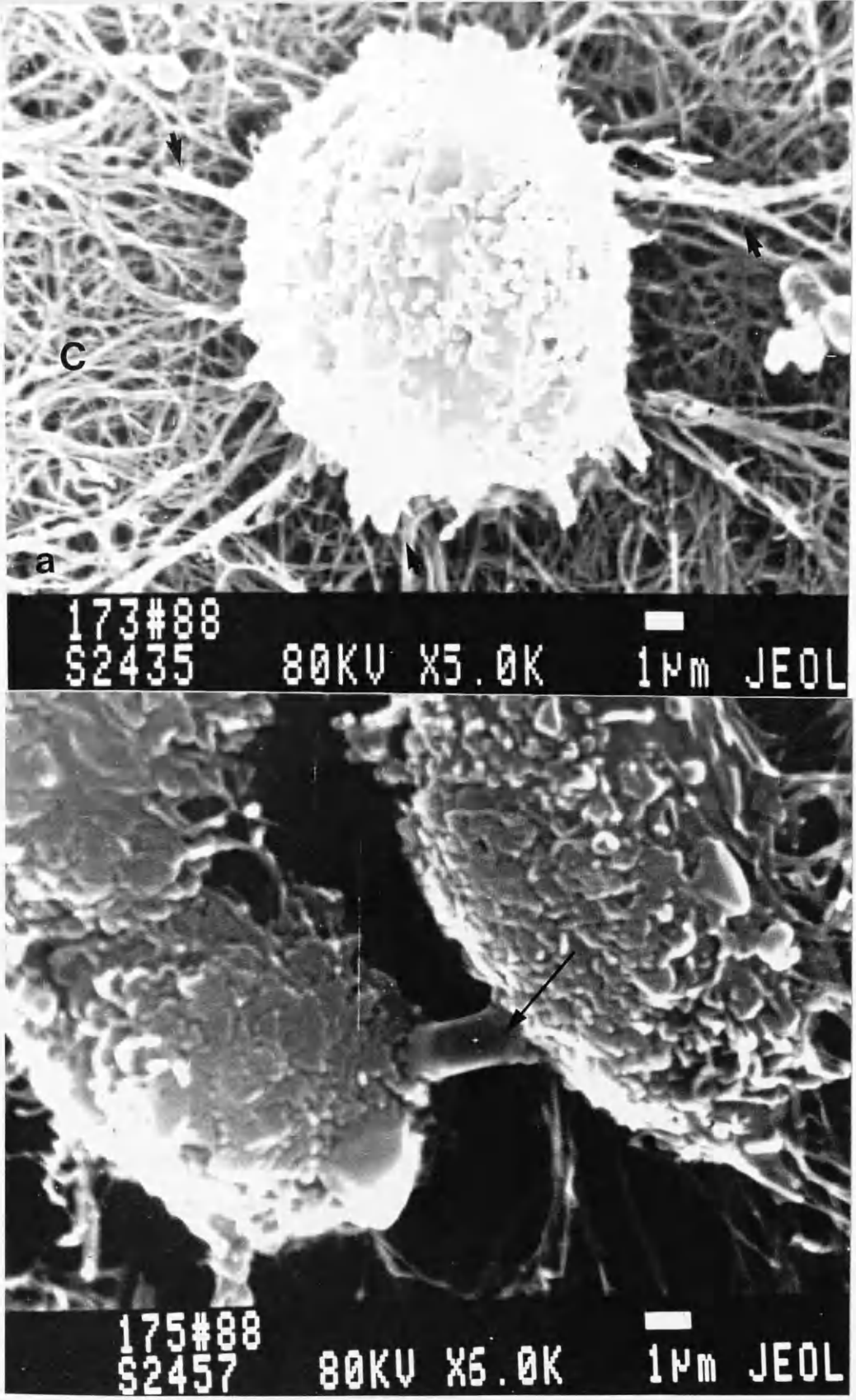



**Plate 4.5:- Scanning electronmicrographs of WIL cells.**

(a). WIL cell grown on plastic substrate for 10 days, shows smooth surface.




(b). The WIL cell grown on collagen substrate (C) in histotypic culture shows formation of surface microvilli and cell-matrix connections (arrows).

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| WIL + b (WIL xenograft derived line) | *7.0 ± 0.2 |

WIL cells in histotypic culture were treated with chemical inducers, conditioned medium was assayed for mucin-like glycoproteins/GAGS as described in Methods. Cells grown in histotypic culture showed induction of mucin-like glycoproteins/GAGS compared to WIL cells grown on conventional plastic substrate. Results are mean ± SEM from at least 3 experiments.

*$P<0.01$ (Mann-Whitney test and Bonferoni adjustment).

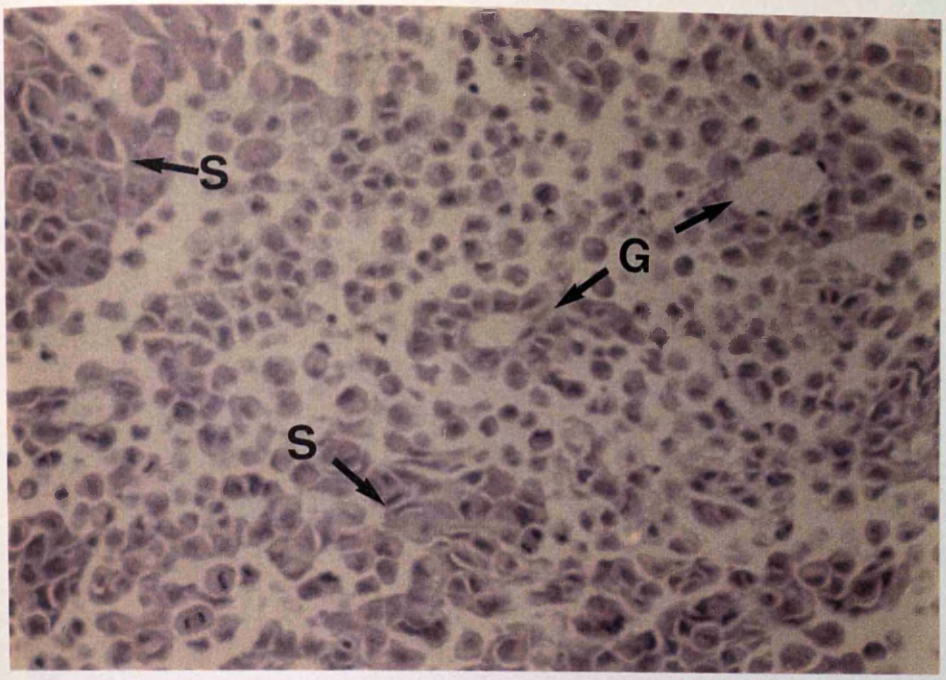


**Plate 4.6:- Effects of in vivo growth on morphology of WIL cells.**

WIL xenograft grown in nude mice shows poorly differentiated cells with some evidence of both squamous (S) and glandular (G) differentiation. $1x10^6$ cells were inoculated s.c into the flank of nude mice and tumour removed after 5 weeks (x 280, H&E).

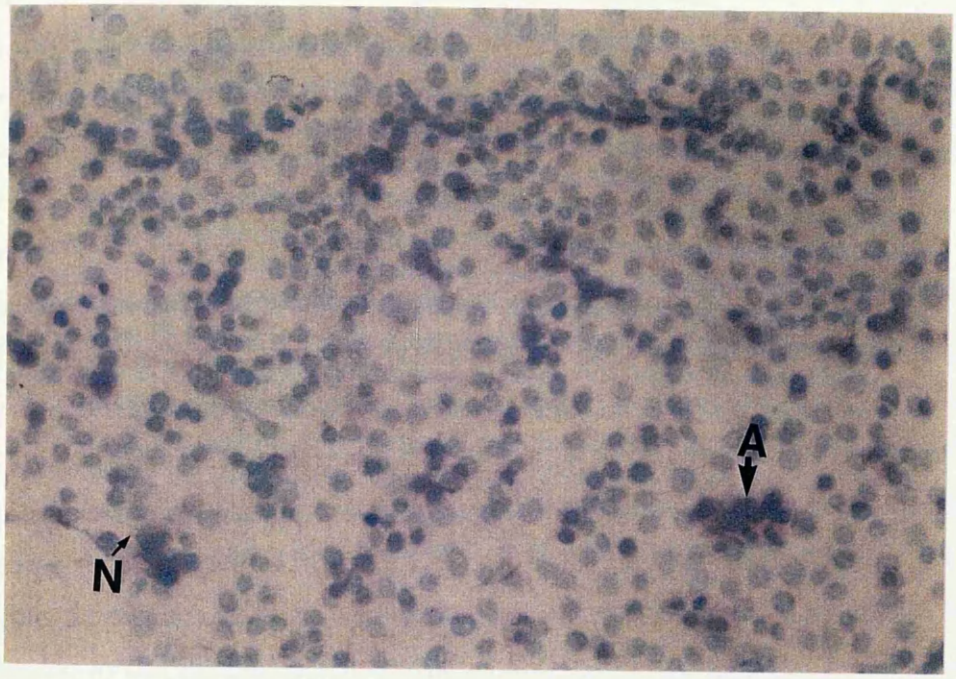


**Plate 4.7:- Expression of mucin in WIL xenograft derived cells in culture.**

WIL xenograft derived cells (WIL + b) in second passage culture stained with PAS-diastase/Alcian blue show positive staining both for acidic (A) and neutral (N) mucins (x 210, PAS/Diastase/Alcian blue).

## 4.4 DISCUSSION

The purpose of this study was to investigate the effects of stromal interaction on the phenotype of NSCLC. The WIL adenocarcinoma cell line was co-cultured with normal human fetal lung fibroblasts for different time periods in the presence and absence of different differentiation inducers, and the effect on the tumour cell morphology, antigenic marker expression, and chemosensitivity were investigated.

Changes were observed in the morphology of co-cultures. A close similarity was observed in morphology of co-culture, mouse lung culture, and the WIL xenograft derived cell line. One explanation is that all of these culture models have similar composition; fibroblasts, and epithelial cells either normal or tumour derived. Therefore, it is conceivable that the morphological changes were due to fibroblastic influence. It seems that the effects were not just physical, but needed live and growing fibroblasts, suggesting a possibility of both physical and biochemical interactions. Similar morphological changes have been found in an alveolar cell carcinoma cell line A549 following fibroblastic interaction [Speirs & Freshney, 1990], where it was proposed that a diffusible growth factor might be responsible. Effects were less obvious on histological examination of co-cultures in filter wells.

Mitogenic effects were observed in tumour cells, following stromal interaction. The stimulation of tumour cell growth by fibroblastic interaction may be due to the release of growth factors from fibroblasts, or it might be that tumour cells stimulate fibroblasts, which in turn synthesize and secrete other substances which are mitogenic to the tumour cells. Proliferation of normal mammary epithelial cells *in vitro* has been shown to be associated with the presence of mammary fibroblasts [Haslam, 1986], suggesting a role of fibroblasts in epithelial cell proliferation. However, others [Delinassios, 1987] have shown negative growth effects in other systems, suggesting that the effects are selective, stimulatory in some cases and inhibitory in others.

There was no difference in the sensitivity of the cells to the cytotoxic drugs vincristine and adriamycin with or without stromal interaction . It is possible that the WIL cell line was not sensitive enough to show any significant differences in chemosensitivity. Stimulation of tumour cell colony formation was also found at very low drug concentrations. It may be a defensive response on the part of tumour cells,

when they are challenged with non-toxic drug concentrations. The exposure to low drug concentration then may trigger a mitogenic response in cells probably via a receptor mechanism, which could be overridden by high drug concentrations, or high drug concentrations may have different mechanisms of cytotoxic effects.

There was no significant effect on the expression of any marker or on the sensitivity to cytotoxic drugs. There could be many reasons for this failure of tumour cells to respond to stromal interaction. Lack of specificity of the markers could be one reason. The WIL cell line was selected as the most suitable available model to represent NSCLC. The cell line had been shown to respond to dexamethasone treatment with a growth reduction both *in vitro* and *in vivo*, and a decrease in plasminogen activator production [McLean *et al.*, 1986]. The other possibility could be the lack of proper inductive micro-environment.

Therefore, it was decided to develop a histotypic tissue culture model system, and then induce the tumour cells, growing under optimal conditions, with agents known to induce phenotypic changes in other systems. The geometry of the histotypic model used in this study has already been described [see above, Figure 4.1 & Plate 4.3]. WIL cells grown in this system were allowed to develop cell-cell interactions of both homotypic (tumour-tumour) and heterotypic (tumour-stromal) nature, cell-matrix (collagen, laminin), and chemical (by chemical inducers) interactions. The cells were allowed to grow in three dimensional culture to recreate polarity and orientation, so that the cells should be receptive. It has been shown that this type of culture system optimises surfactant synthesis in A549 alveolar carcinoma cells [Speirs & Freshney, 1990]. The cultures in the raised position were able to receive nutrients from the basal surface through the filter and the collagen substrate, while the apical surface was exposed to the air-liquid interface, to simulate *in vivo* conditions of respiratory epithelium. Polarization of thyroid cells in similar conditions has been described by Chambard *et al* [1983]. They have demonstrated that thyroid cells growing in a filter well system were able to generate receptors for thyroid stimulating hormone on the basal surface, while the apical surface released thyroglobulin. It has been shown in different studies that the differentiated phenotype can be restored by growing the cells on collagen rather than on conventional plastic substrate. However, most of these studies have examined normal tissues such as tracheal epithelial cells [Wu *et al.*, 1985] or hepatocytes [Ben-

Ze,ev *et al.*, 1988].

The tumour cells grown in histotypic culture showed morphological as well as biochemical changes. There was increased formation of cell surface microvilli compared to the cells grown in conventional monolayer which showed more smooth surfaced cells. Intercellular and cell-matrix communications were more obvious in histotypic cultures. The cells formed connections with other cells as well as with collagen substrate, but only in histotypic cultures.

In addition there was stimulation of mucin-like glycoprotein synthesis in WIL cells grown in histotypic culture. The effects were significant compared to the monolayer cultures. Since microvillus formation and glycoprotein synthesis are seen in normal respiratory cells, this suggests that the histotypic model did alter differentiation. The chemical inducers caused no further changes, suggesting that a component of the model was effective. It is possible that the collagen substrate was responsible.

WIL cells grown as xenografts showed morphological and biochemical changes associated with the differentiated phenotype. There was an increase in synthesis of mucin-like glycoprotein, formation of glandular structures, microvilli, and ultrastructural evidence of mucin-like intracellular inclusions, and a slight decrease in expression of CEA, and HCG by immunohistochemistry. These features were similar to those observed in histotypic culture, suggesting that the model could be used to simulate *in vivo* conditions.

These data indicate that although further changes could not be induced in WIL in this system, it might prove useful in the study of other cell lines.

# CHAPTER FIVE

# EFFECTS OF ONCOGENE TRANSFECTION ON PHENOTYPIC PROPERTIES OF MINK LUNG EPITHELIAL CELLS

**This chapter describes the development and characterization of a model for the study of phenotypic alterations in relation to malignancy, following transfection of an epithelial cell line with human oncogenes.**

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## 5.1 INTRODUCTION

The key events involved in conversion of normal human cells to malignant cells by biological and chemical carcinogens remain unclear. This is in part due to the fact that human cells, particularly of epithelial origin are more resistant to transformation by these agents *in vitro* [DiPaolo, 1983]. The reason for this resistance is as yet unknown. However, it has been proposed that host factors such as chromosomal stability, suppressor genes [Spandidos & Anderson, 1989] and efficient DNA repair system [Topal, 1988] may be responsible.

Cancer has a complex biology *in vivo*, and *in vitro* model systems are useful alternatives for the study of malignant process. Since malignancy cannot be demonstrated *in vitro*, it is useful to use a number of markers associated with malignant cells grown in culture, and which can be used to identify a malignant cell *in vitro*, though it may not imply a causal relationship between these characteristics *in vitro* and malignancy *in vivo*. The major advantage is that a number of malignancy associated parameters (e.g immortalization, lack of contact inhibition, high terminal density, loss of anchorage dependence and loss of serum dependence, altered morphology) can be studied in isolation under defined conditions, though each type of assumed transformed phenotype has to be carefully put into its proper biological setting and the criteria for the transformation have to be meticulously compared with the behaviour of histogenetically analogous normal cells; when this is done properly, it will be found that the phenotypes defined as transformations in laboratory model systems can be used as reliable indicators or marker of the acquisition of essential neoplastic cell features. Transformation in the experimental models deserves the same careful biological, functional, morphological, biochemical, and genetical analysis as tumours in vivo. Because of the vast experimental advantages of the laboratory model systems, this approach will probably be the best avenue towards the distant goal of understanding the biology of the malignant cell.

Transformation is a term applied to describe emergence of the malignant phenotype *in vitro*, however transformation and malignancy are not necessarily equivalent though the events occurring during transformation *in vitro* may be similar to those in carcinogenesis. While the term malignancy has a fairly clear meaning *in vivo*, it is difficult to translate into cell culture conditions. Malignant cells express a number of properties including proliferation, secretion of proteolytic substances, stimulation of angiogenesis, invasion, metastasis, glycolytic metabolism, all of which are also the properties of normal cells, with the only

difference that expression of these properties in malignant cells is inappropriate in space and time. Therefore, it can be concluded that malignant cells are characterized by an inappropriate expression of normal cell properties, rather than de novo expression of tumour specific properties. The term neoplastic transformation, instead of malignancy, has been applied to cells in culture in order to describe the transition of a normal cell into a cell expressing properties associated with malignant cells. However the exact relationship between transformation *in vitro* and malignancy *in vivo* is not fully understood.

The malignancy associated phenotype has been studied, mainly by two approaches; a) the study of malignant cells in culture, following their isolation from tumours, and b) oncogenic transformation of normal cells *in vitro* e.g by transfection. An ideal approach may be the isolation of tumour cells and equivalent normal cells from the same patient and the same tissue, for comparative studies. However this has not been possible in the majority of cases, partly because the normal cells do not grow in culture easily, and secondly if they do, they may not belong to the same lineage, and their position in the lineage is not always clear. Therefore, such an approach though ideal, is not easily available and comparison not justified in all cases, especially, where the genetic background of such normal cells is not known in relation to expression or suppression of oncogenes.

Transformed cells exhibit an altered response to humoral growth controlling factors. The rate of proliferation of normal cells in routine media at low cell density may be as high as that of transformed cells [Buehring and Williams, 1976]. In order to reveal characteristic growth differences between normal and transformed cells they should be cultivated and tested at saturation density or under limiting concentrations of serum or growth factors.

With increasing knowledge of the role of oncogenes in carcinogenesis, a new approach has been based on the transfection of cells with oncogene sequences and this has been shown to induce malignant change in some cell lines.

Most of the previous studies involving the effects of oncogene transfection have been performed with non-epithelial cells, mainly NIH 3T3 fibroblasts. However, of great interest, complexity and clinical relevance are studies utilizing epithelial cells, from which most solid tumours arise, since carcinomas comprise over 80% of human malignancies. In these cells additional features such as cell lineage, differentiation status, and production of and response to endocrine growth factors may be involved in their progression towards autonomy and metastatic phenotype. Even in these limited studies so far investigators have

focused mainly on growth and morphological alterations *in vitro* and tumorigenicity *in vivo* of the transfectants. Relatively little is known about the effects of oncogene transfection on tumour pathology, invasion, and metastasis.

Recently, however, some attempts have been made by Wyllie *et al.* [1987] to analyze pathological changes in tumours produced by c-myc or Ha-ras transfected fibroblast cell lines. The effects of expression of human c-myc and both mutated and normal human Ha-ras, were studied in an aneuploid rat fibroblast line (208F), and activated ras alone in Chinese hamster lung fibroblasts (CHF). In contrast to the parental fibroblasts, cells expressing any of the human oncogenes formed rapidly growing tumours in nude mice. In general Ha-ras transfected cells were more aggressive than those expressing c-myc oncogene. Metastases were found in all tumours expressing oncogenes. Activation of c-abl and c-fos was found in all transfected tumours. These findings have suggested that though transfection of Ha-ras and c-myc itself may be important for transformed phenotype, activation and co-transfection of other oncogenes may also contribute.

The objectives of this study were to investigate the effects of human oncogene transfection on a diploid epithelial cell line. The model system chosen was a cell line of mink lung origin (MvlLu) and three derivative lines transfected with normal c-myc, normal Ha-ras and activated Ha-ras (T24) oncogenes individually. This model was selected because of the potential relevance in the study of carcinomas, particularly the lung carcinoma systems studied in this thesis.

Oncogene transfection was assessed using southern blotting and immunocytochemistry. The cell lines were characterized with respect to lineage growth and invasive characteristics *in vitro* and *in vivo*, and response to cytotoxic drugs. The transfected lines were compared with the parental line. Such studies will provide a baseline for further studies on oncogene interaction and/or modulation of phenotype.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Cell Lines

The parental cell line MvlLu is available from the American Type Tissue Culture Collection (ATCC), Rockville, Md; where it is designated CCL64. For present studies the cell line was obtained from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wilts.

The oncogene transfected cell strains used in this study were obtained from Dr Demetrios Spandidos at the Beatson Institute. The derivation of the plasmids used for transfection has been fully described elsewhere (Spandidos & Wilkie, 1984). From the parental Mv1Lu cell line three sublines were derived following insertion of mutated T(24) and normal human Ha-*ras* and human c-*myc* genes in high expression vectors by calcium phosphate transfection technique [Graham & Van der Eb., 1973]. Each derivative line was expanded from a single clone. The plasmids used for the transfection were pHO6T1, pHO6N1 (Spandidos & Wilkie, 1984), and pMCGM1 (Spandidos, 1985), containing the entire T(24) Ha-ras gene, the entire normal human Ha-ras proto-oncogene, and the entire human c-myc proto-oncogene respectively. In pHO6T1 and pHO6N1 the Ha-ras genes are surrounded by both SV40 and Moloney virus LTR enhancers; whilst in pMCGM1 the Moloney virus sequence is linked to the human c-myc gene. All the plasmids contain the aminoglycoside phosphotransferase (aph) gene conferring resistance to geneticin (G418), which was used in the selection of the lines after transfection.

The cell lines were designated Mv1 (parental), M1 (c-myc transfected), N1 (Ha-ras transfected), and T1 (mutated Ha-ras transfected), as outlined in Table [5.1].

FHO5T1 cell line was derived by transfection of an early passage embryonic Chinese hamster lung fibroblast line (CHL), with the plasmids pHO5T1, which contained the mutationally activated human T24 Ha-ras oncogene ligated to viral LTR enhancing sequences. This cell line was selected as a positive control for ras immunostaining because it has been shown to express the ras specific membrane staining in previous studies [Going et al., 1988].

SCLC cell line H69 and its two derivative lines (see Chapter three-A) were used as positive control for c-myc oncogene expression.

**Table 5.1:- Origin of cell lines.**

| Cell line | [1]Oncogene transfection | [2]Plasmid | Enhancer | Selection | [3]Passage number | [4]Reference |
|---|---|---|---|---|---|---|
| Mv1 | [1]None | None | None | NA | 45-60 | ATCC [CCL64] |
| M1 | c-*myc* (Human) | pMCGM1 | SV40 | G418 | 45-60 | [4]Isolate |
| N1 | Ha-*ras* (Human) | pHO6N1 | SV40 + Molony Virus-LTR | G418 | 45-60 | [4]Isolate |
| T1 | Ha-*ras* (T24) (Human) | pHO6T1 | SV40 + Molony virus LTR | G418 | 45-60 | [4]Isolate |

[1]The parental cell line MvlLu (Mv1) with unknown genomic changes. [2]All plasmids contained aminoglycoside phosphotransferase (aph) gene which confered resistance to geneticin (G418). [3]Approximate passage number in culture during this study. NA: not applicable. [4]Khan et al. [1990].

### 5.2.2 Cytotoxic Drugs

Cytotoxic drugs; cytosine arabinoside and cis-platinum were purchased from Sigma Co. Ltd. Fresh drugs were prepared in culture medium each time.

### 5.2.3 Southern Blotting and Hybridization

Restriction enzyme cleaved DNA was electrophoresed on a 0.7% agarose gel at 1 volt/cm overnight, and transferred to Genescreen (NEN Research products) hybridization membrane by vacuum blotting using the Vacugene (LKB Pharmacia) vacuum blotting unit. DNA was first denatured by washing with 1.5% NaCl, 0.5M NaOH for 4 minutes, subsequently washed with neutralizing solution (1.0M Tris, 2.0M NaCl, pH 5.0) for 4 min and transferred to membrane in 20xSSC under a vacuum of 40 cm $H_2O$ for 1 hour. Membranes were then baked at 80 °C for 2 hours prior to hybridization. Hybridizations were performed according to the methods of Maniatis *et al.* [1982], using random primed [Feinberg and Vogelstein, 1983] $^{32}$P-labelled probes at 42 °C in 5x Denhardts solution with 50% formamide, 10% dextran sulphate, 0.1% SDS and 100 $\mu$g/ml denatured salmon sperm DNA. Membranes were washed at high stringency (0.1xSSC, 0.1% SDS at 65°C) for 1 hour with three changes of wash solution. (These techniques were performed by Dr Jas Lang at the Beatson Institute).

### 5.2.4 Cellular Localization of Ha-ras Protein p21.

The immunohistochemical staining techniques as adapted by Going *et al.* [1988] were employed in this study, the steps are briefly outlined below.

Paraformaldehyde (BDH) stock solution (8%) was prepared in deionized distilled water (DDW) at 70 °C with the addition of 100 mg sodium hydroxide for each 100 ml of solution. The solution was stirred until cleared then filtered and stored at room temperature. Stock solution of 0.05M phosphate buffer pH 7.7 contained 0.0367 M dibasic sodium phosphate ($Na_2HPO_4$) and 0.0133 M monobasic sodium phosphate ($NaH_2PO_4$) in deionized distilled water (DDW). Fresh periodate lysine paraformaldehyde (PLP) solution was prepared in small batches of 200 ml before use by dissolving 2.74 gm of anhydrous sodium metaperiodate in a mixture of 50 ml of 8% paraformaldehyde and 150 ml of phosphate buffer pH 7.7. Subsequent periodate lysine paraformaldehyde dichromate (PLPD) was prepared by dissolving 2.5% w/v of potassium dichromate in PLP diluted with an equal volume of DDW.

Cell suspensions were prepared from monolayer cultures by trypsinization, cells were washed in PBS and spun at 100g for 3 min. Approximately 5x$10^6$ cells

were fixed in PLPD for 15 min at 4 °C, washed in PBS and resuspended in 0.5 ml of 2% low melting temperature agarose (Gibco Ltd, Paisley, Scotland) in PBS at 40 °C. The agarose cell pellet was post-fixed for up to 24 hours in PLPD washed in PBS and processed for paraffin embedding without raising the temperature higher than 56 ° C. Blocks (5x5x4 mm) from xenografts were placed immediately in PLPD and fixed at 4 °C for 24 to 36 hours. Subsequently tissues were washed overnight in running tap water and processed for paraffin sectioning. 4 μm sections were cut and dried at 56 °C for at least 30 min before immunostaining.

An avidin-biotin complex (ABC) technique was employed as adapted by Going *et al.* [1988]. Briefly, the primary antibody Y13-259 (kindly provided by Dr D. Spandidos of Beatson Institute) was applied to sections overnight at 1:50 dilution in tris-buffer isotonic saline (TBS), containing 10% normal goat serum (TBS/NGS). Biotinylated anti-rat antibody (Sigma Co Ltd.) 1/50 in TBS/NGS was applied for 30 min. These stages were each followed by two 5 min washes in tris saline (TS). Streptavidin biotin peroxidase complex (ABC) (Amersham International) 1/200 in TBS was applied for 30 min. After three 5 min TS washes sections were developed with diaminobenzidine-$H_2O_2$ in TBS. Negative controls included omission of Y13-259 from the first stage and its replacement with the same concentration of polyclonal rat immunoglobulin (Sigma Co Ltd.). The non-transfected parental cells and their tumours were included. FHO5T1 cells were used as positive controls to confirm specificity of staining.

### 5.2.5 Cellular Localization of c-myc Protein p62

Mv1 parental cell line was used as a negative control for M1 the c-myc transfected experimental test cell line. The lung carcinoma cell lines (H69; H69V, and H69VZ, see Chapter three-A), and their xenografts were included as positive controls.

Frozen sections, monolayer and cytospin preparations were stained by the alkaline phosphatase, and ABC staining techniques. Slides were incubated in 0.1% triton x-100 in tris saline for 5 min before immunostaining. The primary antibody myc1-9E10 (kindly provided by Dr Demitrius Spandidos of Beatson Institute) was applied at 1:20 dilution in tris-buffer saline for 2 hours at room temperature. Formalin fixed tissue were stained by the avidin-biotin complex peroxidase method. The primary antibody (myc1 9E10) was used at 1:50 dilution in tris-buffer saline overnight at 4 °C.

### 5.2.6 Autoradiography

Methods were adapted from Freshney [1987]. Cultured cells at saturation density were incubated with [$^3$H]-thymidine to label the DNA, washed, fixed and dried. The preparations were coated with melted photosensitive emulsion in the dark and left to expose at 4 °C for 1 week, developed, and examined under the microscope to see the silver grains overlaying the areas where radioisotope was incorporated [see below, Plate 5.11].

Monolayer cultures of cell lines (Mv1, M1, N1, and T1) were grown to saturation density monitored by daily counts in 24 well plates, and daily feeding. Routine medium was then changed to thymidine free medium (DMEM) for 24 hours. $^3$H-thymidine was added to the culture at 5 $\mu$Ci/ml (2Ci/mM) final concentration for 1 hour. They were washed in PBS, trypsinised, and resuspended in a few drops of PBS. The cells were fixed in ice-cold acetic-methanol (1:3) for 10 min, spun and resuspended in fixative at final dilution of 1x10$^5$ cells/ml. Cytospin preparations were made on dry clean glass slides. They were washed in distilled water and finally in 10% cold trichloroacetic acid to extract the acid soluble precursors. They were washed again in distilled water then in methanol, and dried.

Slides were coated with melted photographic emulsion (Ilford L4 diluted 1:1 v/v, with d/w) in dark room, and allowed to dry, then placed in a light-proof slide box with silica gel and sealed with dark vinyl tape, finally wrapped in a black bag and left at 4 °C for 7 days. After 7 days one slide was developed in D19 (Kodak) for 10 min, washed and fixed in photographic fixer (Ilfofix, Ilford) for 4 min, washed in distilled water and placed in hypoclearing agent (Kodak) for 2 min, and then washed with distilled water (5 changes over 5 min). The slides were dried, and examined under the microscope to check for the optimum grain formation. If satisfactory, remainder of the slides were then processed using the same protocol.

Slides were stained with Giemsa, washed, dried, and mounted. The preparations were examined under the microscope (x 10 eyepiece, x 100 oil immersion objective). The number of grains per cell was counted and the specific nuclear localization of grains was confirmed. Cells containing more than 25-30 grains per nucleus were counted as positive. For labelling indices the labelled cells were counted as a proportion of the total cells per field, in 40-60 fields, in total 2500-3000 cells.

### 5.2.7 Bromodeoxyuridine Incorporation Assay

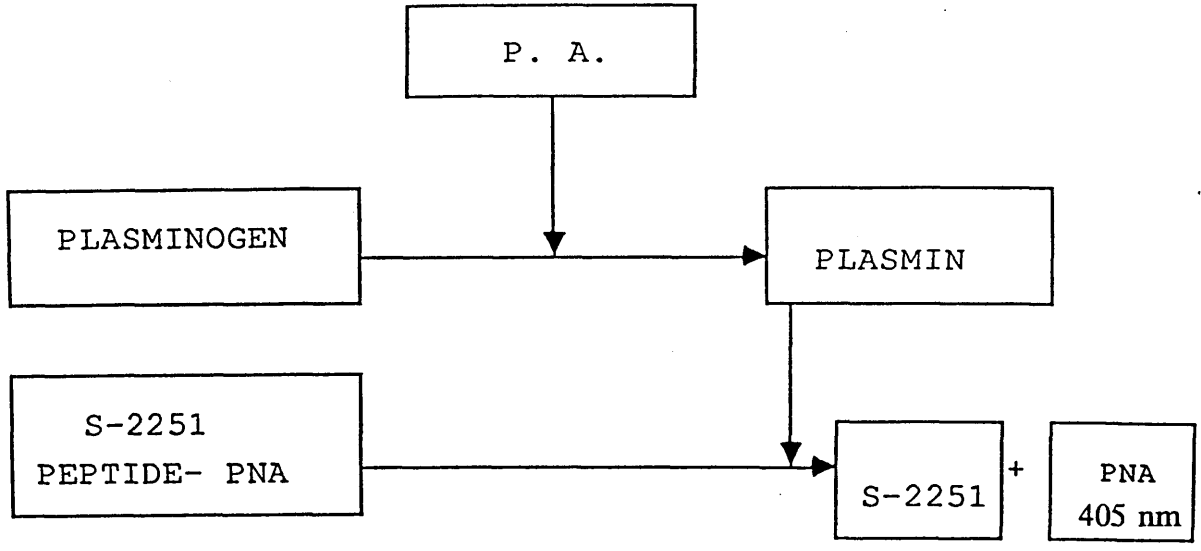
The assay was performed using a commercially available kit (Cell Proliferation Kit) and following the manufacturer's (Amersham International Ltd, Bucks, England) instructions.

Coverslip cultures in 24 well plates were grown to saturation density. Routine medium was replaced by DMEM for 24 hours. Cells were then exposed to labelling reagent, bromodeoxyuridine (BrdUrd) at 1:1000 v/v final dilution in DMEM for 1 hour at 37 °C, washed in PBS three times, and fixed in acid-ethanol (1:3) for 30 min at room temperature, followed by another wash in PBS. Preparations were processed using the indirect immunoperoxidase method as described in the General Methods with following modifications. Non-specific binding sites were blocked with non-immune sheep-serum (1:5 in tris buffer) for 20 min before application of each antibody. Pre-diluted mouse monoclonal primary antibody (anti-BrdUrd) was applied for 60 min at room temperature. Peroxidase linked secondary antibody (sheep anti-mouse-HRP) was applied for 30 min at room temperature. Slides were washed in saline, and incubated with 3'-3'-diaminobenzidine (DAB) solution containing nickel and cobalt as colour intensifier, for 10 min. Background nuclei were stained with neutral red. The coverslip preparations were then dehydrated and mounted. 2500-3000 cells from the representative areas of the replicate coverslips were counted in each assay (x 10 eyepiece, x 100 oil immersion objective). The labelling index (LI) was expressed as a percentage of total cells for each cell line.

### 5.2.8 Plasminogen Activator Assay

The chromogenic assay for measuring plasminogen activator (PA) activity adapted in this study was a modification of that developed by Whur *et al.* [1980].

The assay is essentially an amidolytic reaction which is a two step process, resulting in release of p-nitroanaline from a synthetic chromogenic substrate. In the first step, PA in the test sample (cell/medium, tissue/fluid) converts plasminogen into plasmin which in turn cleaves the chromogenic substrate S-2251 (H-D-Valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride), releasing p-nitroanaline the absorbance of which is measured at 405 nm, which is used as a function of PA in the test sample [Figure 5.1].




PA = Plasminogen activator
S-2251 = Substrate
PNA = Para-nitroaniline

**Figure 5.1:- Schematic representation of plasminogen activator assay.**

PA

H-D-Val-Leu-Lys-pNA ----------→ H-D-Val-Leu-Lys-OH + pNA

The cells were plated at $1x10^3$/ml/well in 24 well plates, and grown to confluence at 37 °C, 2% $CO_2$ and 98% air, in humid incubator. Routine culture medium was replaced with serum-free medium for 3 days followed by 24 hours incubation in serum free phenol red free medium, to remove possible absorbance of phenol red and also to reduce the inhibitory effects of serum factors on PA.

Conditioned medium was saved after 24 hours, centrifuged at 100g for 5 min and transferred into 24 well plates again to be assayed in the same way as cellular PA. The monolayer cells were washed 3 times with PBS. Test samples (cells/medium) were incubated with 500 µl of reaction mixture which consisted of 5 mM chromogenic substrate S-2251 (KabiVitrum) 5cu/ml of plasminogen (KabiVitrum), 1.5 mg/ml poly-D-lysine, in HBSS. The reaction was then allowed to run for up to 2 hours when the assay was terminated with the addition of 500 µl of 5% acetic acid in each well. The absorbance was read at 405 nm using a spectrophotometer (Gilford 250), which gave a measure of p-nitroanaline produced as a function of PA released by cells. Cell number per well was determined by electronic counting of the single cell suspension following trypsinization. Protein content per well was also determined by the method of Lowry *et al.* [1951].

Each set of readings was blanked against the corresponding plasminogen free controls and the net change in absorbance attributable to the activation of plasminogen was calibrated against serial dilutions of a urokinase standard curve. A reagent blank was included, by excluding the source of PA (cells/or medium) from the reaction mixture. The enzyme activity was expressed as plough units (PU) per million cells or PU per mg protein, and also as PU per ml of medium in case of secreted PA.

### 5.2.9 Other Methods

All other methods used in this Chapter including tissue culture, chromosome analysis, LDH isoenzyme analysis, invasion *in vitro*, cytotoxicity assays, various animal experiments, invasion and metastasis *in vivo*, histological procedures, and immunohistochemical techniques were performed as described in General Methods, more specific details are provided with the figure legends, where appropriate.

**Table 5.2:- The confirmation of exogenous oncogene incorporation into the host genome and its detection at different levels of expression.**

| Cell line | Oncogene | Southern blotting | [1]ICC staining | [2]Phenotypic transformations | [3]Remarks |
|---|---|---|---|---|---|
| Mv1 | None | - | - | - | Control |
| M1 | c-*myc* | + | + | + | Mildly transformed |
| N1 | Ha-*ras* | + | + | ++ | Moderately transformed |
| T1 | Ha-*ras* (T24) | + | + | +++ | Strongly transformed |

[1]Immunocytoochemical staining for Ha-*ras* and c-*myc* proteins, in both cultured cells and tumours produced from the cells in nude mice. [2]Morphological and growth transformations in both *in vitro* and *in vivo*. [3]Classified on the basis of the degree of phenotypic transformations.

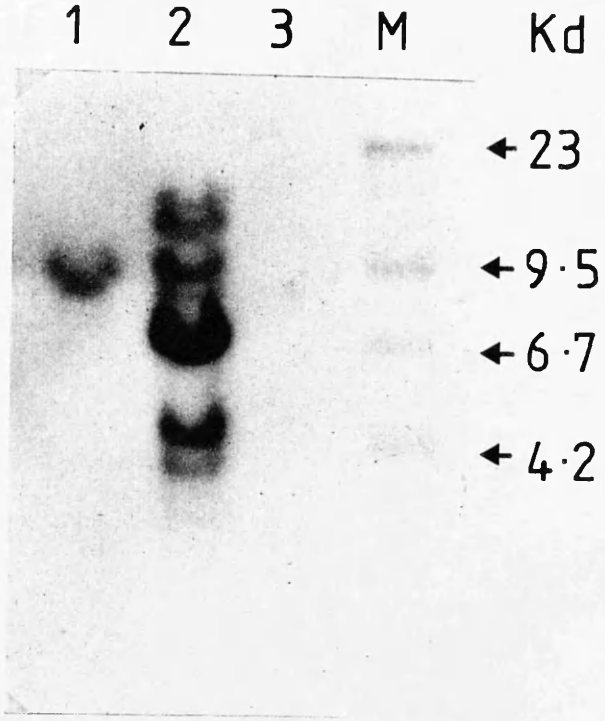
**Plate 5.1:- Southern Blot Analysis of The Cell lines.**

**(a). Expression of Ha-ras in the cell lines N1 and T1**

Southern blot analysis of Ha-ras transfected cell lines hybridised with $^{32}p$ labelled Ha-ras probe isolated by sma I/SST I cleavage of PT24C3 and spanning exons 1-4. The 6.6 Kd human specific Bam HI fragment encompassing the Ha-ras gene was observed in cell line N1 (track 2). An Ha-ras specific fragment of higher molecular weight was observed in cell line T1 resulting from a probable integration of Ha-ras sequence within the 6.6 Kd Bam fragment with endogenous mink lung sequences (track 1). Parental cell line Mvl shows no hybridisation to the human Ha-ras probe (track 3).

**(b). Expression of c-myc in cell line M1.**

Southern blot analysis of c-myc transfected cell line M1 hybridised with $^{32}p$ labelled human c-myc probe isolated by PVU II/Bg1 II cleavage of PMC41 and spanning exon 2. The 8 Kd human specific Hind III/EcoRI fragment encompassing the entire human c-myc gene was observed in cell line M1 (track 1) and was absent from parental cell line Mvl (track 2).


1
2
3
M
Kd
23
9·5
6·7
4·2

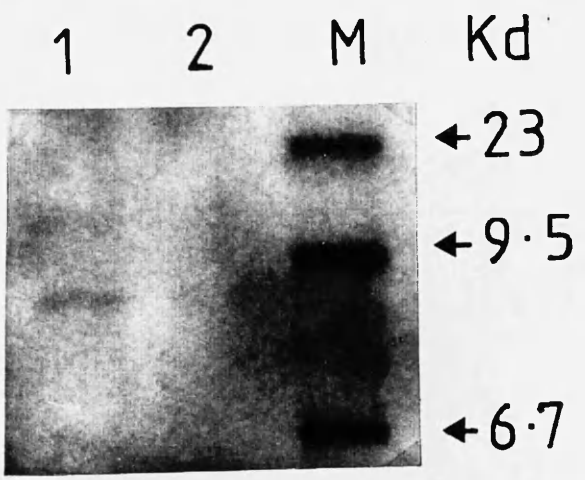
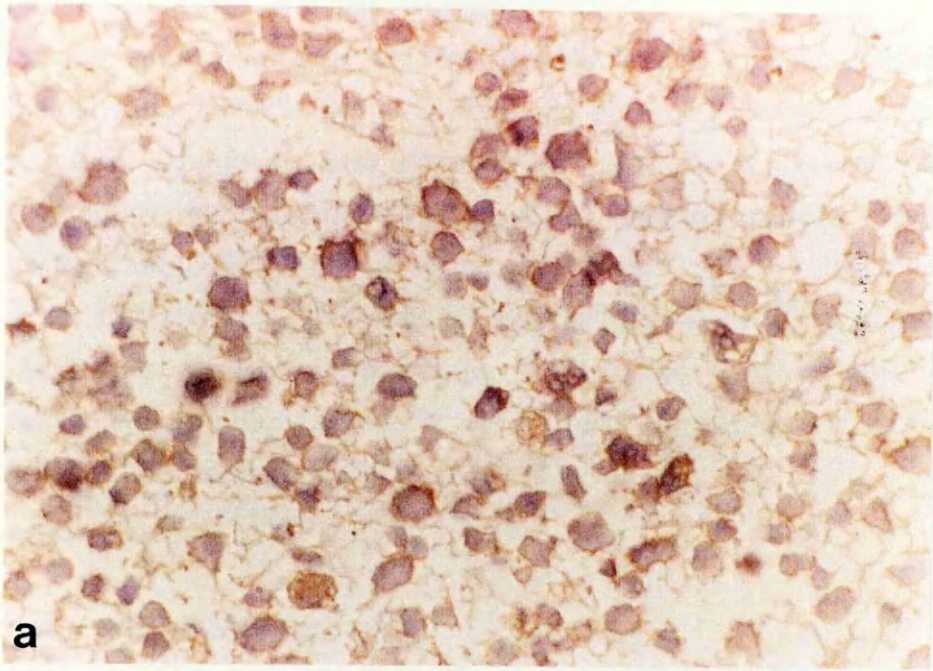

1
2
M
Kd
23
9·5
6·7

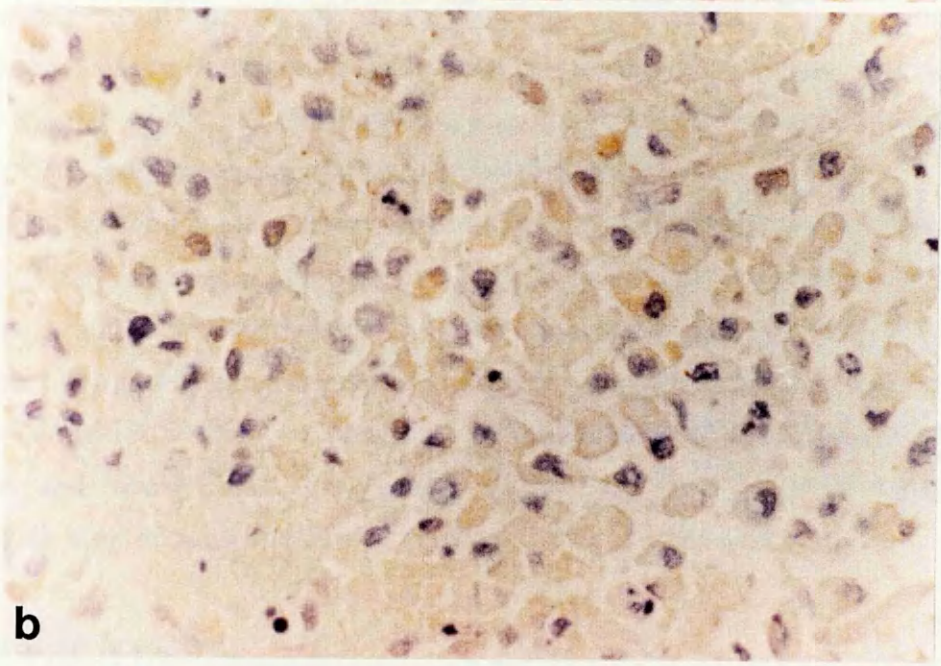
**Plate 5.2:- Ras p21 specific immunostaining**

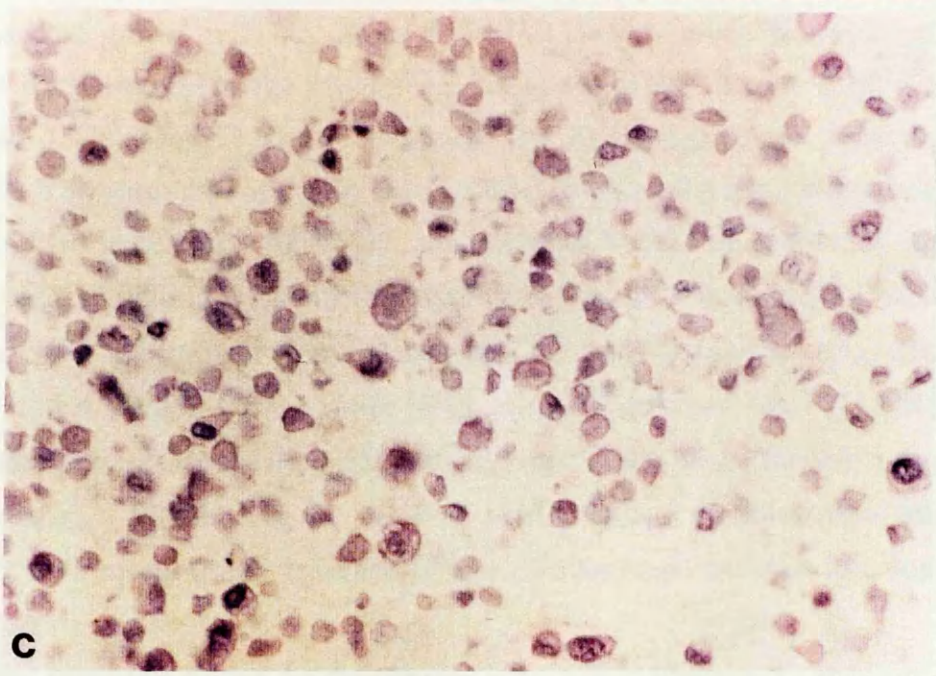
(a). FHO5T1 cells showing membrane specific staining, using Y13-259 antibody (X 360, avidin biotin technique).

(b). The Ha-ras transfected cells (T1) grown as tumour were stained as (a) showing focal membrane staining.

(c). Primary antibody replaced by non-immune rat immunoglobulin.


a


b


c

#### 5.3.1.3 Cellular Localization of c-myc Product

Both nuclear and cytoplasmic staining was observed in positive control lung carcinoma cell lines, H69V and H69VZ [Plate 5.3 a] when primary antibody 9E10 was applied for 2 hours at room temperature with either of the methods used. Specific staining was not observed either in the parental cell line (Mvl) or in c-myc transfectant (M1). However when antibody was applied overnight a few cells were stained positive, with both nuclear as well as cytoplasmic staining, but only at the expense of background staining [Plate 5.3 b]. Replacement of 9E10 with non-immune mouse serum or monoclonal control at the same concentration abolished staining [Plate 5.3 c].

### 5.3.2 CHARACTERISATION IN VITRO

Following confirmation of the oncogene transfection, this section investigates the validity of selection system used in transfection assays, species and lineage identity of the cell lines, and the effects of oncogene transfection on morphology, and growth characteristics of transfectants *in vitro*.

Results of *in vitro* studies showed that oncogene transfections of mink lung epithelial cells had imparted a number of malignancy associated properties to the transfectants, which were absent in the non-transfected parental cell line, including morphological changes, enhanced growth rates, high labelling indices at saturation densities, acquisition of anchorage independent growth, less serum dependence, and invasiveness. Only minor differences were found in c-myc transfectants compared with the Ha-ras oncogenes transfected cell lines.

#### 5.3.2.1 Geneticin Sensitivity

Cells were grown in 25 $cm^2$ flasks as well as cloned (monolayer cloning), in presence and absence of the drug. The drug sensitivity was tested both at early [Table 3.5 a] as well as at late [Table 3.5 b] passage levels following transfection. The parental line (Mvl) was sensitive while all the transfected lines were resistant to geneticin. 100% kill was observed in Mvl cells both at low (200 $\mu$g/ml) and at high (800 $\mu$g/ml) drug concentration. A few colonies seen in the parental line ( 200 $\mu$g/ml) after 2 weeks were followed further but no growth was found after 4 weeks. On the other hand, all the transfected lines (M1, N1, and T1) exhibited resistance to geneticin, and no effect was observed when colonies were kept in the drug for up to 4 weeks, instead colonies grew and formed a monolayer culture in presence of drug. A significant increase in colony

**Plate 5.3:- The c-myc specific immunostaining**

(a). H69V tumour tissue showing both nuclear and cytoplasmic staining for c-myc protein, using 9E10 antibody (X 360, avidin biotin technique).

(b). Tumours produced by M1 processed as (a) showing occasional staining, both nuclear and cytoplasmic.

(c). Primary antibody replaced with monoclonal control or non-immune mouse serum.

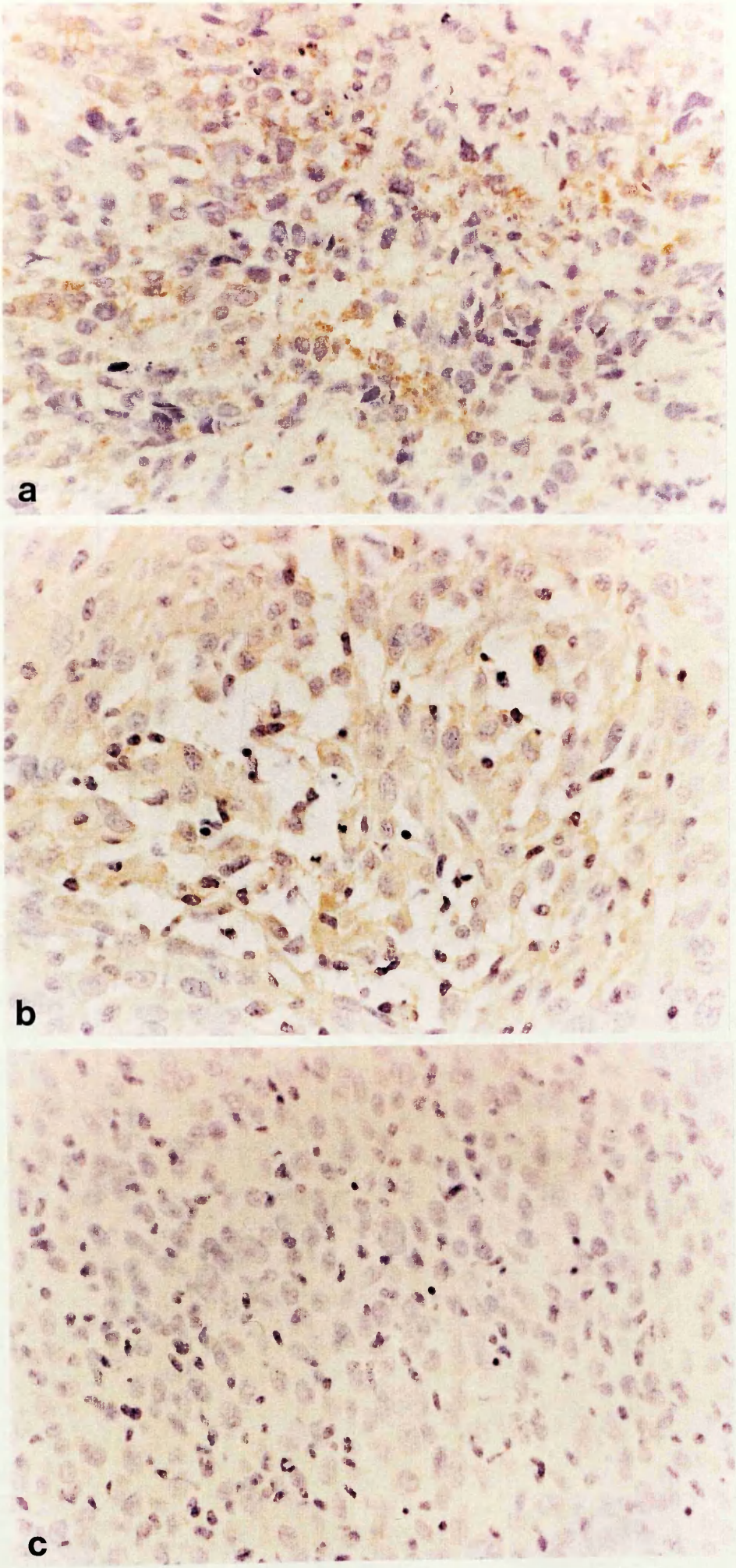

a
b
c

**Table 5.3a:- Sensitivity of the cell lines to geneticin (G418) in early passage culture (monolayer cloning).**

| Cell line | Geneticin [ug/ml] | | | Remarks |
|---|---|---|---|---|
| | [0] | [200] | [800] | |
| Mv1 | 158 ± 3 (63%)— | 0 (0%) | 0 (0%) | Sensitive |
| M1 | 164 ± 6 (66%)— | 155 ± 5 (62%)— | 160 ± 4 (64%)— | Resistant |
| N1 | 201 + 3 (80%)— | 191 + 4 (76%)— | 101 + 4 (40%)— | Resistant |
| T1 | 209 + 4 (84%)— | 217 + 2 (87%)— | 191 + 4 (76%)— | Resistant |

Sensitivity was tested using fresh cultures in their second passage, after taking out of freezer. Data are number of colonies (Mean+SEM) from 3 independent experiments. The figures in parentheses are— percentage values determined by dividing the number of colonies formed by total number of cells seeded, and multiplying by 100.

**Table 5.3b:- Sensitivity of the cell lines to geneticin (G418) in late passage cultures (monolayer cloning).**

| Cell line | Geneticin [ug/ml] | | | Remarks |
|---|---|---|---|---|
| | [0] | [200] | [800] | |
| Mv1 | 152 ± 6 (60%) | 0 (0%) | 0 (0%) | Sensitive |
| M1 | 157 ± 9 (63%) | 155 ± 5 (62%) | 156 ± 7 (62%) | Resistant |
| N1 | 199 ± 5 (80%) | 187 ± 3 (75%) | 90 ± 4 (36%) | Resistant |
| T1 | 207 ± 4 (83%) | 209 ± 3 (84%) | 191 ± 4 (76%) | Resistant |

Sensitivity was tested in late cultures in their 12-14 passage, as in previous table.

size was noticed in N1 cell line following drug exposure.

### 5.3.2.2 Species of Origin of The Cell Lines

#### (A) Chromosome Analysis

Chromosome analysis of cell lines showed a diploid chromosome number of 30 [Table 5.4, Figure 5.2].

**Table 5.4:- Chromosome number of the lines.**

| *NO | Mvl | M1 | N1 | T1 |
|---|---|---|---|---|
| N=30 | 70 | 67 | 60 | 77 |
| N=30 | 30 | 33 | 40 | 23 |

*NO: Chromosome number. A Pearson chi-square test was not statistically significant (P=0.15) indicating that the chromosome number of the four cell strains was the same.

A diploid chromosome number of around 30, a modal chromosome number of 30 and morphology of the chromosomes confirmed the mink origin of the cell lines. No obvious differences were observed among the strains in terms of their gross chromosomal morphologies, or chromosome number. Detailed karyotypic analysis of the cell strains by G-banding was attempted, but preliminary results did not show any differences (data not shown).

#### (B) Lactate Dehydrogenase Isoenzyme Analysis

LDH-isoenzymes revealed characteristic bands specific to the mink lung cell lines, which were absent from the cell lines of other species [see Plate 3.2, Figure 3.1].

### 5.3.2.3 Lineage Identification

#### (A) Morphological Properties

When grown in **sparse** culture there were no obvious differences among the lines. Both the parental as well as transformants showed multipolar flattened cells. When observed at **confluence**, Mvl cells grew as flattened cells exhibiting contact inhibition. M1 appeared very similar to the parental line. However, N1 and T1 showed distinct morphological changes, with cells attaining a bipolar or

spindle shaped appearance. Their growth was also very fast and they reached confluence earlier than the parental line, started at the same cell density. The morphological phenotypes were strikingly different in the dense cultures, the parental line formed contact inhibited monolayer [Plate 5.4 a], while the transfected cells formed characteristic whorls [Plate 5.4 b, c, & d]. The differences were always more pronounced in mutated ras transformed cells compared with other transformed lines.

**(B) Cytological Studies**

Examination of the centrifuged or whole mount preparations of monolayer cells stained with Giemsa showed an epithelial appearance in all the lines. Mvl [Plate 5.5] and M1 cell lines were polygonal, with large central round nuclei, and prominent nucleoli, while T1 [Plate 5.5] and N1 were spindle shaped.

**(C) Loss of Contact Inhibition**

The parental cell line, Mvl, grew as a typical contact-inhibited monolayer of cells [Plate 5.6], while all the transfected cell lines showed piling up of more spindle shaped fibroblastic type cells, as shown in T1 [Plate 5.6].

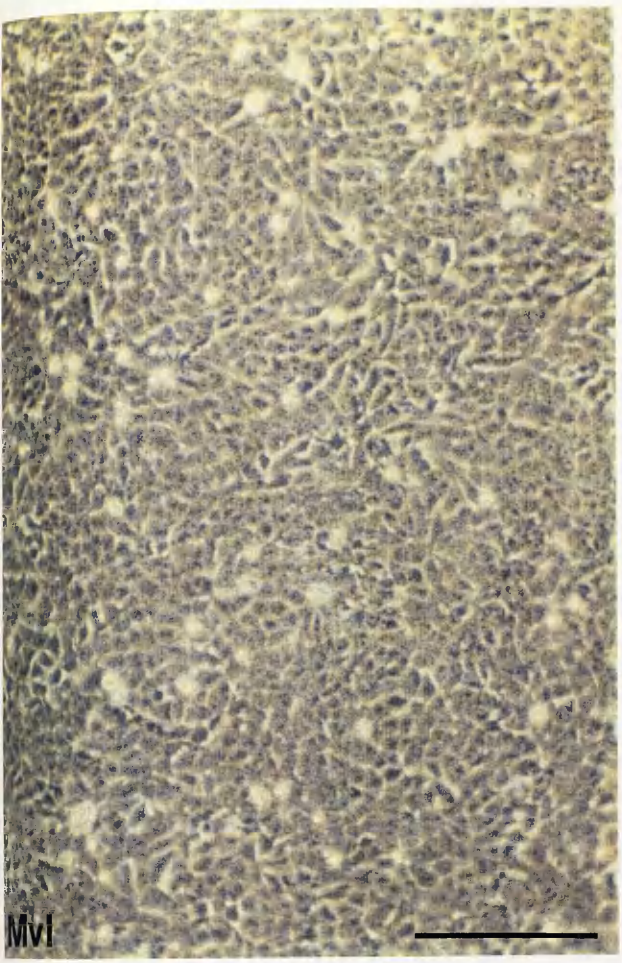
### 5.3.2.4 Growth Characteristics of The Cell Lines

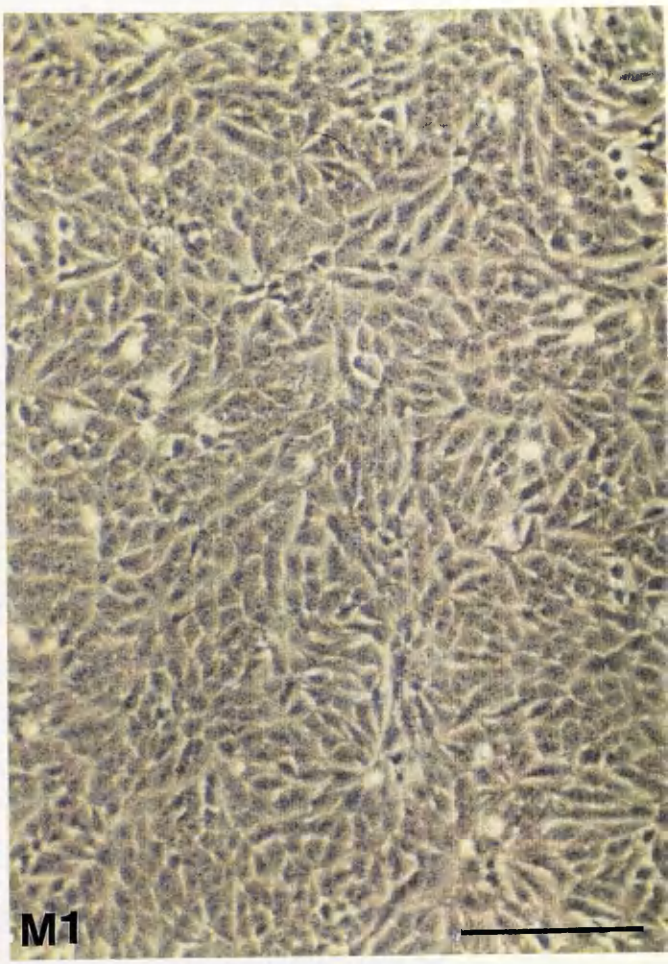
**(A) Growth Rates**

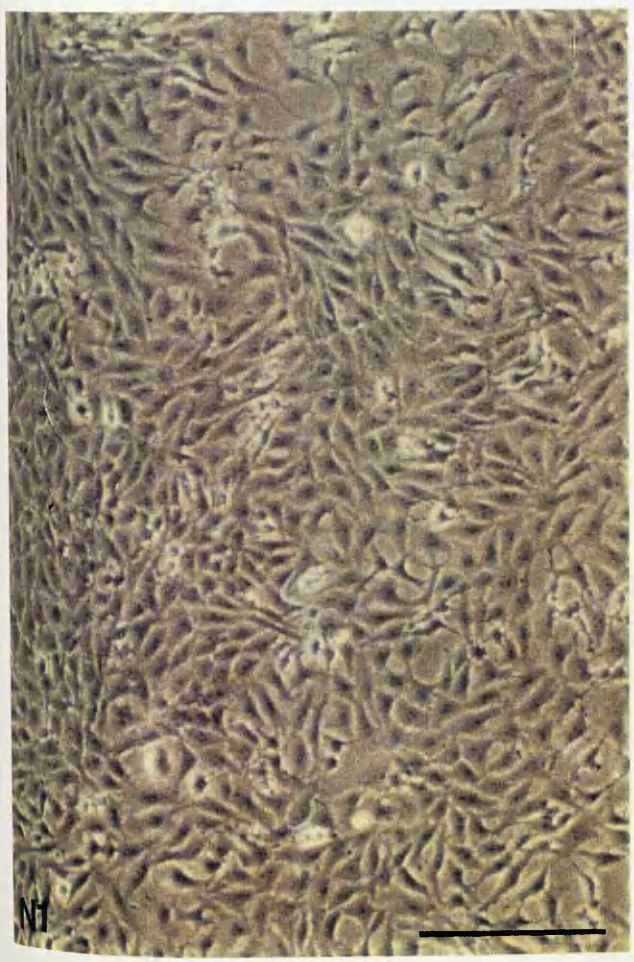
A growth curve was generated [Figure 5.3] for each cell line, by plating $1x10^3$ cells/well in 24 well plates and counting at 24 hours intervals. The cell number was significantly higher in the transformed than in the parental cells throughout the growth period, but the differences were most distinct at the saturation densities. The numerical data are shown in Table 5.5. Under routine culture conditions, all the cell lines grew rapidly with average doubling times of under 23 hours, a short lag period of around less than a day, and a relatively steeper log-phase of around 8 days duration. The plateau was reached after approximately 9 days in all cell lines under the culture conditions as described above. However, the population doubling time (DT) of the parental line was relatively longer than those of transfected lines. The doubling time was significantly reduced in transfected cell lines, especially in T1, compared to nontransfected line, and the saturation densities of the transfectants were increased relative to their nontransfected counterpart.

**Plate 5.4:- Morphology of the cell lines in culture.**

Mvl grows with a typical epithelial morphology. M1 also shows epithelioid morphology with some spindle cells. N1 and T1 show a more marked spindle cell morphology, and the changes being most prominent in T1 (X 10 objective, phase-contrast, scale bar= 200 $\mu$m).


Mvl


M1


N1


T1

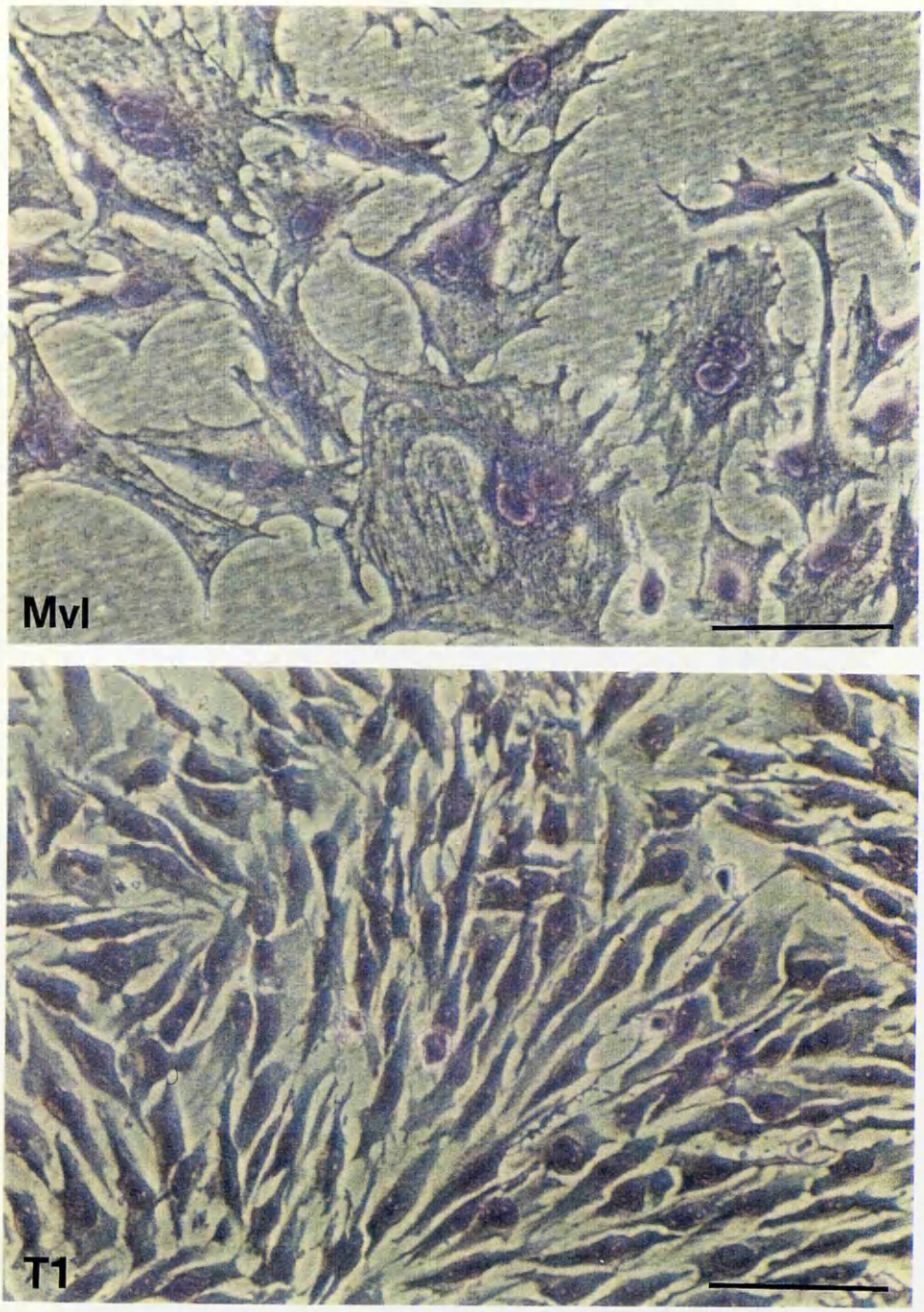



**Plate 5.5:- Effects of oncogene transfection on cytology of transfectant**

Sub-confluent cultures fixed in methanol 48 hours after subculture, showing significant morphological differences. Compared to parental line Mvl (large, multipolar, epithelioid cells) the activated Ha-ras transfected line T1 shows a more marked spindle shaped morphology. M1 cells were similar to Mvl and N1 to T1 (X 20 objective, Giemsa stain, bar scale = 100 $\mu$m)

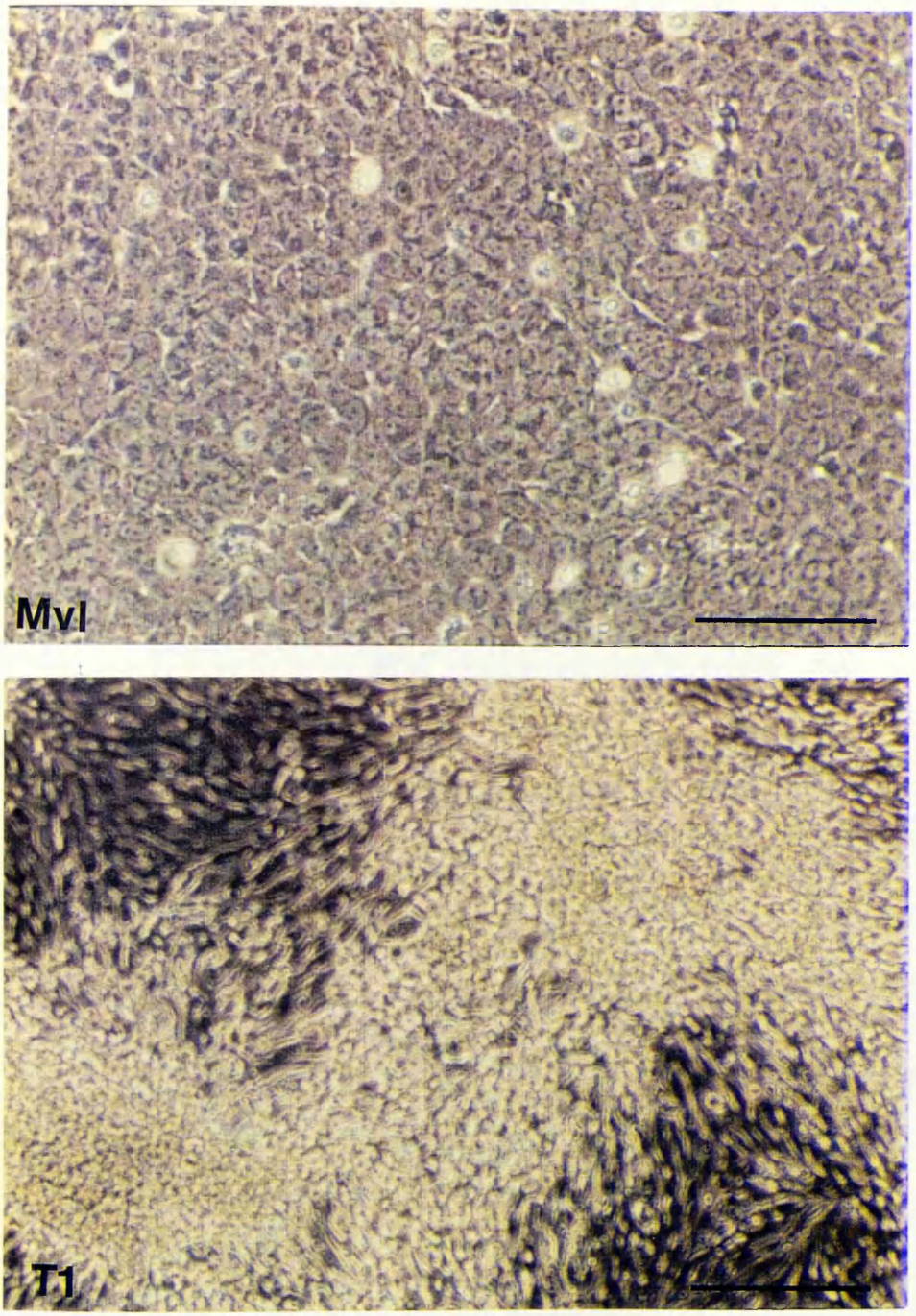



**Plate 5.6:- Effects of oncogene transfection on contact inhibition.**

Post-confluent cultures of Mv1 cells forming a contact inhibited monolayer, and the Ha-ras transfected line T1 showing evidence of multilayering with loss of contact inhibition (X 20 objective, phase-contrast, bar scale = 100 $\mu$m)

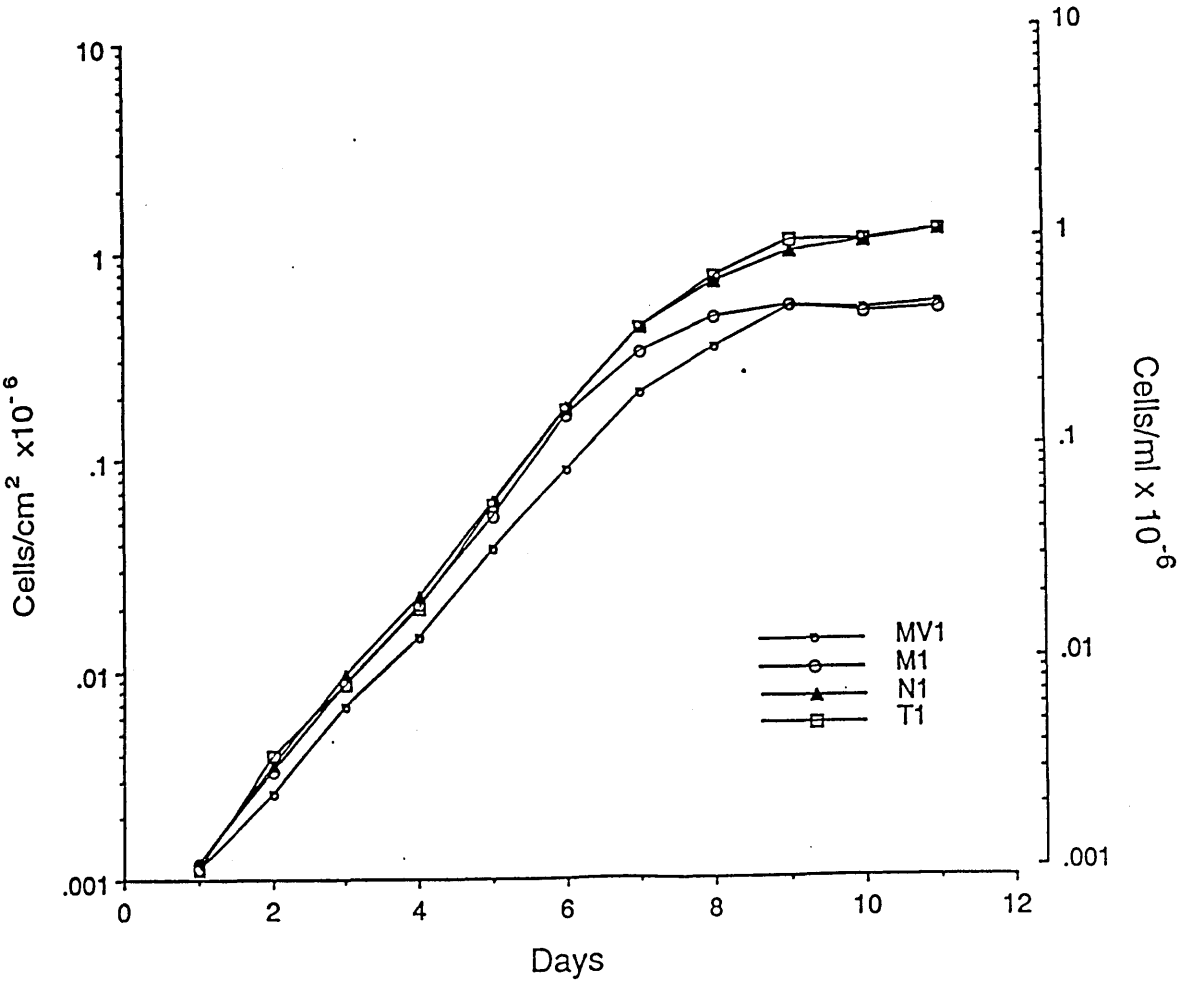



**Figure 5.3:- Growth curves of cell lines in vitro.**

$5x10^2$ cells were plated per well in 24 well plates at day zero. Cells were trypsinised and counted from a minimum of 3 wells daily, and using an electrical particle counter. Medium was changed with fresh on alternate days in early growth period i.e from 1-5 days, and daily thereafter. Each point represents mean ± SEM of 4 replicates from one experiment similar results were obtained from three other experiments, repeated on different occasions.

**Table 5.5:- Growth characteristics in vitro, following oncogene transfection of mink lung cell line Mv1.**

| Cell line | Oncogene transfection | Population Doubling time (Hours) | Saturation Density (Cells/$cm^2$) $x10^{-5}$ |
|---|---|---|---|
| Mv1 | None | 22.8 ± 1.0 | 3.9 ± 0.4 |
| M1 | c-myc | 19.1 ± 0.7 | 6.4 ± 1.0 |
| N1 | Ha-ras | 16.9 ± 1.3 | $^{**}$9.3 ± 0.7 |
| T1 | Ha-ras (T24) | $^{*}$15.7 ± 1.3 | $^{**}$9.8 ± 0.5 |

Cells were grown for 12 days in F10:DMEM containing 10% FBS and in 24 well plates. Cells were counted from three wells and fed daily. Data are mean ± SEM from at least 4 independent experiments.

$^{*}P<0.02$, $^{**}P<0.004$ (Mann-Whitney test with Bonferoni adjustment).

## (B) Serum Dependence

### *Plating Efficiency in Monolayer Culture*

The ras transfected cells always showed a relative increase in their ability to clone in monolayer in the absence of serum, compared with the non-transfected line [Table 5.6]. However, all lines cloned poorly in the absence of serum, especially Mv1, and M1. The growth of transfected lines was not affected as much as that of the parental line when grown at high cell concentrations ($1x10^5$/ml) in 25 $cm^2$ flasks. However, the growth of all the cell lines was reduced significantly by increasing the passage number in serum free conditions. Increasing the serum concentration to 0.5% increased the cloning efficiency although the number was still higher in the transfected lines by over three fold. The plating efficiency of transfected lines showed only a modest increase when serum concentration was raised from 10% to 20%, an increase in the mean size of colony was noticed at 20% serum especially in N1 line [Plate 5.7].

### *Anchorage Dependence*

While the plating efficiency in monolayer showed only a modest increase in the transfected lines, cloning in suspension with 10% serum gave a low efficiency in Mv1, and both N1 and T1 showed a marked increase, 7-10 fold in N1 and 10-20 fold in T1 [Table 5.7]. A more modest increase of 2-3 fold was also observed in M1. The small number of colonies formed in Mvl plates were of the same size as M1 and T1, while N1 cell gave numerous smaller colonies [Plate 5.8]. Slight inhibition of colony formation was observed in N1 and T1 in contrast to an apparent stimulation of colonies in Mv1 and M1 when serum concentration was raised from 10% to 20% [Table 5.7]. M1 and T1 gave larger sized colonies while N1 gave numerous but smaller sized colonies. This finding was consistent, but in contrast to monolayer cloning. When cloned in medium containing 0.5% serum, Mv1 cells died within 10 days of cloning. While the transfected cells did survive, though they could not form colonies, they were alive and non-dividing or quiescent, and were able to be stained with MTT dye even after 3 weeks in agar suspension. When cloned in serum free medium none of the cell lines was able to form colonies. The cells died completely both in Mv1 and M1, although a few single and non-dividing cells were still able to reduce the MTT, after 3-4 weeks in agar culture.

### *Saturation Densities*

Cells were plated in 24 well plates at a density of $1x10^3$ cells/well in routine culture medium (10% FBS). The cells were trypsinised and counted

from at least 3 wells/treatment on alternate days until the cell number reached the plateau usually at 10th. to 12th. day. The peak cell number for each cell was determined and compared with control. In parallel experiments, saturation densities were found in different serum concentrations (0%, 0.5%, 10%, and 20%). Saturation densities in regular monolayer cultures were higher in the transfected lines than in non-transfected cells, and were maximum at 10% FBS, while the parental line still showed a further increase at 20% FBS [Figure 5.4]. All transfected lines showed a higher cell number in limiting serum concentrations (0.5%) with T1 showing least dependence. Terminal cell numbers in the absence of serum were extremely low but were still highest in T1 and higher in N1 and M1 than in Mv1.

### 5.3.2.5 Effects of Dexamethasone on Cloning Efficiencies of The Cell Lines.

The effect of dexamethasone ($1x10^6$ M) on monolayer cloning was stimulatory. Though the effect on the number of colonies was only modest [Table 5.8], a significant increase in size of colonies was noticed as seen with 20% serum, and striking finding was a marked increase in size of colony in N1, an effect opposite to that in agar [see above, Plate 5.7].

Dexamethasone also showed stimulatory effects on the growth of cells in agar [Plate 5.9; Table 5.8]. A marked increase in size of colonies was noticed in all lines [Plate 5.10], except N1 which showed numerous, but still very small colonies, a feature opposite to that found in monolayer. Mvl and M1 showed an intermediate response.

### 5.3.2.6 Labelling Indices of The Cell Lines

Labelling indices (LI) were determined at saturation densities both by conventional autoradiography and by BrdUrd incorporation assays. A good correlation was found between the values determined by these two different techniques [Table 5.9]. There were consistent differences among the different cell lines [Plate 5.11]. The indices increased both in N1 and T1 cell lines by approximately 4 fold, but there was only slight increase in M1 cell line. These differences were consistent with the growth of cell strains in culture [see Table 5.5], where T1 and N1 showed a high growth rate compared with the parental line and the M1 was intermediate relative to other lines.

**Table 5.6:- Effect of serum concentration on plating efficiency in monolayer.**

| Cell line | Plating efficiency (%) | | | |
|---|---|---|---|---|
| | 0% FBS[a] | 0.5% FBS | 10% FBS | 20% FBS |
| Mv1 | 0.0 | 2.8 + 1.4[b] | 59.2 ± 6.1 | 70.6 ± 10.0 |
| M1 | 0.0 | 9.3 ± 1.4 | 67.5 ± 6.8 | 81.1 ± 10.1 |
| *N1 | 0.1 ± 0.1 | 7.3 ± 0.9 | 75.3 ± 4.5 | 89.0 ± 6.8 |
| *T1 | 0.1 ± 0.1 | 7.7 ± 3.9 | 81.9 ± 7.7 | 90.1 ± 7.2 |

[a]FBS Foetal bovine serum. [b]Values are mean ± SEM of three independent experiments, each containing at least 4 replicates.

*$P<0.008$ (Analysis of variance and Bonferoni adjustment).

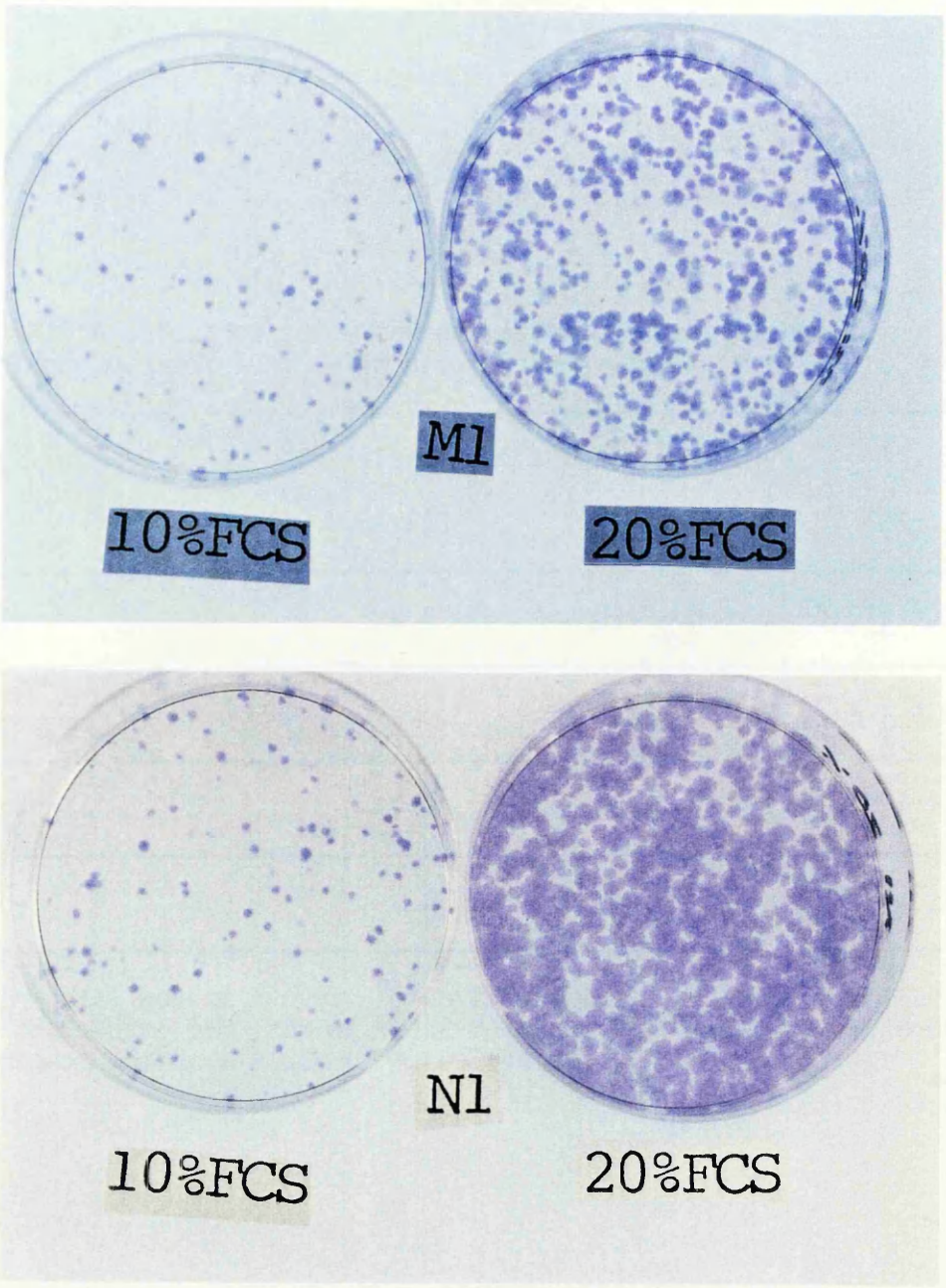



**Plate 5.7:- Effects of serum concentration on plating efficiency and colony size of the cell lines.**

A single cell suspension in culture medium was seeded into 60 mm petri dishes with 500 cells per dish in 5 ml medium with varying serum concentration (0%, 0.5%, 10%, and 20%), and colonies fixed after 2 weeks. M1 and N1 showing an increase in colony number and size in 20% serum compared to 10% serum. Mvl and T1 were similar to M1. N1 cells show an increase in size in 20% serum, in contrast to a small size colony formation in agar (see Plate 5.8). Similar effects were seen when the cells were grown in 10% serum containing 1 $\mu$M dexamethasone.

**Table 5.7:- Effect of serum concentration on the cloning efficiency of mink lung cells growing in semisolid medium.**

| Cell line | Cloning efficiency (%) | | | |
|---|---|---|---|---|
| | 0% FBS | 0.5% FBS | 10% FBS | [a]20% FBS |
| Mv1 | 0.0 | 0.0 | 0.1 ± 0.0 | 0.5 ± 0.3 |
| *M1 | 0.0 | 0.0 | 0.7 ± 0.1 | 5.4 ± 0.6 |
| *N1 | 0.0 | 0.0 | 16.2 ± 2.2 | 10.3 ± 1.3 |
| *T1 | 0.0 | 0.0 | 22.6 ± 2.0 | 10.7 ± 1.8 |

FBS= Foetal bovine serum. [a]Significant increase in colony size. Data are mean ± SEM from at least 3 independent experiments, each experiment with a minimum of 4 replicates.

*$P<0.050$ (Mann-Whitney test)

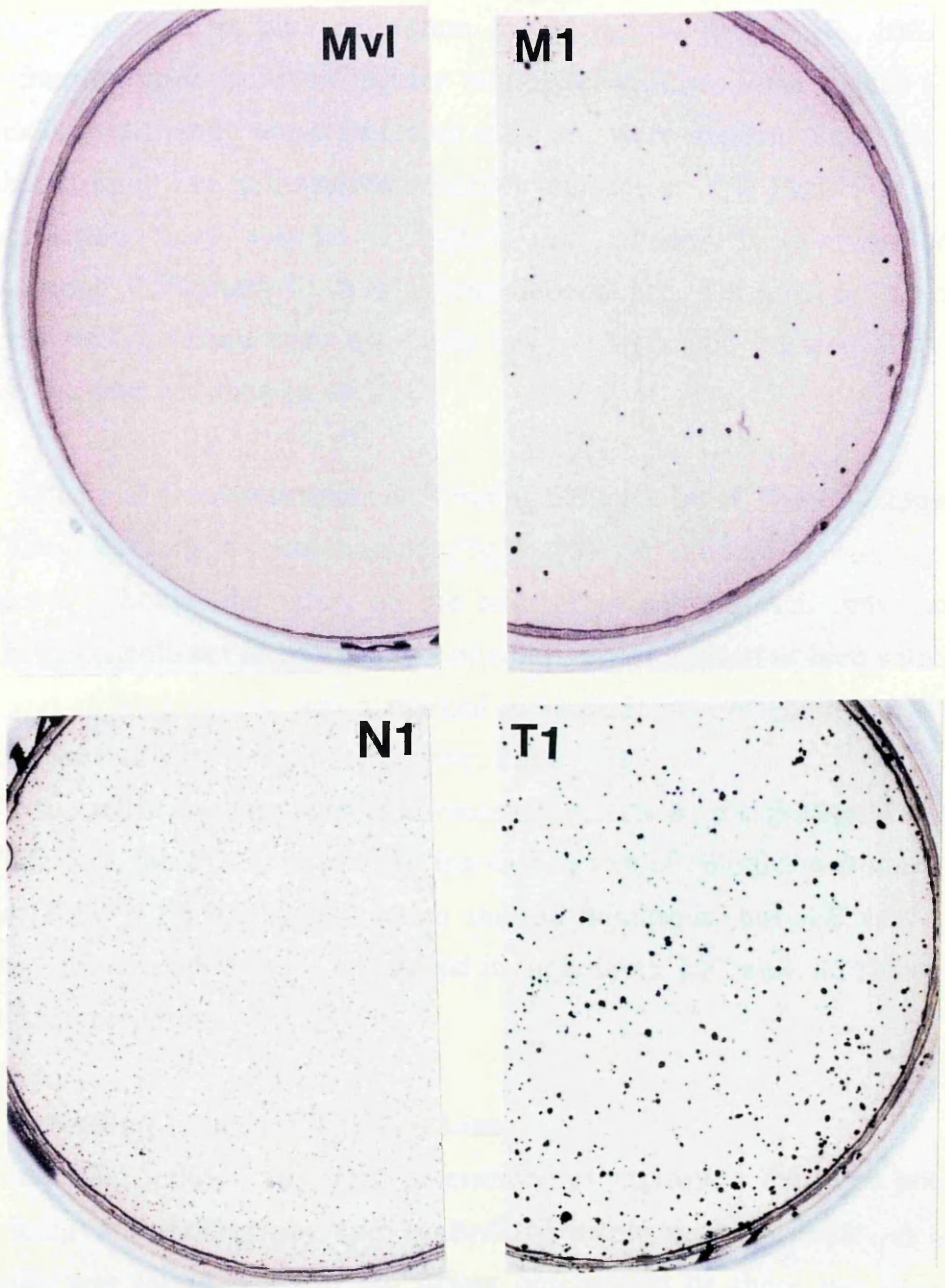



**Plate 5.8:- Clones of cells in soft agar.**

$1x10^4$ cells were plated per dish in 0.3% agar on a 1% agar underlay in a 35 mm dish, and colonies stained with MTT after 3 weeks. There is no colony formation in Mv1. All transfected cell lines show colony formation reaching a maximum with T1. N1 formed numerous small sized colonies, while M1 formed fewer but still large size colonies.

from at least 3 wells/treatment on alternate days until the cell number reached the plateau usually at 10th. to 12th. day. The peak cell number for each cell was determined and compared with control. In parallel experiments, saturation densities were found in different serum concentrations (0%, 0.5%, 10%, and 20%). Saturation densities in regular monolayer cultures were higher in the transfected lines than in non-transfected cells, and were maximum at 10% FBS, while the parental line still showed a further increase at 20% FBS [Figure 5.4]. All transfected lines showed a higher cell number in limiting serum concentrations (0.5%) with T1 showing least dependence. Terminal cell numbers in the absence of serum were extremely low but were still highest in T1 and higher in N1 and M1 than in Mv1.

### 5.3.2.5 Effects of Dexamethasone on Cloning Efficiencies of The Cell Lines.

The effect of dexamethasone ($1x10^{6}$ M) on monolayer cloning was stimulatory. Though the effect on the number of colonies was only modest [Table 5.8], a significant increase in size of colonies was noticed as seen with 20% serum, and striking finding was a marked increase in size of colony in N1, an effect opposite to that in agar [see above, Plate 5.7].

Dexamethasone also showed stimulatory effects on the growth of cells in agar [Plate 5.9; Table 5.8]. A marked increase in size of colonies was noticed in all lines [Plate 5.10], except N1 which showed numerous, but still very small colonies, a feature opposite to that found in monolayer. Mvl and M1 showed an intermediate response.

### 5.3.2.6 Labelling Indices of The Cell Lines

Labelling indices (LI) were determined at saturation densities both by conventional autoradiography and by BrdUrd incorporation assays. A good correlation was found between the values determined by these two different techniques [Table 5.9]. There were consistent differences among the different cell lines [Plate 5.11]. The indices increased both in N1 and T1 cell lines by approximately 4 fold, but there was only slight increase in M1 cell line. These differences were consistent with the growth of cell strains in culture [see Table 5.5], where T1 and N1 showed a high growth rate compared with the parental line and the M1 was intermediate relative to other lines.

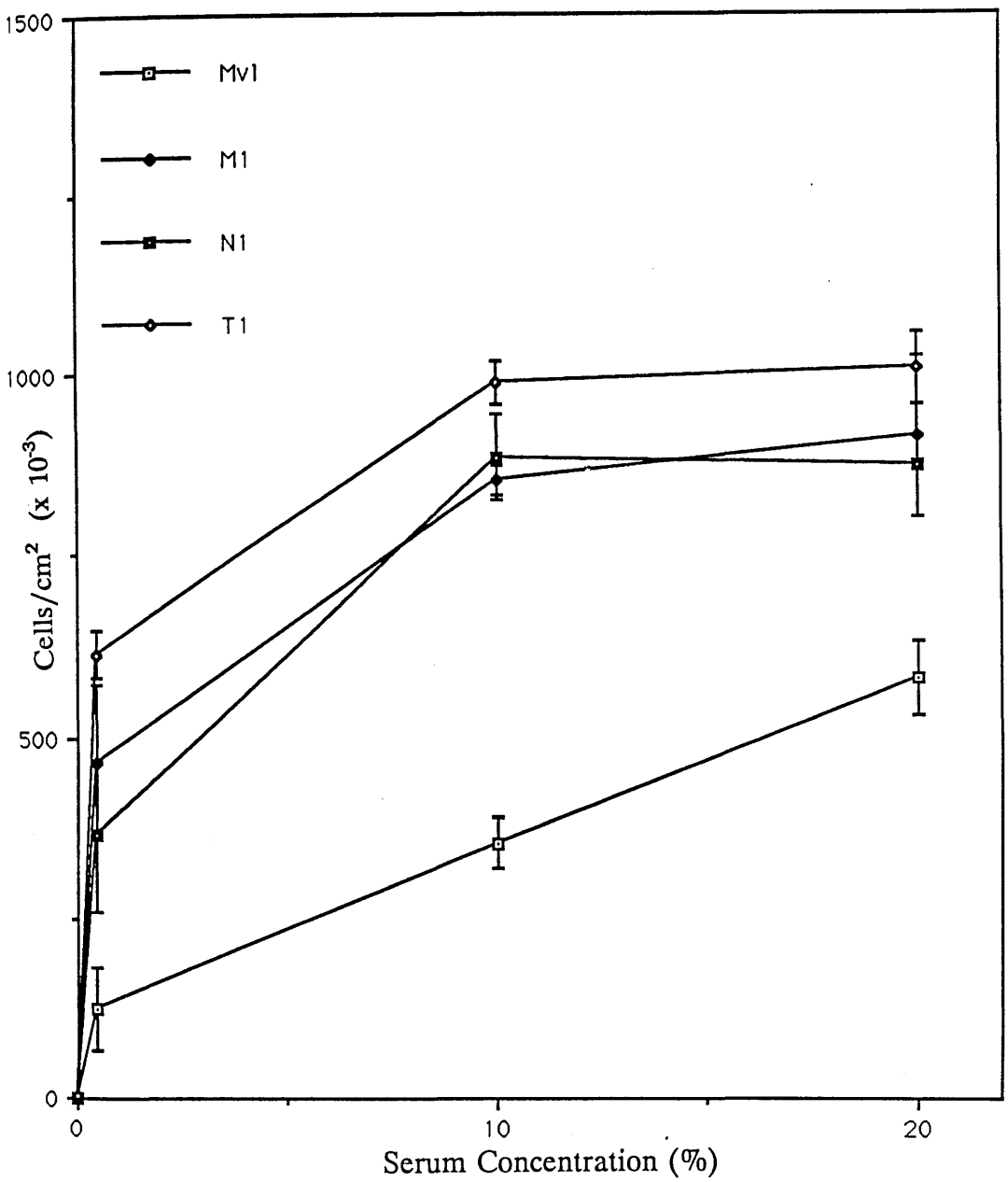



**Figure 5.4:- Effects of serum concentration on terminal cell density.**

$10^3$ cells were seeded per well in 24 well plates on day 0, with medium containing varying serum concentration (0%, 0.5%, 10%, and 20%). Cells counted daily from wells with 10% serum (see Figure 5.3), and cell number for each serum concentration determined at day 10-12. Each data point represents mean ± SEM (bars) from 3 experiments.

**Table 5.8:- Effects of dexamethasone on colony forming efficiencies of cell lines in monolayer and in semisolid medium.**

| Cell line | Monolayer Cloning (%) | | Suspension Cloning (%) | |
|---|---|---|---|---|
| | (-Dex) | (+Dex) | (-Dex) | (+Dex) |
| Mv1 | 59 ± 6 | 74 ± 9 | 0.1 ± 0.0 | 0.5 ± 0.0 |
| M1 | 68 ± 7 | 79 ± 11 | 0.7 ± 0.1 | 7.8 ± 1.2 |
| N1 | 75 ± 5 | 95 ± 13 | 16.7 ± 2.2 | 23.6 ± 6.0 |
| T1 | 82 ± 8 | 96 ± 7 | 22.6 ± 2.0 | 28.1 ± 8.0 |

Cells were grown in F10:DMEM containing 10% serum with (+Dex) or without (-Dex) dexamethasone (1.0 uM). Cells (250-500) were plated in 60 mm petri dishes with 5 ml medium for monolayer cloning, and $10^4$ cells/ml 0.3 % agar for suspension cloning as outlined in General Method.

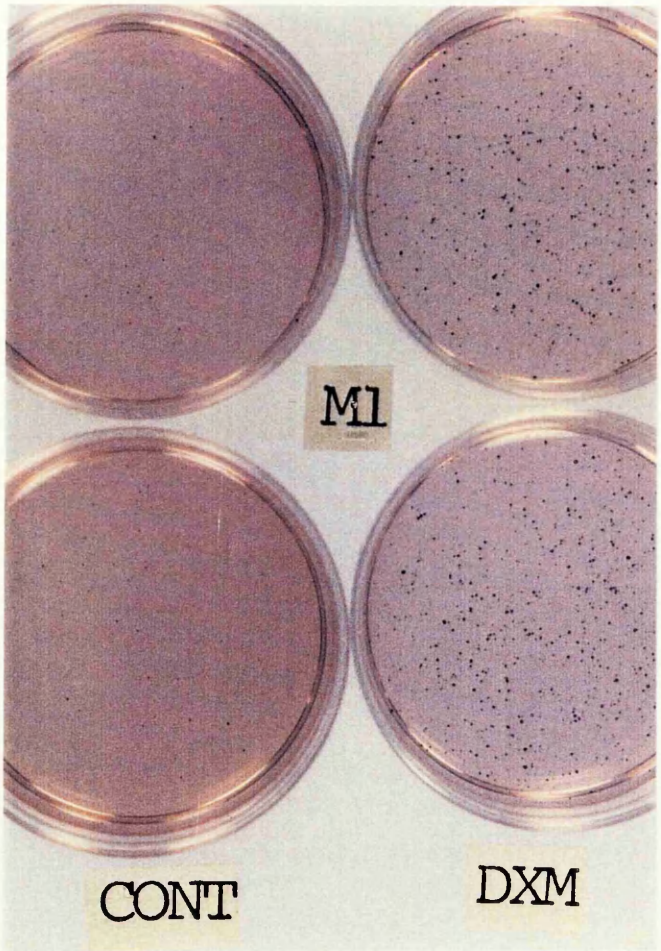
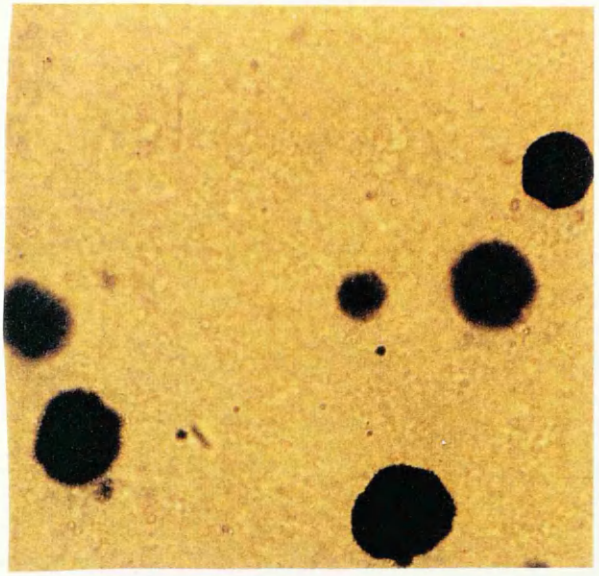



**Plate 5.9:- Effect of dexamethasone on colony number in agar.**

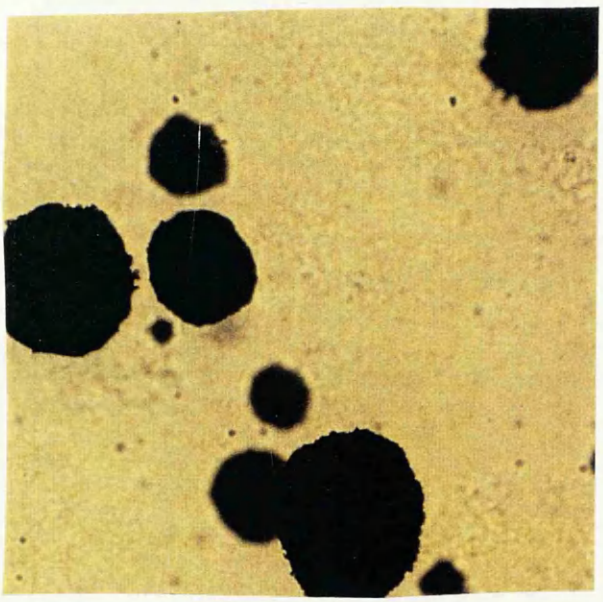
M1 cells $10^4$/ml 0.3% agar plated with 10% serum without dexamethasone (CONT) or 10% serum containing 1 $\mu$M dexamethasone (DXM). Duplicate plates show increased number of colonies in the presence of dexamethasone. Colonies stained with MTT after 3 weeks. Similar results were observed in Mvl and T1 lines, while N1 formed numerous but still small size colonies (see above, Plate 5.8)

**Plate 5.10:- Effects of serum and/or dexamethasone on agar cloning.**

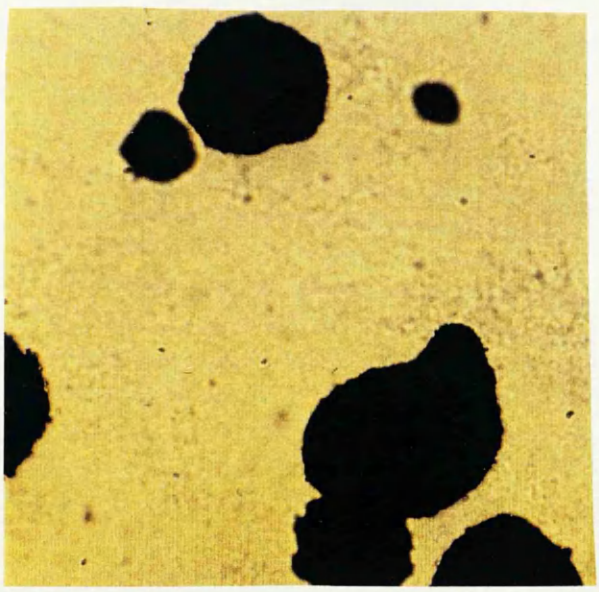
T1 colony size 2 weeks after cloning in 0.3% agar in 10% serum (a), 20% serum (b), and 10% serum with 1 $\mu$M dexamethasone (c). Both increasing serum concentration and the addition of 1 $\mu$M dexamethasone resulted in an increase in colony size. Similar results were seen in M1 (see above, Plate 5.9) and to a lesser extent in Mvl. There was no effect on colony size in N1 (see above, Plate 5.9).



a.



b.



c.

**Table 5.9:- Labelling indices of the cell lines at saturation density.**

| Cell line | [a]LABELLING INDICES (%) Autoradiography ($^3$H-thymidine) | Bromodeoxyuridine (BrdUrd assay) | *P-Value |
|---|---|---|---|
| Mv1 | 6.3 ± 0.6 | 4.7 ± 0.3 | |
| M1 | 6.6 ± 0.8 | 5.5 ± 0.2 | >0.05 |
| N1 | 22.3 ± 0.1 | 15.9 ± 0.7 | <0.001 |
| T1 | 24.0 ± 1.3 | 27.0 ± 1.4 | <0.001 |

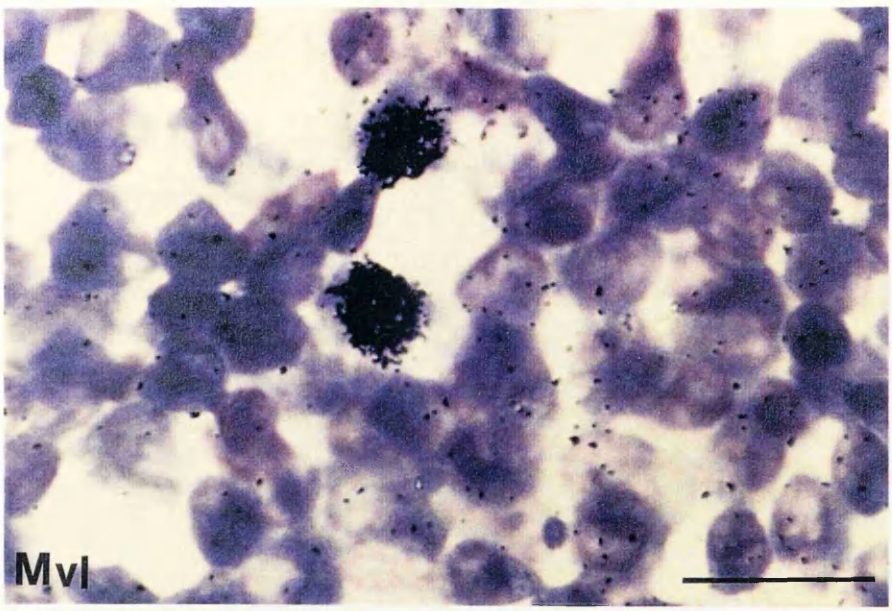
[a]Cells were exposed to the labelling reagent ($^3$H-thymidine or Bromodeoxyuridine) for 1 hour at 37 $^{o}$C.

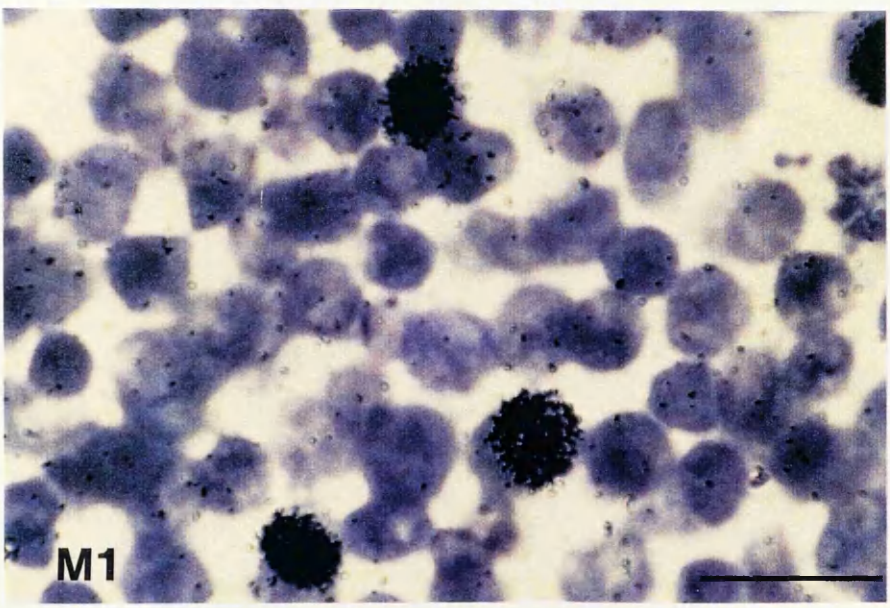
*Statistical analysis was carried out using analysis of variance technique with Bonferoni adjustment.

**Plate 5.11:- Autoradiogrphy**

Cells labelled with $^{3}H$-thymidine (5.0 $\mu$Ci/ml) for 1 hour in DMEM medium, were fixed, and coated with radiosensitive emulsion, and incubated in the dark for 1 week before developing. Photomicrographs of labelled cells from representative fields of Mv1, M1, and T1 are shown. Labelled cells are more frequent in both M1 and T1 than in the parental line (see Table 5.8), with T1 showing greatest number, and N1 appeared as T1.

(X 40, Giemsa, scale bar= 50 $\mu$m).


Mvl


M1

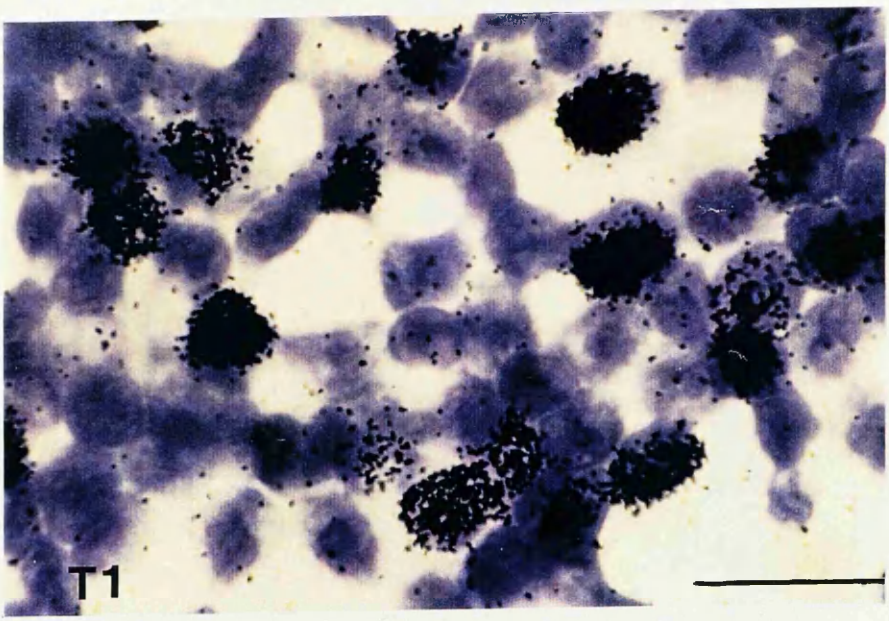
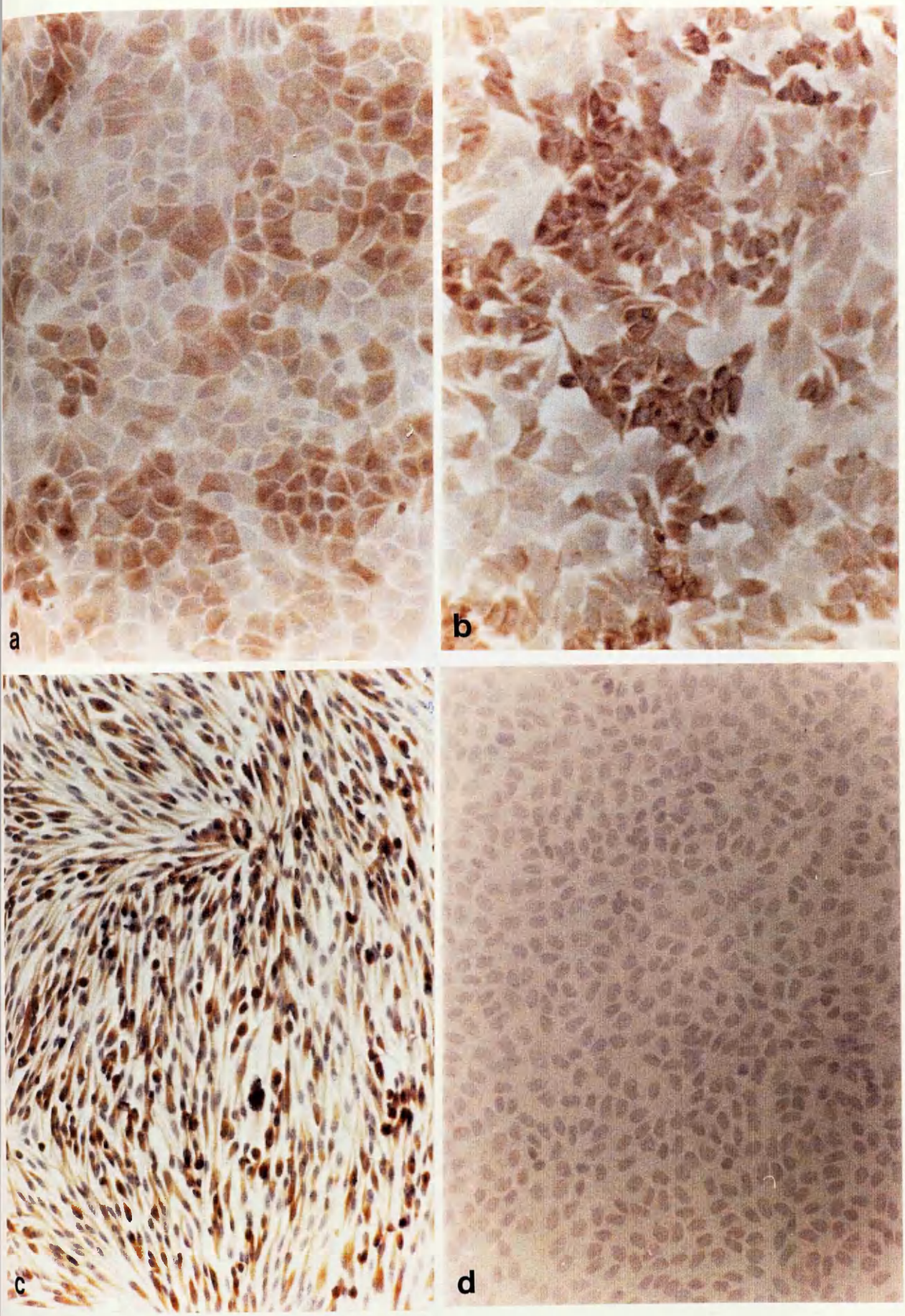

T1

**Plate 5.12:- Expression of cytokeratins in monolayer cultures**

Positive staining for broad range basic cytokeratins is demonstrated in Mv1 (a), M1 (b), and T1 (c). N1 appeared as T1. Staining was abolished when primary antibody was excluded in Mvl (d). (X 225, AE3 antibody: indirect IP).


a
b
c
d

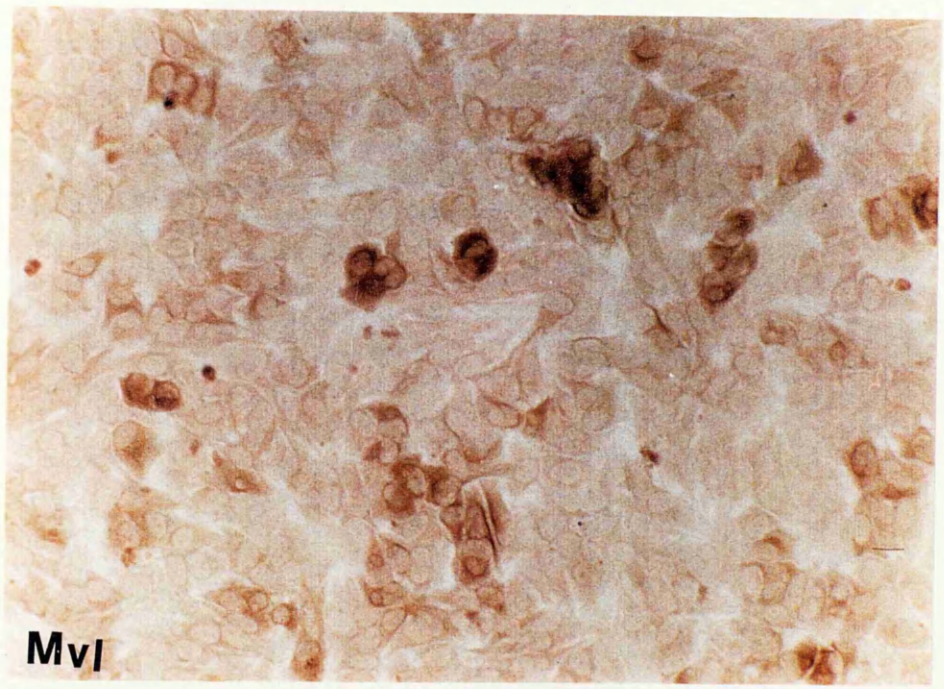


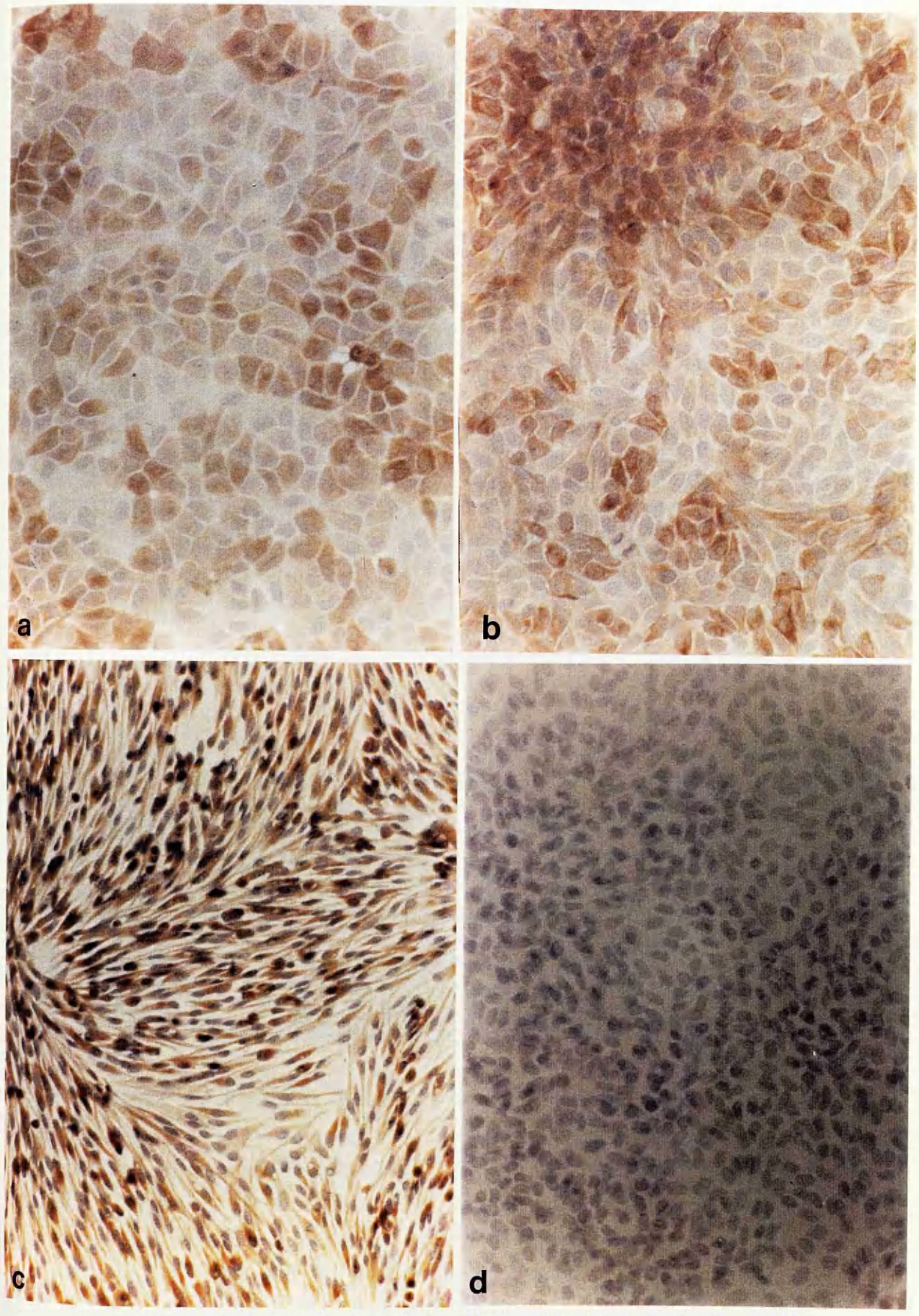

**Plate 5.13:- Expression of low molecular weight cytokeratin in cell lines.**

Example of focal positivity for low molecular weight cytokeratin in Mvl, consistent with glandular differentiation. Similar results were observed in other cell lines (X 255, CAM5.2 antibody: indirect IP).

**Plate 5.14:- Expression of vimentin in monolayer cultures.**

Positive staining for vimentin is demonstrated in cell line Mvl (a), M1 (b) and T1 (c). N1 appeared as T1. Staining was abolished when primary antibody was replaced with non-immune serum in Mv1 (d).

(X 225, anti-vimentin antibody: indirect IP)).


a
b
c
d

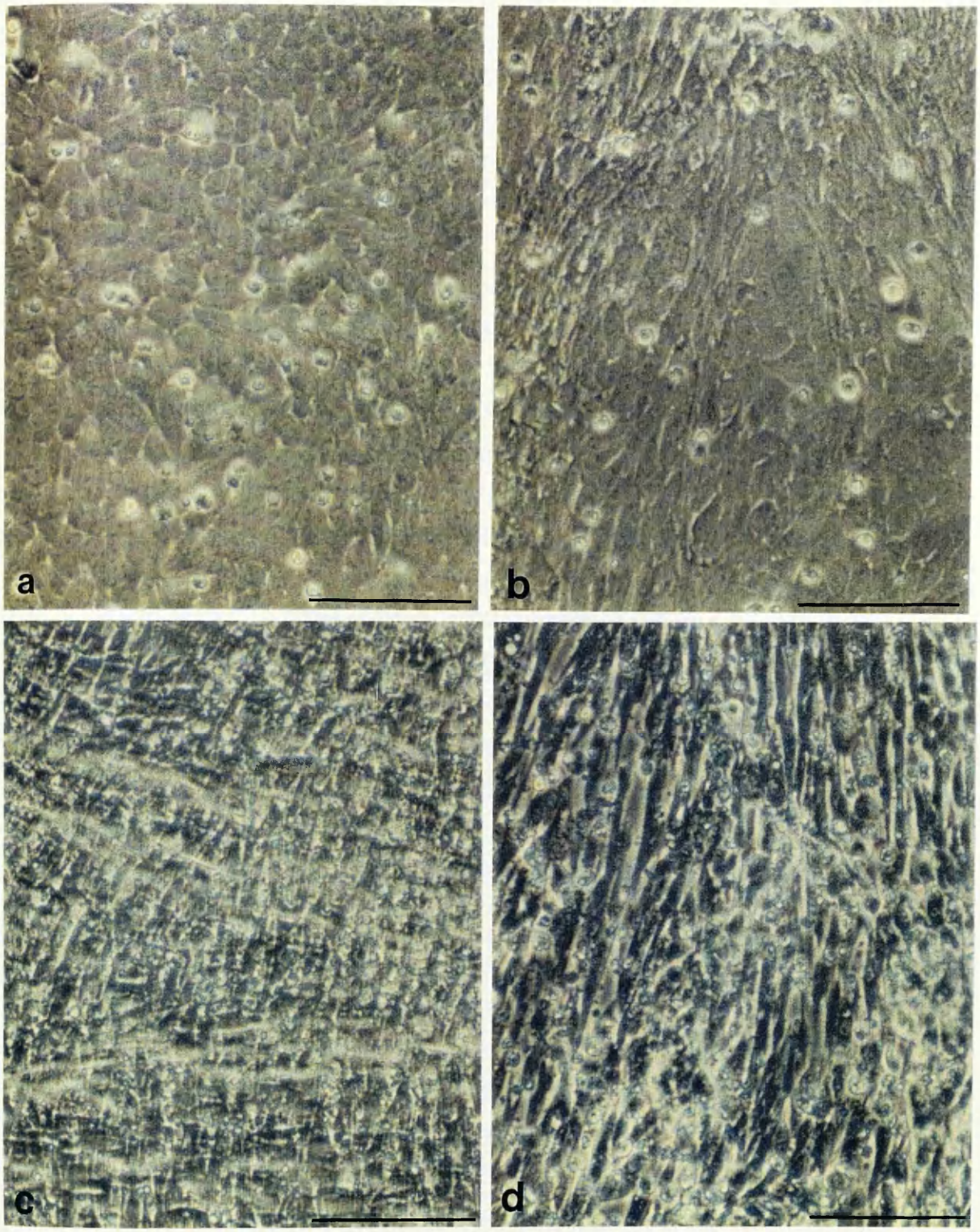



**Plate 5.15:- Effects of stromal interaction on morphology of cell lines.**

Morphology of fibroblast co-culture with Mv1 (a), M1 (b), N1 (c), and T1 (d) cells in 50:50 ratio, 3 weeks after seeding. $2x10^4$ cells/ml were plated in 25 $cm^2$ flasks, with 5 ml culture medium. Mvl shows no morphological effects in presence of fibroblasts, and M1 shows only slight changes. In contrast N1 and T1 show intermingling of the cells with fibroblasts giving different culture morphology compared to the cultures without fibroblasts (see above, Plate 5.4) (X 20 objective, phase-contrast, scale bar= 100 $\mu$m).

### 5.3.2.7 Immunohistochemical Marker Expression

#### (A) Cytokeratin Intermediate Filament Proteins

All cell lines stained positively [Plate 5.12] with a broad spectrum anti-cytokeratin monoclonal antibody AE3, which recognizes almost all of the known basic, type-II cytokeratins (type 1-8 according to Moll's catalogue, [Moll *et al.*, 1982].

Cell lines were negative when stained with AE1 another broadly specific monoclonal antibody against type-I or acidic cytokeratins of Moll's catalogue 3, 5, 12, and 19 [Moll *et al.*, 1982].

Focal positivity (around 10%) was observed in cells stained with CAM5.2 [Plate 5.13], which demonstrates low molecular weight (39kD, 42kD, and 45kD) cytokeratins [Moll *et al.*, 1982).

Approximately, 40%-50% of all cell lines showed positive staining for vimentin, an intermediate filament protein more commonly expressed by the cells of non-epithelial origin. The pattern of staining was different from that of cytokeratin, with prominent positivity in cell processes [Plate 5.14].

#### (B) Membrane Specific Antigens

Positive staining with EMA was focal and observed in less than 5% of cells. There was no difference in the staining of different cell lines.
Staining was similar with HMFG 1 and 2.

Immunopositivity with $\alpha$,and $\beta$-HCG was demonstrated in less than 5% of cells.

### 5.3.2.8 Ultrastructural Features

The presence of intermediate filaments, poorly developed adhesion specializations, microvillus profiles, and general cell to cell relationships were indicative of epithelial differentiation. These findings were present in all cell lines. The ras transfected cells showed more lysosomes then the other lines, but this feature was not consistent, and other lines also occasionally showed similar features (see below).

### 5.3.2.9 Effects of Stromal Interaction

In preliminary studies co-culture with human fetal lung fibroblasts did not produce any significant differences, in terms of cellular morphology [plate 5.15 a,b,c,d]. Cells growing in 50:50 co-culture grew normally first with fibroblasts intervening, but later on fibroblasts were invaded by epithelial cells, and growth of fibroblasts was reduced with time with their ultimate replacement by the test cells.

### 5.3.3 RESPONSE OF THE TRANSFECTANTS TO THE CYTOTOXIC DRUGS

This section describes the potential effects of oncogene transfection on the response of mink epithelial cells to cytotoxic drugs; cisplatin and cytosine arabinoside. The specific aims were to investigate whether transfection of lung epithelial cells with human oncogene could alter the cellular response to cytotoxic drugs *in vitro*, either by altering growth rate or by genetic change conferring an altered cellular phenotype with different response to cytotoxic drugs. Studies undertaken in this section showed varying response of transfectants to cytotoxic drugs depending upon the type of oncogene involved.

#### 5.3.3.1 Chemosensitivity Studies

Chemosensitivity data of cell lines are summarised in Table 5.10 and the dose response curves of different cell lines to cisplatin [Figure 5.5a] and ara-c [Figure 5.5b] are also shown. The normal ras transfected cell line was significantly more resistant to ara-c, compared with the parental cell line or other transfected lines. There were no significant differences in the sensitivities of the other transfected cell lines, relative to the parental cell line, to either ara-c or cisplatin.

### 5.3.4 INVASIVENESS *IN VITRO* AND PLASMINOGEN ACTIVATOR ACTIVITY OF THE TRANSFECTANTS

The proteolytic activity and invasion are malignancy associated properties. Plasminogen activator activity and invasive behaviour of the cells, before and after transfection were investigated in this section. *In vitro* invasion assays were performed using fragments of embryonic chick heart in confrontation with cells in tissue culture, while plasminogen activator activity of cells was determined using a chromogenic assay.

#### 5.3.4.1 Invasiveness *In Vitro*

Loose adhesion of the cells to the chick heart fragments could be detected within first 6 hours in all of the cell lines used, and firm adhesions within 24 hours.

Invading cells were readily identified in all sections because their nuclei were considerably larger and more intensively stained with haematoxylin than those of the host tissues. Only minor differences in the rate of attachment and adhesion between transformed and control cells were found. All the lines tested

**Table 5.10:- Sensitivity of cells to cytotoxic drugs.**

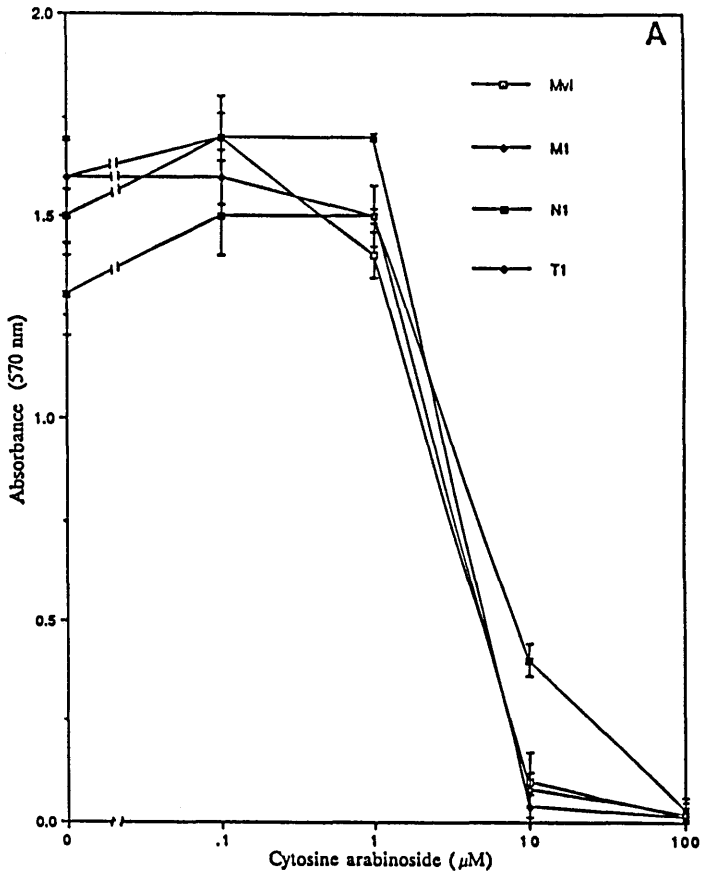
| CELL LINE | [a]$ID_{50}$ VALUES (M) OF CYTOTOXIC DRUGS | |
|---|---|---|
| | CISPLATIN ($1x10^{-7}M$) | CYTOSINE ARABINOSIDE ($1x10^{-6}M$) |
| Mv1 | 4.1 ± 0.5 | 3.3 ± 0.1 |
| M1 | 4.6 ± 0.6 | 3.1 ± 0.1 |
| N1 | 3.3 ± 0.3 | *5.4 ± 0.3 |
| T1 | 5.0 ± 0.2 | 2.9 ± 0.1 |

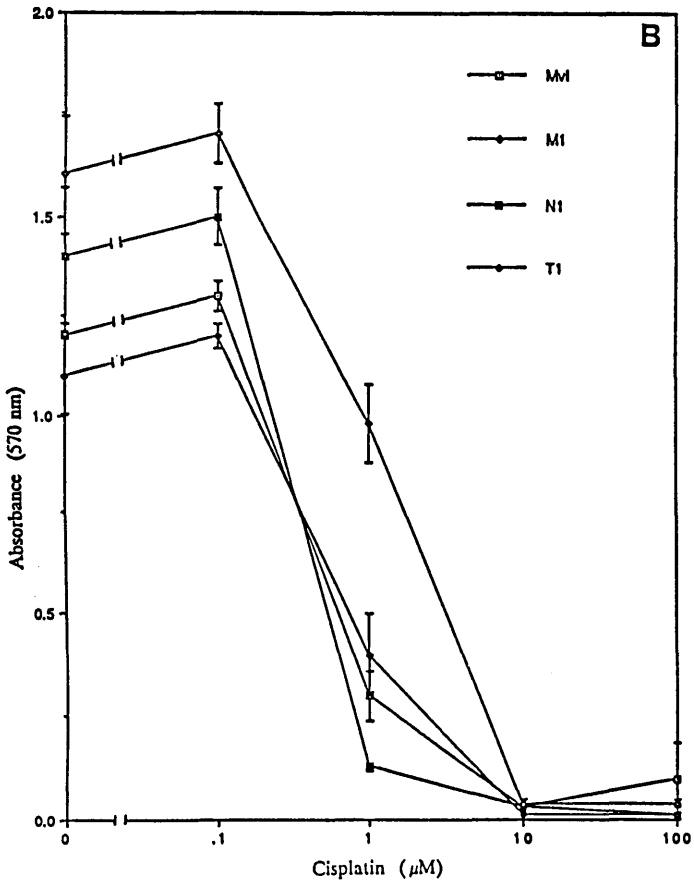
[a]The ID50 value is defined as the concentration of drug which gives a 50% reduction in absorbance compared to that in drug free control. Values represent arithmetic mean ± SEM from at least 3 independent observations.

*$P<0.02$ (Mann-Whitney test)

**Figure 5.5:- Cytotoxicity curves of cell lines exposed to cytosine arabinoside and cisplatin for 48 hour.**

Curves show relative absorbance of Mv1, M1, N1 and T1 cell lines, following a 48 hours exposure to increasing concentrations of cytosine arabinoside (A) and cisplatin (B), by MTT assay. Cells were allowed to recover in drug free medium for 3 days. Each point is the mean ± SEM (bars) of 3 replicate values, from one experiment, similar curves were obtained from three other experiments (see Table 5.9).


A
MvI
M1
N1
T1
Absorbance (570 nm)
2.0
1.5
1.0
0.5
0.0
0
.1
1
10
100
Cytosine arabinoside (μM)


B
MvI
M1
N1
T1
Absorbance (570 nm)
2.0
1.5
1.0
0.5
0.0
0
.1
1
10
100
Cisplatin (μM)

were invasive with minor differences in the degree and the pattern of the process. Invasion was progressive in time from grade I through to IV [Table 5.11] with complete replacement of PHF after 7-10 days.

The unexpected invasive behaviour of the Mv1 cell line (parental) was an interesting finding in this study. Though slightly slower in progression, these cells were equally invasive within the first week of confrontation. Following 24 hours of incubation [Plate 5.16] cell aggregates were found lying at the edge of the PHF or forming a layer of cells on the rounded edge of heart fragment, this was scored as grade-I invasion. The cells showed grade-II invasion on day 2 and on day 4 grade-IIb when clumps or strips of cells were observed invading into PHF. After day 7 [Plate 5.17] they reached deep into heart fragment scoring grade-IV invasion. Most of the heart fragment was destroyed by day 10.

Under similar conditions, M1 cells attached rapidly scoring grade-II on day 1 [Plate 5.16], and showed a grade-III invasion on day 2. They exhibited an infiltrated pattern of growth and invasion through the outer fibroblastic layer. Histologically, whorls or clumps or strands of cells were seen advancing and invading PHF as continuous sheet of cells, maintaining the characteristic tissue culture pattern of growth and morphology. This was called massive or bulk invasion. As shown in plate 5.17, by day 7 the whole fragment was replaced almost by test cells.

N1 and T1 invariably showed very rapid invasion, destruction and replacement of the cardiac cells. As shown in plate 5.16, within 24 hours of confrontation cells penetrated the outer fibroblastic layer (grade II). Thereafter replacement of the host tissue by the test cells took place rapidly (grade III by day 2), and grade IV by day 4. By day 7 the tissue fragment consisted mainly of the invading tumour cells [Plate 5.17]. The nuclei of the normal heart cells next to the advancing invading cells were smaller and more compact in appearance, and these disappeared rapidly leaving only a network of interstitial fibres.

Following complete or nearly complete replacement of the heart fragment by these cells central necrosis appeared after 7 to 10 days. Both N1 and T1 were scored as the most aggressive in three successive experiments.

### 5.3.4.2 Cell Proliferation and Invasion

There was no correlation between invasiveness and the mitotic activity of the cells, in chick heart invasion assays. Though all lines were invasive, Mv1 showed lower mitotic activity compared to the transfectants (data not shown).

**Table 5.11:- Grading of the cell lines for in vitro invasiveness.**

| | Grading After | | | | |
|---|---|---|---|---|---|
| Cell line | 24 hrs | 2 days | 4 days | 7 days | 10 days |
| Mv1 | I | II | IIb | IV | V |
| M1 | II | III | IV | V | V |
| N1 | II | III | IV | V | V |
| T1 | II | III | IV | V | V |

Confronted samples were processed at day 1, 2, 4, 7 & 10 after co-culture of test cells with chick heart fragments, for evaluation of invasion as described in General Methods.

**Plate 5.16:- Invasion of chick heart fragments in vitro at day 1 after co-culture.**

Histological sections from confronting cultures of mink lung cell lines with pre-cultured heart fragments (PHF) set up as described in General Methods and grown for 10 days on a gyratory shaker. Samples were collected at day 1 and day 7 ( see below, Figure 5.17) fixed sectioned and stained with H&E and by immunoperoxidase for anti-chick heart antigen, with the avidin biotin technique. Mv1 cells are on the surface of PHF but attached firmly (grade I), M1 has partially replaced the heart fragment (grade II), Both N1 and T1 have shown similar effects to M1.

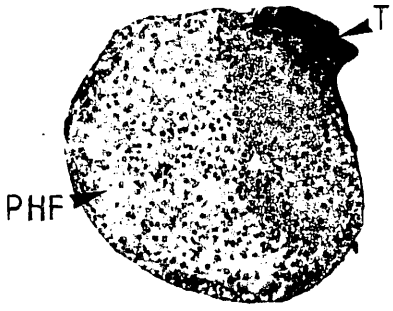
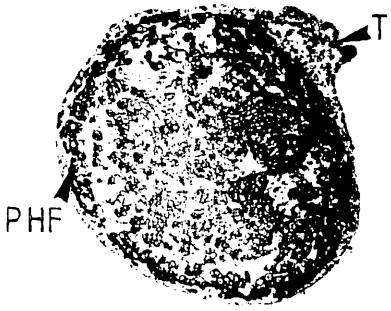
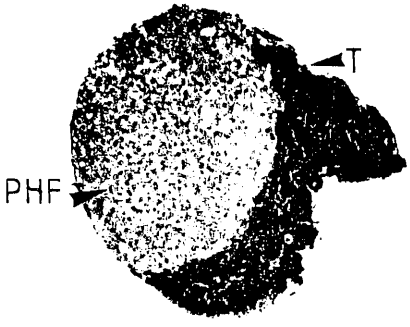
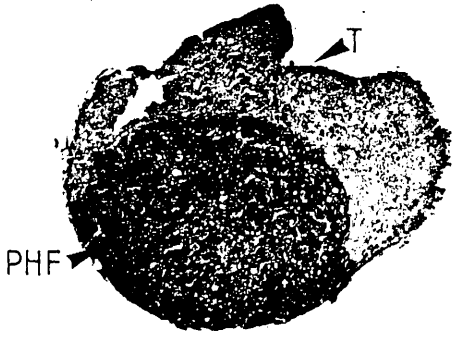
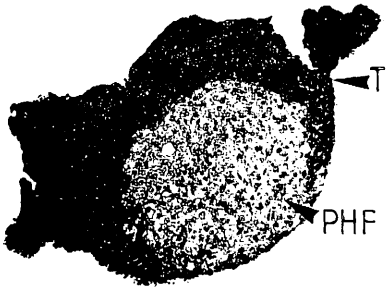
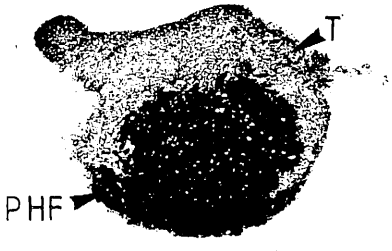
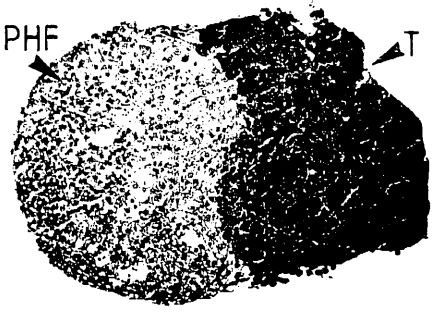
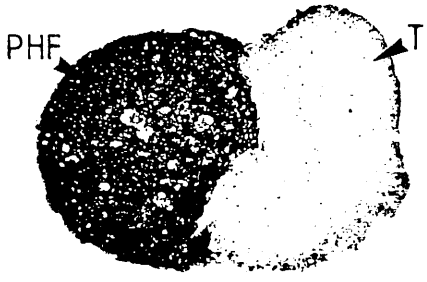
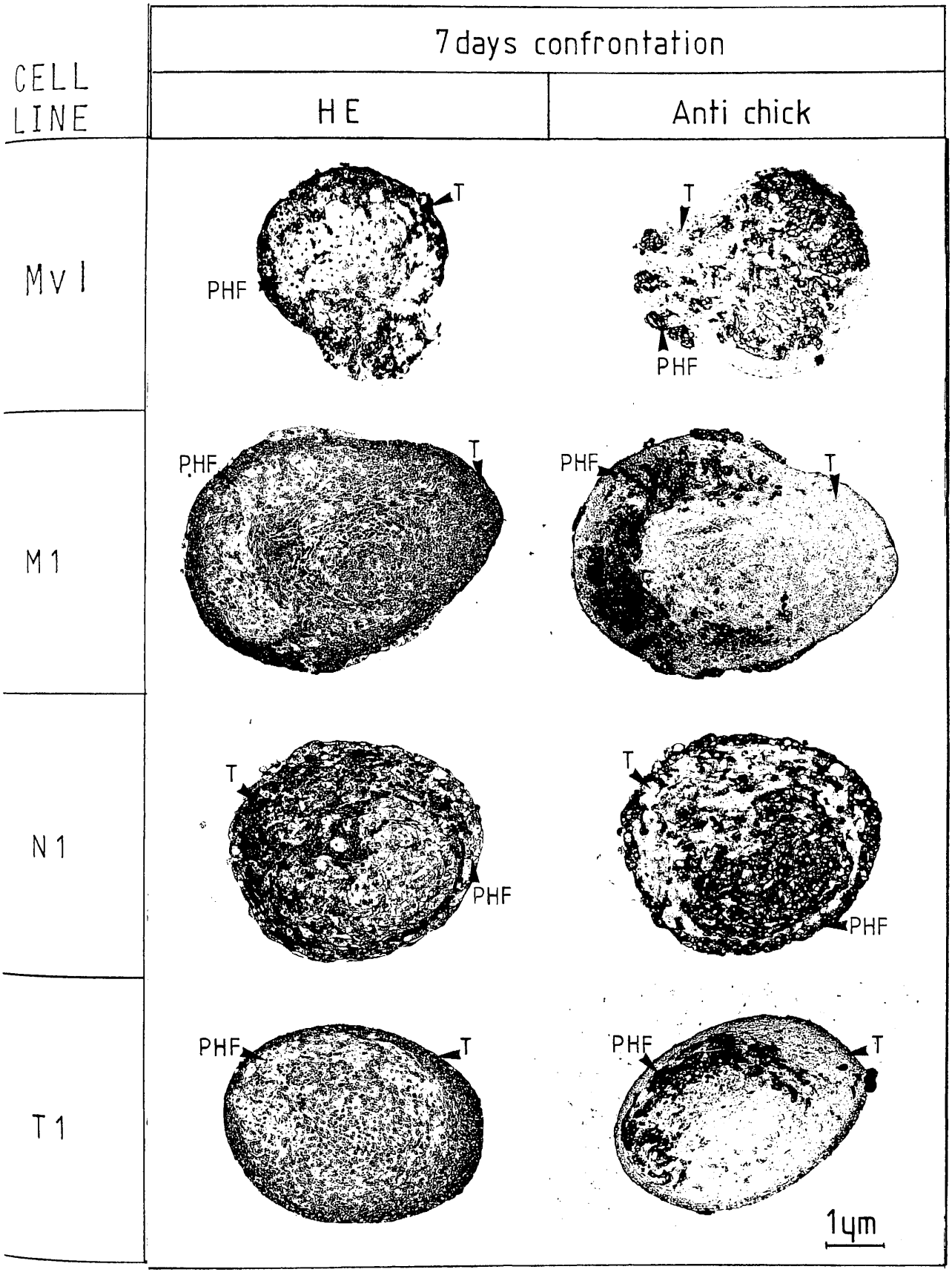
| CELL LINE | 1 day confrontation | |
|---|---|---|
| | H E | Anti chick |
| Mv l |   |   |
| M 1 |   |   |
| N 1 |   |   |
| T 1 |   |   |

**Plate 5.17:- Invasion of chick heart fragment in vitro at day 7 after co-culture.**

All four cell lines show significant invasion. Explanation as for Figure 5.16.


7 days confrontation
CELL LINE
HE
Anti chick
Mv1
M1
N1
T1
T
PHF
1µm

#### 5.3.4.3 Plasminogen Activator Activity

A low PA activity was observed in all lines [Table 5.12]. There was no significant increase in transfected lines compared to the parental line. A significantly low activity was found in N1 line, while T1 expressed only slightly increased activity. All lines had undetectable levels of secreted PA. A reasonable correlation was found for values obtained by both cell count and protein estimation.

### 5.3.5 CHARACTERISATION *IN VIVO*

As discussed in previous sections, cultured cells may acquire a number of transformed characters following oncogene transfection. One of these characters is tumorigenicity, that is the ability of the cells to grow as tumours when implanted into appropriate animals. The development of tumours implies that cells *in vitro* can undergo transformation similar to that occurring in course of carcinogenesis *in vivo*. Tumorigenicity is a manifestation of a series of changes which progress in one direction towards more malignant phenotype.

This section describes *in vivo* studies of the cell lines, and their comparison with *in vitro* growth properties as described in previous section. *In vivo* studies included: tumorigenicity of cell lines in nude mice, observation and evaluation of the tumour growth patterns, gross morphological properties during tumour development, tumour histology, immunohistochemistry, and ultrastructure features.

In present studies, the pattern of cell growth *in vitro* was nicely correlated with their growth as tumour in athymic nude mice, demonstrating that growth *in vitro* can be used as predictive test for *in vivo* growth of cell as tumours.

#### 5.3.5.1 Tumorigenicity

The **Mv1** cell line without oncogene transfection produced only 20% tumours. Negative cases were observed for over 16 weeks after injection, and showed no abnormality at autopsy. Tumours grew with a typical growth pattern [Figure 5.6 a]. An initial steep curve representing a rapid growth was followed by a steady phase showing a pause in growth, and then a slow but gradually rising growth rate. Doubling time was relatively shorter during the first phase compared to the second half of growth. Only one tumour could be assessed by histology and passage, because of either the death of the animal or necrosis of the tumour in the other two cases. The average latency period was around two

**Table 5.12:- Plasminogen activator (PA) activity.**

| | PA [a]PU/1x10$^6$ cells | | PA PU/mg protein | | *P-Value |
|---|---|---|---|---|---|
| Cell line | Cellular | Secreted | Cellular | Secreted | |
| MvlLu | 0.283 ± 0.150[b] | 0 | 0.387 ± 0.287 | 0 | |
| M1 | 0.345 ± 0.215 | 0 | 0.404 ± 0.248 | 0 | $p>.05$ |
| N1 | 0.059 ± 0.047 | 0 | 0.045 ± 0.025 | 0 | $p<.05$ |
| T1 | 0.335 ± 0.188 | 0 | 0.410 ± 0.286 | 0 | $p>.05$ |

[a]Plough units. [b]Values are mean ± SEM of 3 separate experiments. Activity was significantly low in N1.

*Statistical analysis was carried out by analysis of variance.

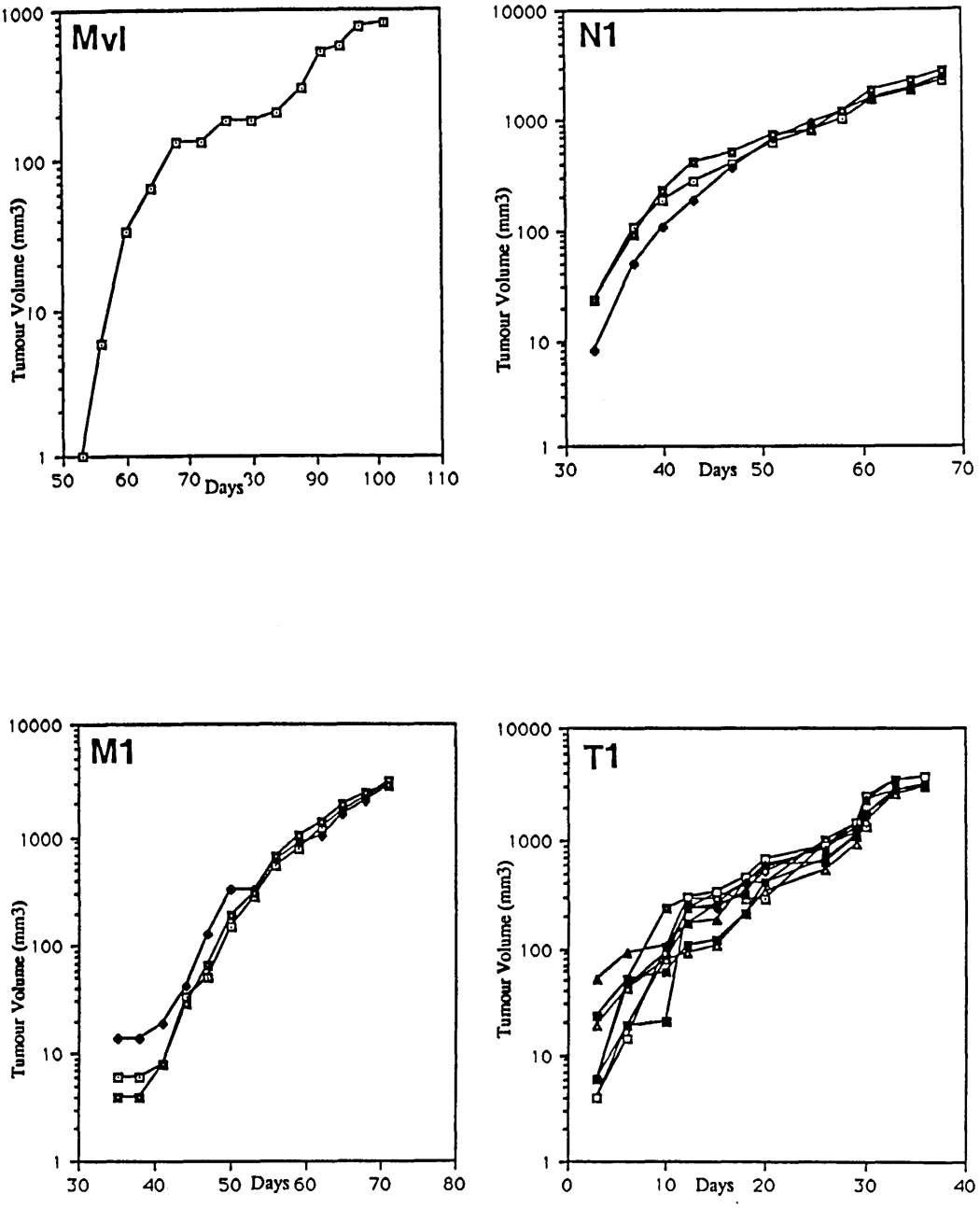



**Figure 5.6:- Growth curves of primary xenografts of cell lines in nude mice.**

$1x10^6$ cells of each cell line (Mvl, M1, N1, & T1) were inoculated subcutaneously into the flank of each mouse at day zero. Tumour measurements were carried out twice weekly, starting, when tumours reached a volume around 14 to 19 $mm^3$. Volume was calculated from two diameters by calliper measurements as outlined in General Methods.

months, and the tumour which could be assessed reached a maximum volume of less than 1 $cm^3$ after 6 weeks [Table 5.13].

However, the single tumour when passaged grew with 100% take and a short latency period around three weeks. The growth pattern though not identical [Figure 5.7 a] was of the same type as described above but more steep with shorter doubling times. Transplants reached a significantly larger size within approximately 5 weeks. The growth was significantly enhanced in passaged tumours [Table 5.14]

The **M1** cells transfected with the c-myc human proto-oncogene yielded rather slow growing tumours, though faster than Mvl, after an average latency period of six weeks and with 100% take rate. The pattern of tumour growth remained the same as in Mv1 [Figure 5.6 b], with a shorter doubling time during the first half than during the second half of tumour growth [Table 5.13]. These tumours were relatively larger in size than parental, but smaller than the tumours produced by ras transfected cell lines.

All passaged tumours grew with 100% take rate, a shorter latency period, with doubling times and final tumour volumes similar to primary tumours [Table 5.14]. The growth curves showed the same growth pattern as primary tumours [Figure 5.7 b]. The passaged tumours showed significantly more aggressive behaviour compared either to parental tumour or M1 tumours during primary growth, but their growth was less aggressive than both of N1 and T1.

The **N1** cell line expressing the normal human Ha-ras gene produced rapidly growing tumours in all animals with 100% take after an average latency period of around 5 weeks, tumours reached a larger size within a relatively shorter period, compared to the parental tumours (4-fold). The tumours showed a gradually increasing growth pattern [Figure 5.6 c]. Doubling time was shorter in the first than in the second half of the growth [Table 5.13].

After the first passage [Table 5.14] the tumours grew significantly more rapidly compared to parental or M1 tumours, with a short latency period of around 1 week and 100% take rate. Doubling times were reduced significantly. Growth was faster in passaged than in primary tumours [Figure 5.7 c], and they reached a mean diameter significantly greater than the parental tumours.

The **T1** cells bearing the T24 Ha-ras human oncogene yielded rapidly growing tumours in all animals with 100% take rate and very short latent period. They grew with a sharp progression reaching a greater volume in relatively shorter time. Tumour doubling times were significantly shorter during primary growth [Table 5.13]. The growth was faster and progressive [Figure 5.6 d].

**Table 5.13:- Growth characteristics of primary tumours.**

| Cell line | [a]Tumorigenicity | Take rate (%) | [b]Latency period (days) | cDuration of observation (days) | [d]Tumour bearing time (days) | [e]Doubling time in days [50-100] ($mm^3$) | [e]Doubling time in days [400-800] ($mm^3$) | Maximum volume (mm3) x10-$^3$ |
|---|---|---|---|---|---|---|---|---|
| Mv1 | 3/14 | 21.4 | 60±3 (n=3) | 101 (n=1) | 41 (n=1) | 4 (n=1) | 9 (n=1) | 0.8 (n=1) |
| M1 | 15/15 | 100.0 | 40±5 (n=9) | 74±5 (n=9) | 34±4 (n=9) | 3±1 (n=9 | 6±1 (n=9) | 2±0 (n=6) |
| N1 | 15/15 | 100.0 | 32±1 (n=9) | 68±1 (n=9) | 36±1 (n=9) | 2±0 (n=9) | 7±1 (n=9) | 5±1 (n=6) |
| T1 | 16/16 | 100.0 | 7±1 (n=9) | 33±2 (n=9) | 26±1 (n=9) | 2±0 (n=9) | 4±0 (n=9) | 3±0 (n=9) |

[a]Number of mice with tumour/total number of mice inoculated. [b]Latent period was counted from the day of implantation to the day when tumour reached a volume of>33 $mm^3$. [c]Time between injection of cells and death of animal. [d]Time of the observation minus latency period. [e]Doubling time was determined for early (50-100 $mm^3$) and late stage (400-800 $mm^3$) growth.

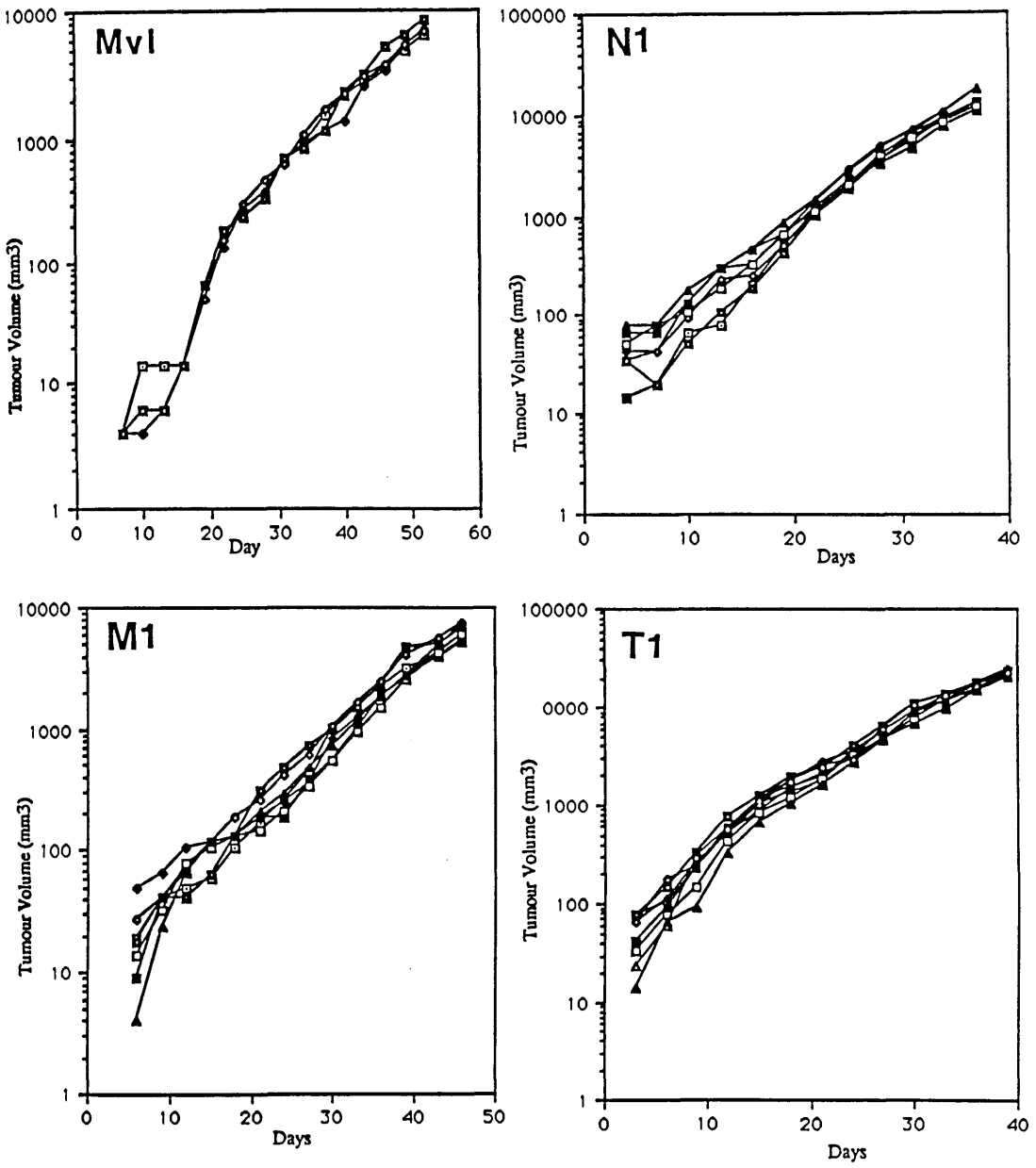



**Figure 5.7:- Growth curves of transplanted tumours after first passage.**

A tumour fragment approximately 2x2x1 mm was transplanted subcutaneously into the flank of each mouse at day zero. Size was measured twice per week. Volume was calculated from two diameters by calliper measurements as out lined in General Methods.

**Table 5.14:- Growth characteristics of passaged tumours.**

| Cell line | [a]Tumorigenicity | Take rate (%) | [b]Latency period (days) | [c]Duration of observation (days) | [d]Tumour bearing time (days) | [e]Doubling time in days [100 - 800] ($mm^3$) | [e]Doubling time in days [1000 - 4000] ($mm^3$) | Maximum volume ($mm^3$) $x10^{-3}$ |
|---|---|---|---|---|---|---|---|---|
| Mv1 | 6/6 | 100 | 19.5±0.5 (n=6) | 52 (4/6) | 33 (4/6) | 4.1 ± 0.3 (n=4) | 6.7 ± 0.3 (n=4) | 7.0 ± 0 (4/6) |
| M1 | 10/10 | 100 | 11.4 ± 0.8 (n=10) | 46 (7/10) | 35 (7/10) | 4.5 ± 0.3 (n=7) | 4.8 ± 0.1 (n=7) | 8.0 ± 1 (7/10) |
| N1 | 10/10 | 100 | 8.5 ± 0.8 (n=10) | 36 (8/10) | 27.5 (8/10) | 3.3 ± 0.2 (n=8) | 3.2 ± 0.3 (n=8) | 12.0 ± 1 (8/10) |
| T1 | 12/12 | 100 | 5.0 ± 0.6 (n=12) | 39 (9/12) | 34 (9/12) | 2.4 ± 0.1 (n=10) | 4.2 ± 0.2 (n=10) | 22.0 ± 4 (9/12) |

[a]Number of mice with tumour/total number of mice inoculated. [b]Latent period was counted from the day of transplantation to the day when tumour reached a volume of>65$mm^3$. [c]Time between transplantation of tumour and death of animal. [d]Time of observation minus latency period. [e]Doubling time was determined for early (100-800 $mm^3$) and late stage (1000-4000 $mm^3$) growth.

The latent period remained the same during first passage [Table 5.14] with a take rate of 100%, and shorter doubling, tumours gained significantly greater volume compared to the parental tumour, with over 20-fold difference. The growth pattern was sharper in the first than in second half of the tumour growth [Figure 5.7 d]. Growth of the T1 tumours was most aggressive in both primary and transplant tumours. The tumour showed irregular growth with macroscopic evidence of invasion [see Plate 5.23].

Statistical analysis of data during primary tumour growth showed that the latency period of T1 was significantly shorter than Mv1 ($P<0.0001$), M1 ($P<0.008$), N1 ($P<0.009$). Doubling time during first half of growth in primary tumours of Mv1 was significantly longer than M1 ($P<0.008$), N1 and T1 ($P<0.005$). Doubling time of M1 was significantly longer than either N1 or T1 ($P<0.006$). Doubling time during second half of the growth in Mv1 was also significantly more longer than M1 ($P<0.008$), T1 ($P<0.001$), and the tumour doubling time of T1 was significantly shorter than M1 ($P<0.008$) and N1 ($P<0.006$). Maximum tumour volume of Mv1 was significantly smaller than either N1 or T1 tumours ($P<0.04$).

Statistical analysis of passaged tumours showed that the latent period of Mv1 was significantly longer than T1 ($P<0.0001$), N1 ($P<0.001$) and M1 ($P<0.001$). The latent period of T1 was also significantly shorter than M1 ($P<0.0001$) and N1 ($P<0.003$). The doubling time of Mv1 during the first half of growth was significantly longer than N1 and T1 ($P<0.008$). T1 was shorter than M1 ($P<0.0001$). Doubling time of Mv1 was longer than M1 ($P<0.008$), N1 (0.004) and T1 ($P<0.005$) during second half of growth. Maximum tumour volume of Mv1 was less than N1 ($P<0.008$), and T1 ($P<0.0001$), and M1 was significantly less than N1 ($P<0.008$) and T1 (0.0001). [Mann-Whitney test with Bonferoni adjustment].

### 5.3.5.2 Morphological Features

Grossly the **Mv1** tumour showed circumscribed margins with a smooth surface [Plate 5.18 a]. The skin was attenuated over the tumour with sparse blood vessels passing from the skin into the tumour mass. It showed little attachment to underlying structures, and was enucleated easily during dissection. There was no evidence of necrosis, ulceration or inflammation. The tumour was firm in consistency. On section the tumour was firm and showed little attachment to the underlying structures. Skin attachment was relatively firm with small blood vessels and connective tissue attached to tumour. There was minimal evidence

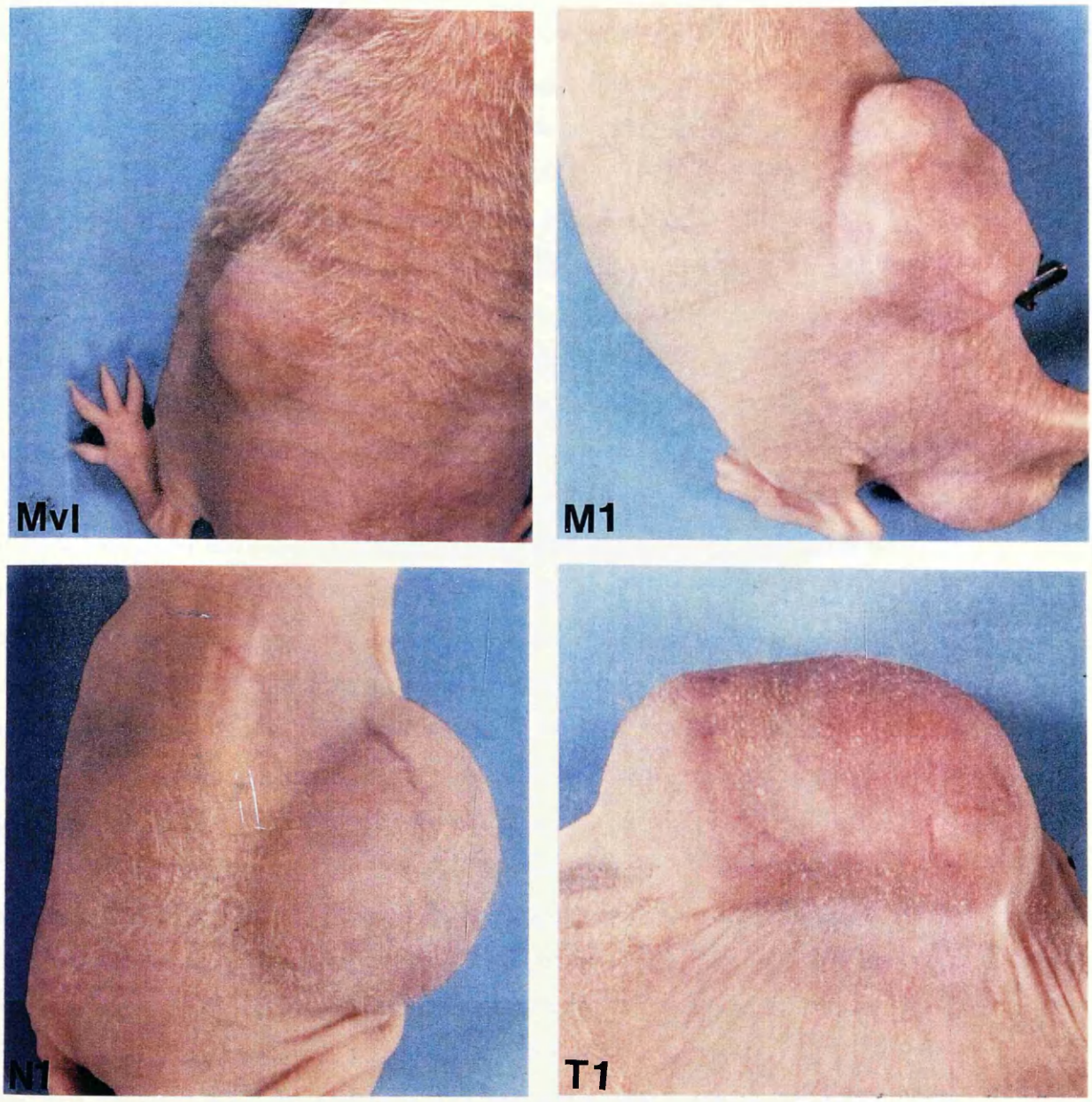



**Plate 5.18:- Nude mice bearing primary tumours of different cell lines.**

$1x10^6$ cells were inoculated s.c in each mouse. Tumour sizes 30 days after the first appearance of tumour nodule at the site of cell inoculation.

of central necrosis. Histologically, the tumour was undifferentiated, with mild to moderate nuclear pleomorphism and a low mitotic rate [Plate 5.19 a]. Central necrosis was present. There was no evidence of local invasion in the single tumour during primary growth, and no lymph node or distant metastases were identified. Transplants showed similar features with undifferentiated cells forming sheets or plates in between necrotic material [see Plate 5.20].

Grossly, the **M1** tumours grew gradually with well circumscribed margins [plate 5.18 b] and firm attachment. Again the skin over the tumour was attenuated. Tumours were attached firmly to the underlying tissues, and could not be easily enucleated by dissection. Transplants grew faster than primary tumours, with irregular margins, frequent ulceration, and an increase in vasculature in the overlying skin. The cut surface of the tumour showed no evidence of gross cavitation. Tumours were also attached firmly to both the skin and underlying tissues, the margins were more irregular, and it was difficult to remove the tumour from its bed completely. Histologically all tumours were undifferentiated, with moderate nuclear pleomorphism [Plate 5.19 b]. Mitoses were moderately frequent. The transplanted tumours showed similar features, but with increased pleomorphism, and central necrosis with sheets of viable tumour cells in between the necrotic material.

Grossly the **N1** tumours grew as irregular masses at the site of injection with a nodular surface, ill defined margins and prominent blood vessels [Plate 5.18 c]. The skin over the tumour was firmly attached, giving a nodular appearance. Tumour was also firmly attached to underlying tissues. Early necrosis and ulceration was not observed in primary tumours. After the first passage tumours grew much faster to a relatively bigger size in a shorter time, with evidence of early necrosis of the central tumour tissue. The cut surface was firm with some evidence of central necrosis. Histology of N1 [Plate 5.19 c] revealed a pleomorphic, undifferentiated tumour with giant cells, many mitotic figures, apoptotic bodies and prominent vascular elements. Transplants also showed similar picture but with more necrosis.

Grossly growth of the **T1** tumour was very irregular in outline with ill-defined margins and nodular surface appearance [Plate 5.18 d]. Overlying skin had prominent blood vessels passing across or into the tumour. There was macroscopic evidence of infiltration into both superficial and deep structures. Ulceration was often present [see Plate 5.24]. Transplanted tumours showed similar features, but they grew to a larger size in shorter time, with more ulceration. The cut surface of the tumour showed areas of necrosis. There was

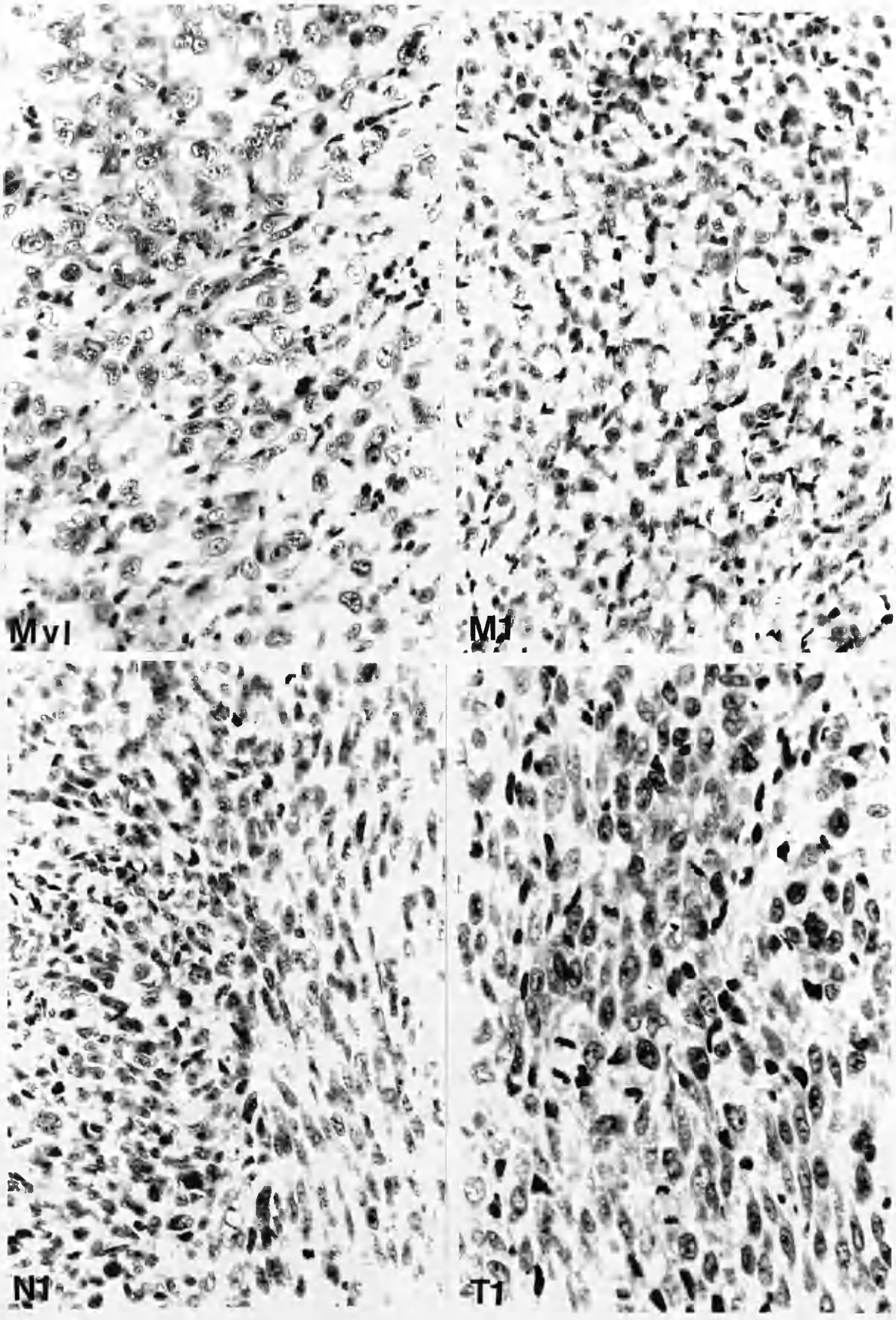



**Plate 5.19:- Histology of xenografts.**

All tumours were poorly differentiated, consisting of sheets of large nucleolated cells. Pleomorphism was more marked and mitoses more abundant in N1 and T1 (X 360, H&E).

firm attachment to skin and underlying tissues. Histologically, the tumour was again undifferentiated and showed significant pleomorphism [Plate 5.19 d]. Necrotic areas were frequent, lying between sheets of tumour cells [Plate 5.20]. Tumours had a high mitotic rate, and frequent abnormal mitoses. Transplanted tumours showed similar features, necrosis and pleomorphism being most marked.

### 5.3.5.3 Quantitative Study of Cell Turnover

Mitotic and apoptotic figures [Plate 5.21] were counted in each tumour. Numerical data are shown in table 5.15. There was no significant difference between primary and passaged tumours in all transfected tumours, and therefore, combined data are shown, except Mvl, which showed significant differences between primary and passaged tumours.

During primary growth, in **Mv1** a low level of mitotic activity was observed, and apoptoses were not frequent. However, in first passage (Mvl-P) the mitotic index was significantly higher, but the apoptotic index was of the same order as primary tumour.

**M1** tumours also had a moderately increased mitotic and apoptotic rate, in both primary and passaged tumours. The mitotic index was significantly higher compared to the parental line tumours, but there was no significant difference in their apoptotic indices.

**N1** had significantly higher mitotic, and apoptotic indices compared to the parental line tumours. The mitotic and the apoptotic indices were also significantly higher than M1 tumours.

**T1** had the highest indices for both the mitotic and apoptotic figures. It had significantly increased number of mitoses and apoptoses compared to the parental line tumours. The mitotic index was significantly higher than M1, but not the N1. Apoptotic index was higher than all other tumours.

### 5.3.5.4 Antigenic Marker Expression

All tumours were immunopositive with cytokeratins [Plate 5.22 a,b ]. They also stained focally for EMA and HMFG1&2, and all tumours were positive for vimentin [Plate 5.22 c]. The intensity of the staining was not as strong as in monolayer cultures, but the cellular patterns or the staining were the same both *in vitro* and *in vivo* in all cell lines. There was no significant difference among the lines in terms of their staining characteristics.

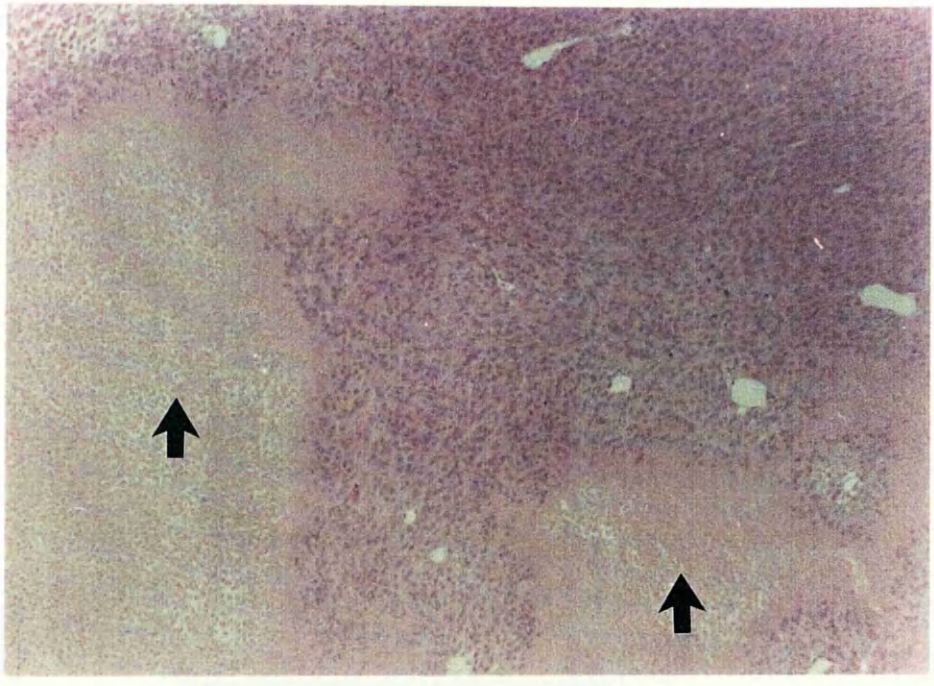


**Plate 5.20:- Tumour necrosis**

T1 tumour showing significant areas of necrosis (arrows) (X 144, H&E).

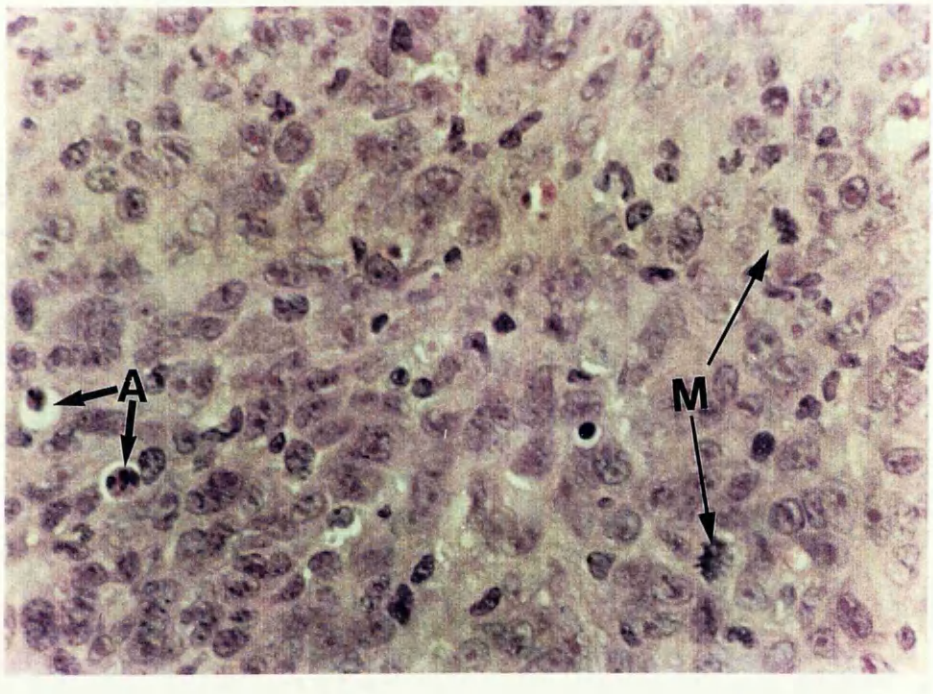



**Plate 5.21:- Typical mitotic and apoptotic figures.**

T1 tumour showing mitotic (M) and apoptotic (A) figures (X 360, H&E).

**Table 5.15:- The mitotic (MI) and apoptotic (AP) indices of tumours, produced by the different cell lines.**

| Cell line | MI | AI |
|---|---|---|
| Mv1 | 1.2 ± 0.2 | 3.0 ± 0.3 |
| Mv1-P | *2.8 ± 0.2 | **3.1 ± 0.2 |
| M1 | *2.5 ± 0.3 | **3.3 ± 0.3 |
| N1 | *4.0 ± 0.3 | *4.1 ± 0.2 |
| T1 | *4.1 ± 0.2 | *7.1 ± 0.3 |

For each tumour the mean of mitotic and apoptotic figures in 12 high power fields (X 40) per slide were determined from 3 slides of the single parental tumour (Mv1), 4 from Mv1 tumours in first passage in vivo (Mv1-P), and 6 slides of M1, N1, and T1 from tumours grown at different times.

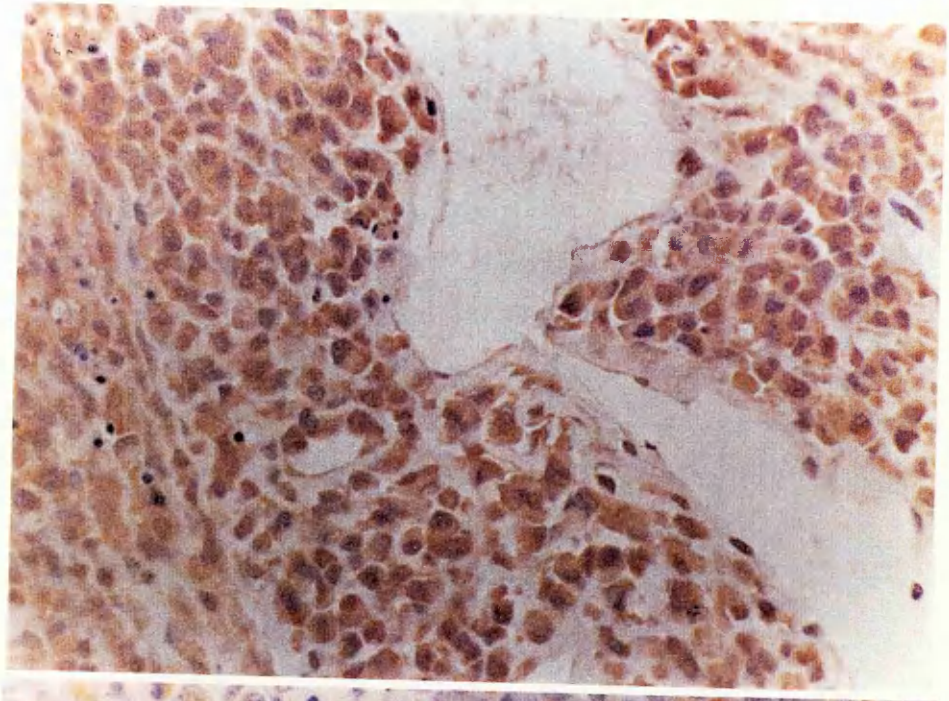
* $P<0.0001$, ** $P>0.05$ (analysis of variance and Bonferoni adjustment).

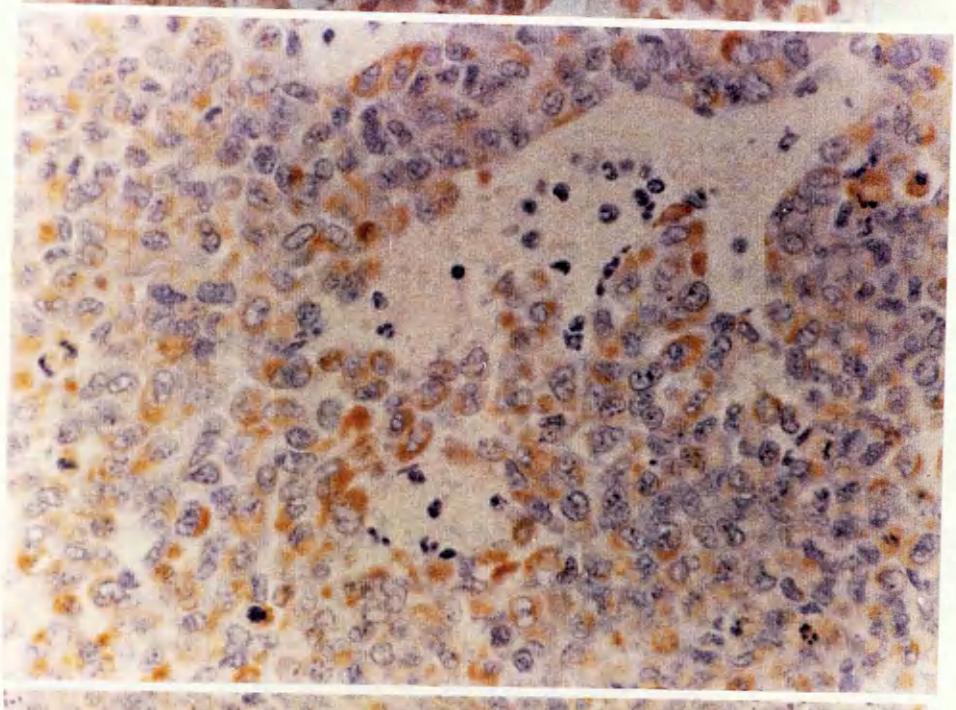
**Plate 5.22:- Expression of immunohistochemical markers in tumours.**

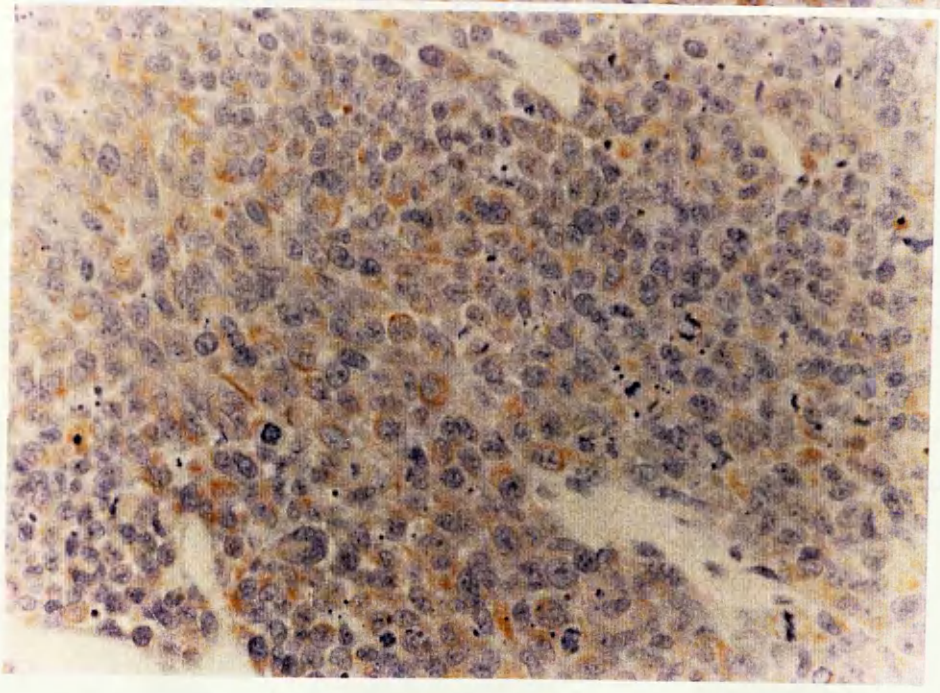
(a). T1 tumour showing immunopositivity for broad spectrum basic cytokeratins (X 360, antibody AE3: indirect IP).

(b). T1 tumours showing more focal positivity for simple glandular cytokeratins (X 360, CAM5.2: indirect IP).

(c). T1 tumour cells also show immunopositive staining for vimentin (X 360, anti-vimentin, indirect IP).







#### 5.3.5.5 Ultrastructural Features of The Tumours

Tumours showed features similar to those found in their parental cell lines in culture, including microvillus profiles [Plate 5.23 a], intermediate filament like structures [Plate 5.32 b], poorly developed adhesion specializations [Plate 5.23 c], and epithelial type cell to cell relationships [Plate 5.23 d].

Only minor ultrastructural differences were observed in the transfected cell lines compared to the non-transfected line. T1 showed more lysosomal activity [Plate 5.23 e], and glycogen contents. However, these features were not consistent, and could be seen occasionally in the parental cells as well. There was a good correlation between *in vitro* and *in vivo* ultrastructural features of the cell lines and their tumours.

### 5.3.6 INVASION AND METASTASIS

*In vivo* studies were followed further to investigate the invasive and metastatic behaviour of the cell lines, as these are the most definitive markers of the advanced malignant phenotype, to determine whether oncogene transfection would result in increased expression of transformed phenotypes such as invasion, and metastasis. Although several assays have been described for the study of invasion and metastasis, none is without shortcomings [Mareel *et al*, 1983]. In the present study, invasion and metastasis *in vivo* were investigated by subcutaneous inoculation of cells in athymic nude mice.

#### 5.3.6.1 Gross Morphological Analysis of Invasion

Tumours produced by all the transfected lines showed macroscopic evidence of invasion. However, the tumours engendered by T1 line exhibited more aggressive behaviour compared with those produced by other lines, especially in terms of growth pattern, being irregular and spreading into the surrounding tissue planes, intimate adherence to skin and underlying tissue, and frequency of skin ulceration [Plate 5.24]. The single tumour formed by the parental line did not show any obvious sign of macroscopic invasion, but only formed a small nodule attached to skin, fairly mobile over the underlying tissues. All lines showed aggressive behaviour during first passage *in vivo*. No enlarged lymph nodes were found in any of the tumour bearing animal.

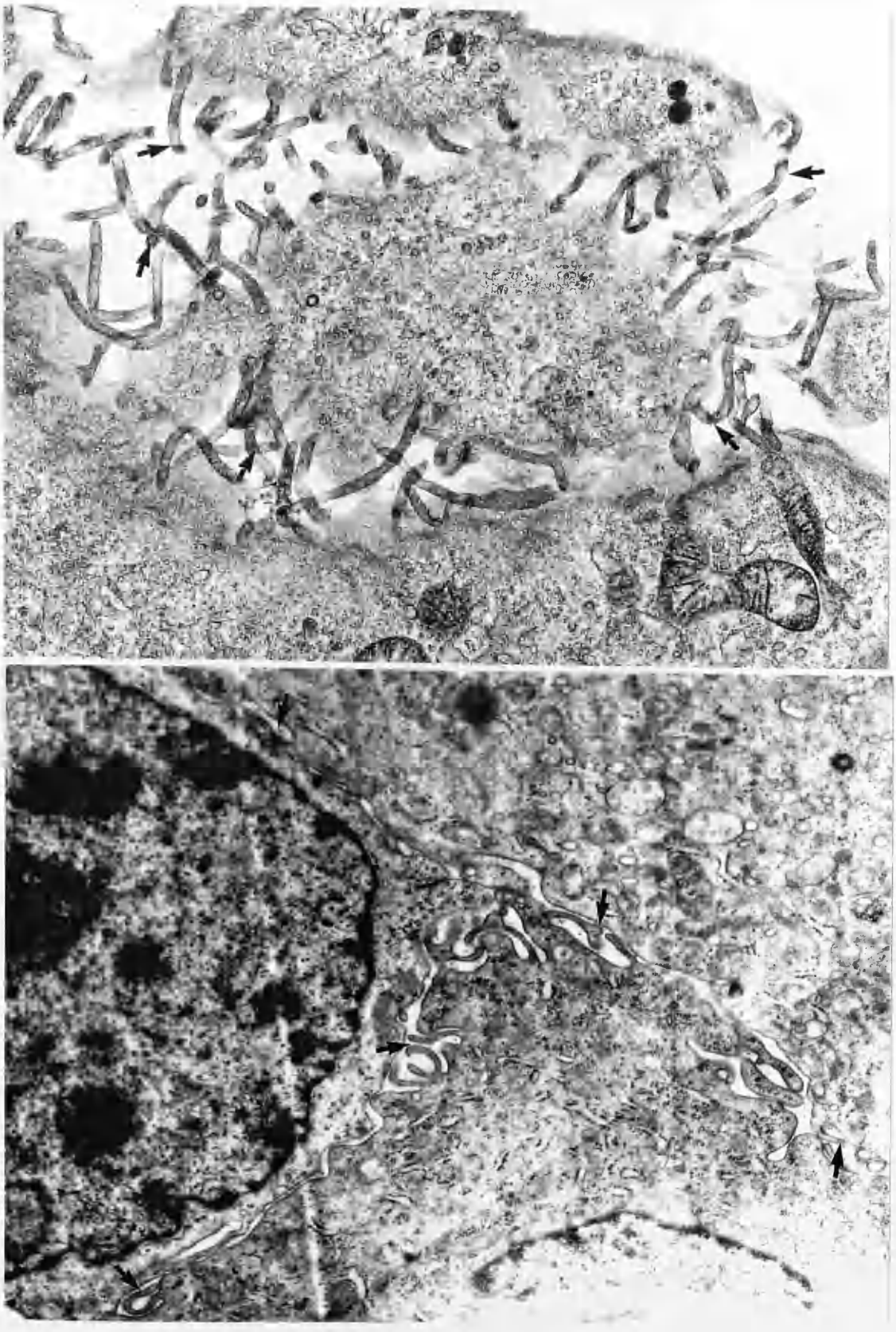
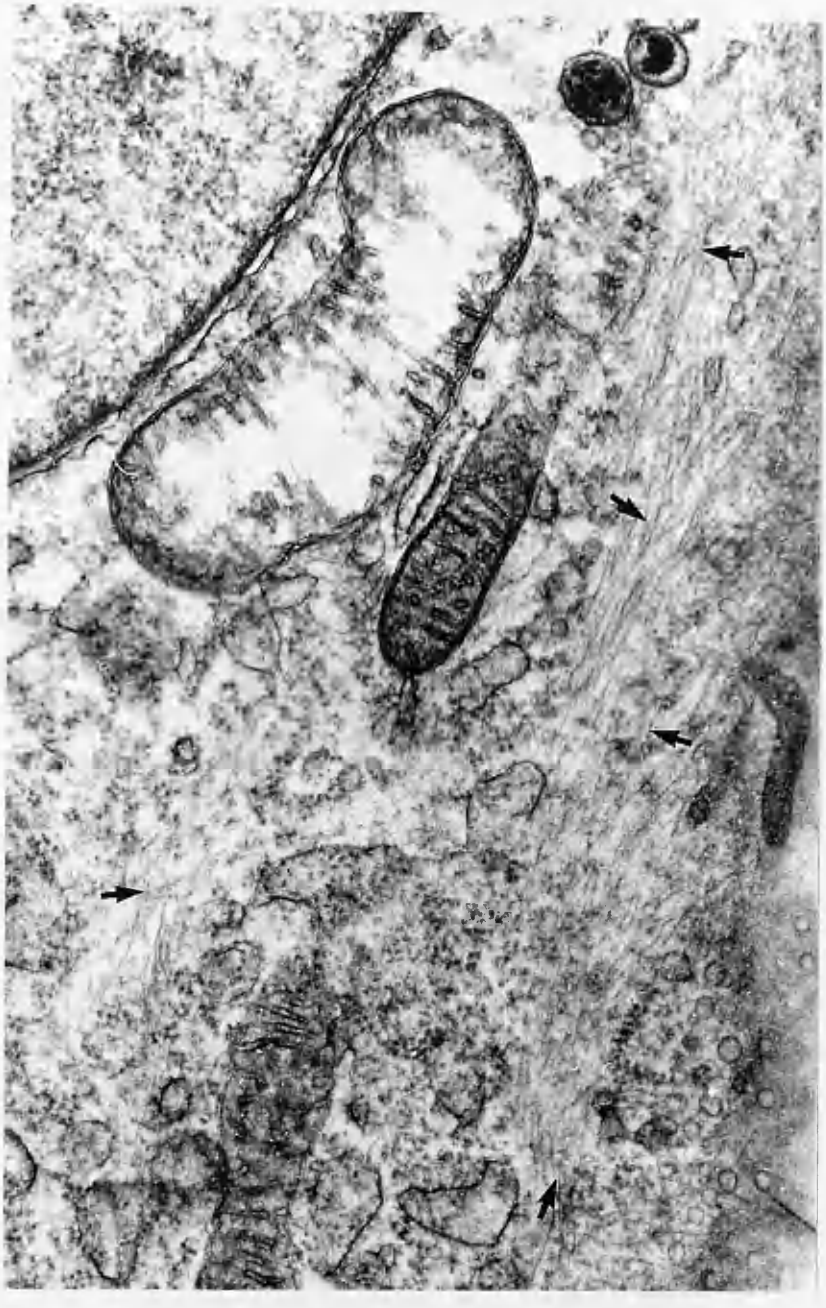
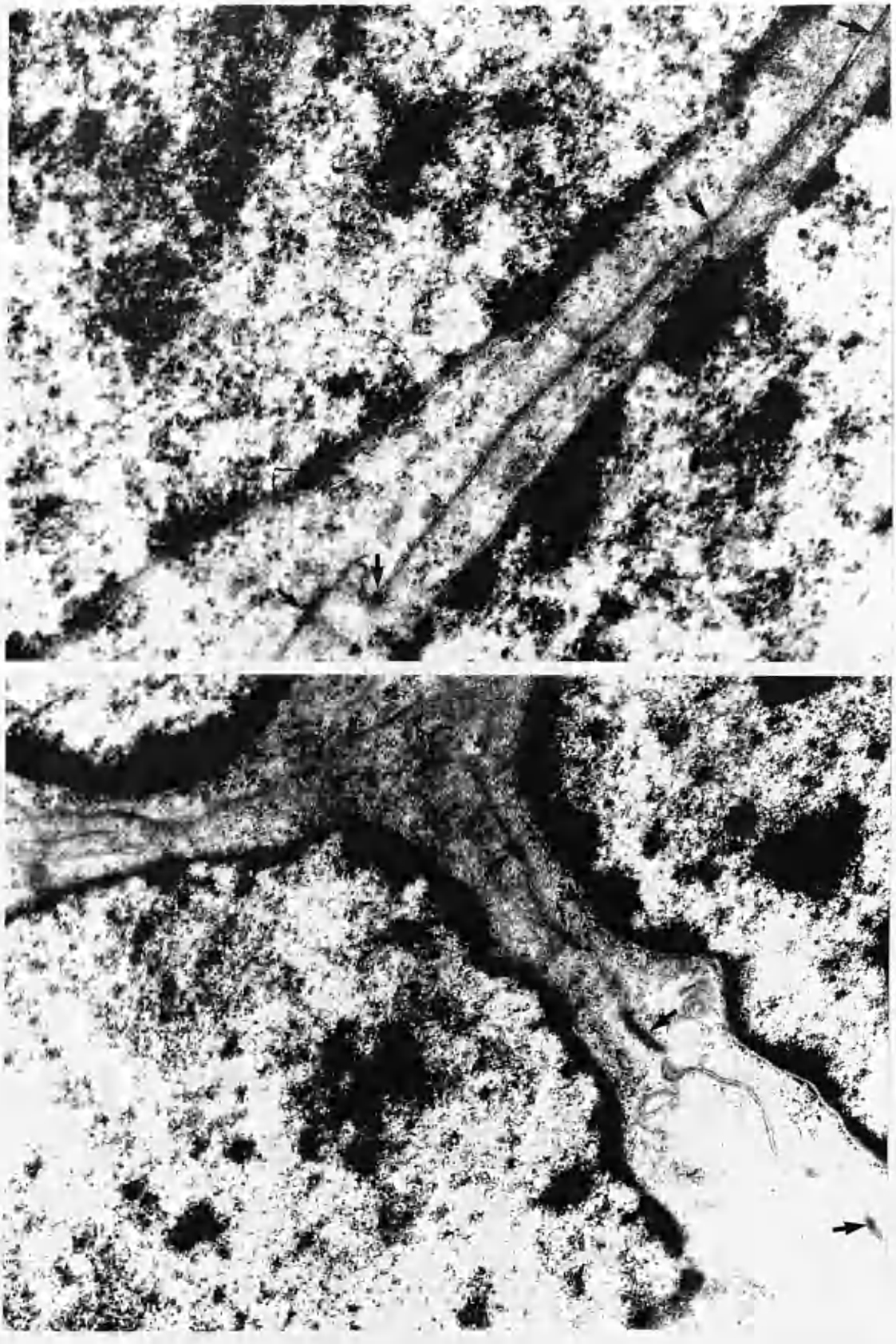


**Plate 5.23:- Ultrastructural features of cell lines in vitro and in vivo.**

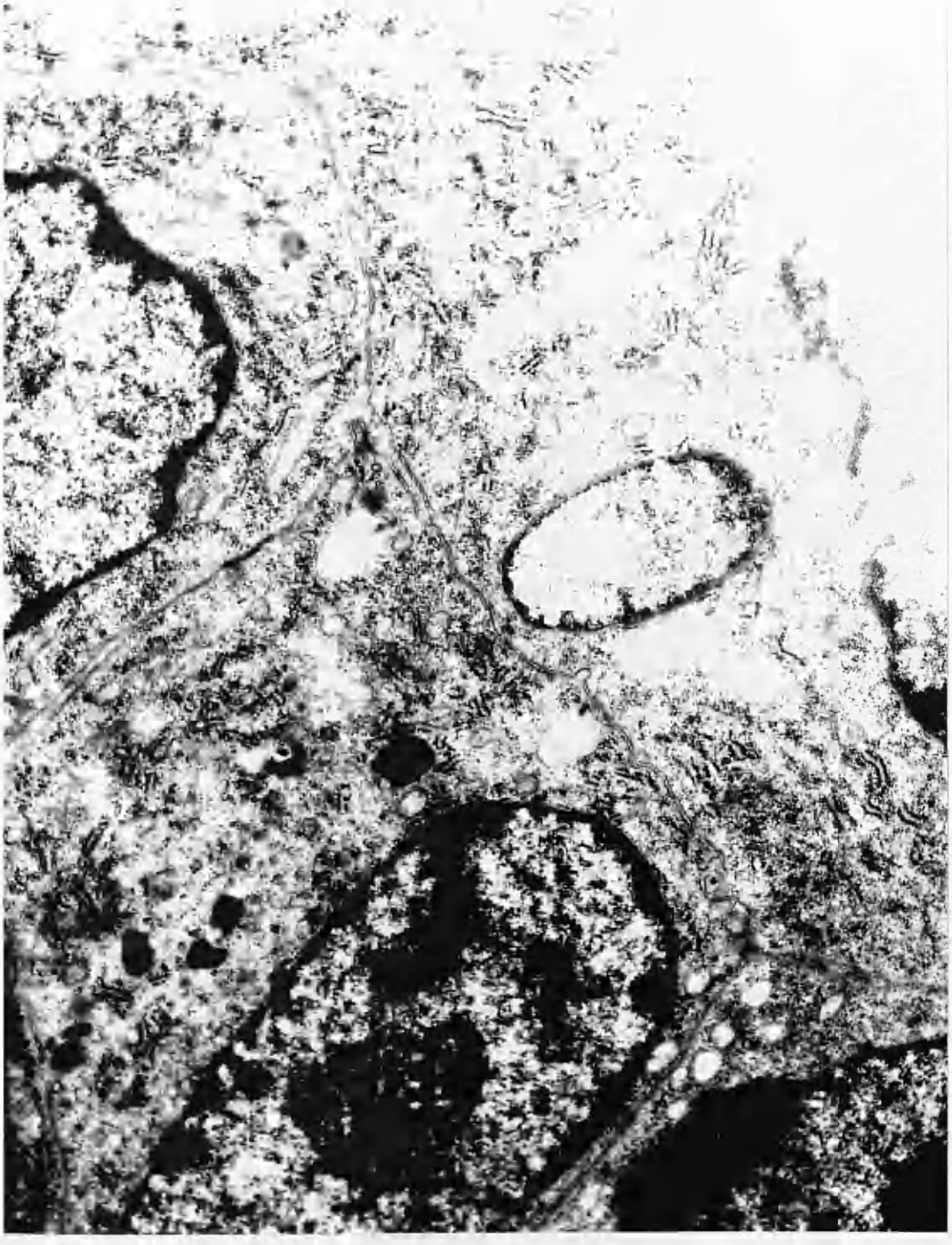
(a). Electronmicrographs showing microvillus structures (arrows) in Mv1 both in vitro (above x 7500) and in vivo (below x 14750) (uranyl actate and lead citrate stain).



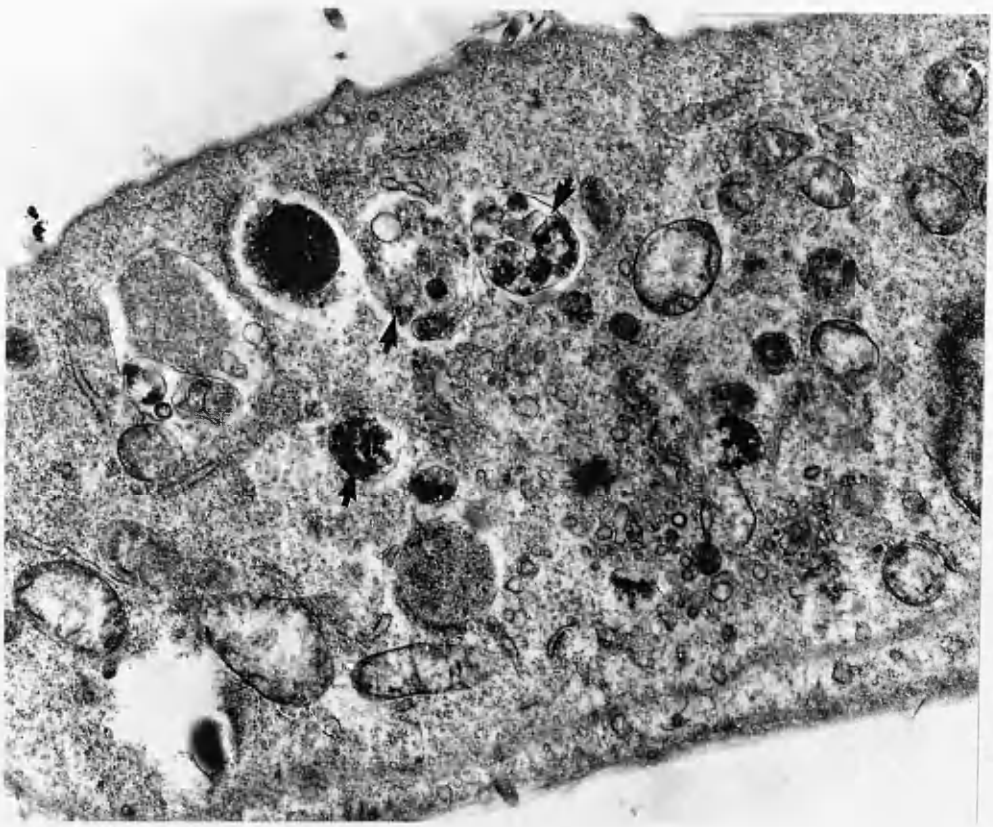
(b). Electronmicrograph demonstrating cytoplasmic intermediate filaments (arrows) in Mv1 (x 18000, uranyl actate and lead citrate stain).



(c). Electronmicrographs of Mv1 (above) and M1 (below) showing moderately developed adhesion specializations, the arrows indicating desmosome like structures [above x 55000, below x 24500, uranyl actate and lead citrate stain].



(d). Electronmicrograph of T1 tumour showing cell-cell relationship of epithelial nature. Five cells are in view showing their membrane attachments (x 18750, uranyl actate and lead citrate stain).



(e). Electron micrograph of T1 showing secondary lysosomal structures (arrows) (x 16875, uranyl acetate and lead citrate stain).

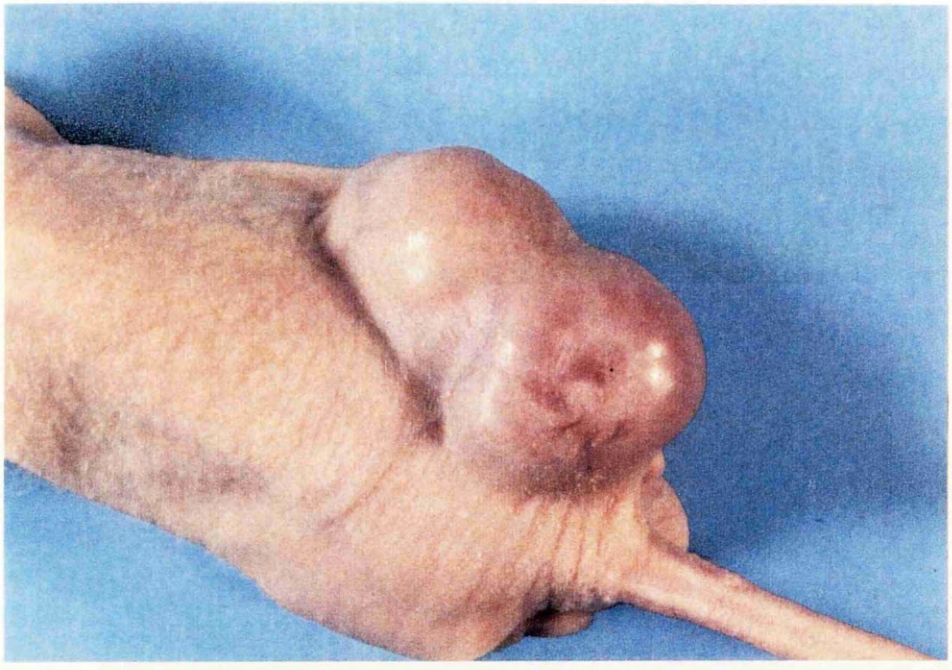


**Plate 5.24:- Gross morphology of the T1 tumour**

Nude mouse bearing T1 tumour, showing irregular outline, nodular surface, and early ulceration.

### 5.3.6.2 Histological Analyses of Invasion

Microscopically, the single tumour nodule formed by the parental line showed minimal infiltration of surrounding structures with well a defined edge during primary growth [Plate 5.25 a], but it was equally as invasive after first passage as the transfected tumours, showing invasion into all surrounding tissues as above. The tumours produced by all transfected cells showed invasion into surrounding host tissues including overlying skin and its appendages [Plate 5.25 b], subcutaneous fat [Plate 5.25 c], and deep into muscles [Plate 5.25 d]. The tumours produced by M1 showed evidence of local invasion into the surrounding structures, but were not as aggressive as ras transfectants. Both N1 and T1 showed extensive infiltration into host tissues, but T1 was always more aggressive.

All transplanted tumours were more invasive than the primary tumours. Tumour cells were seen in blood vessels but without evidence of vessel wall damage or adherence which made any further interpretation difficult.

There was no evidence of metastatic spread to local lymph nodes or distant sites, including lung, liver, and brain, at either macroscopic or microscopic level.

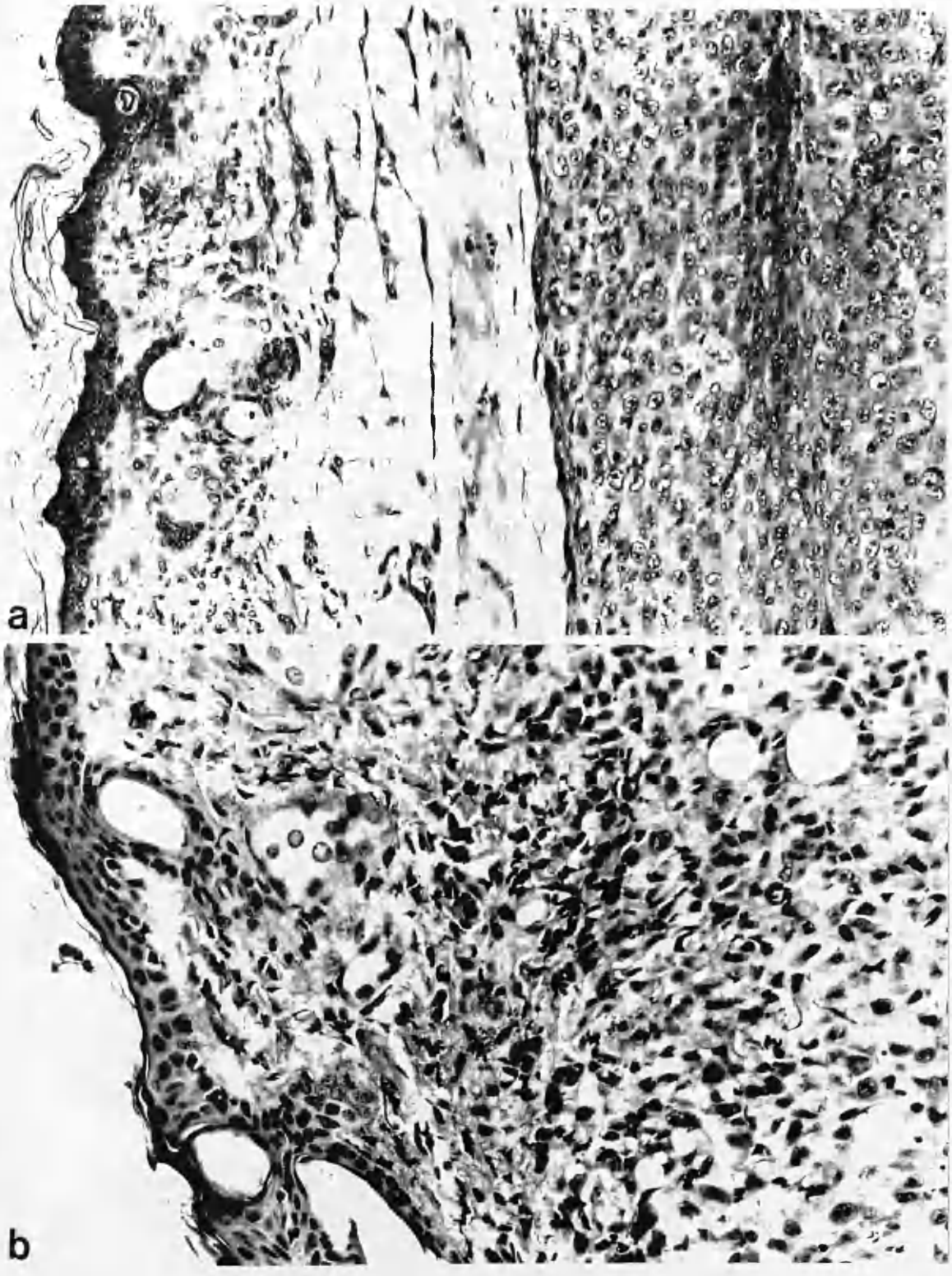
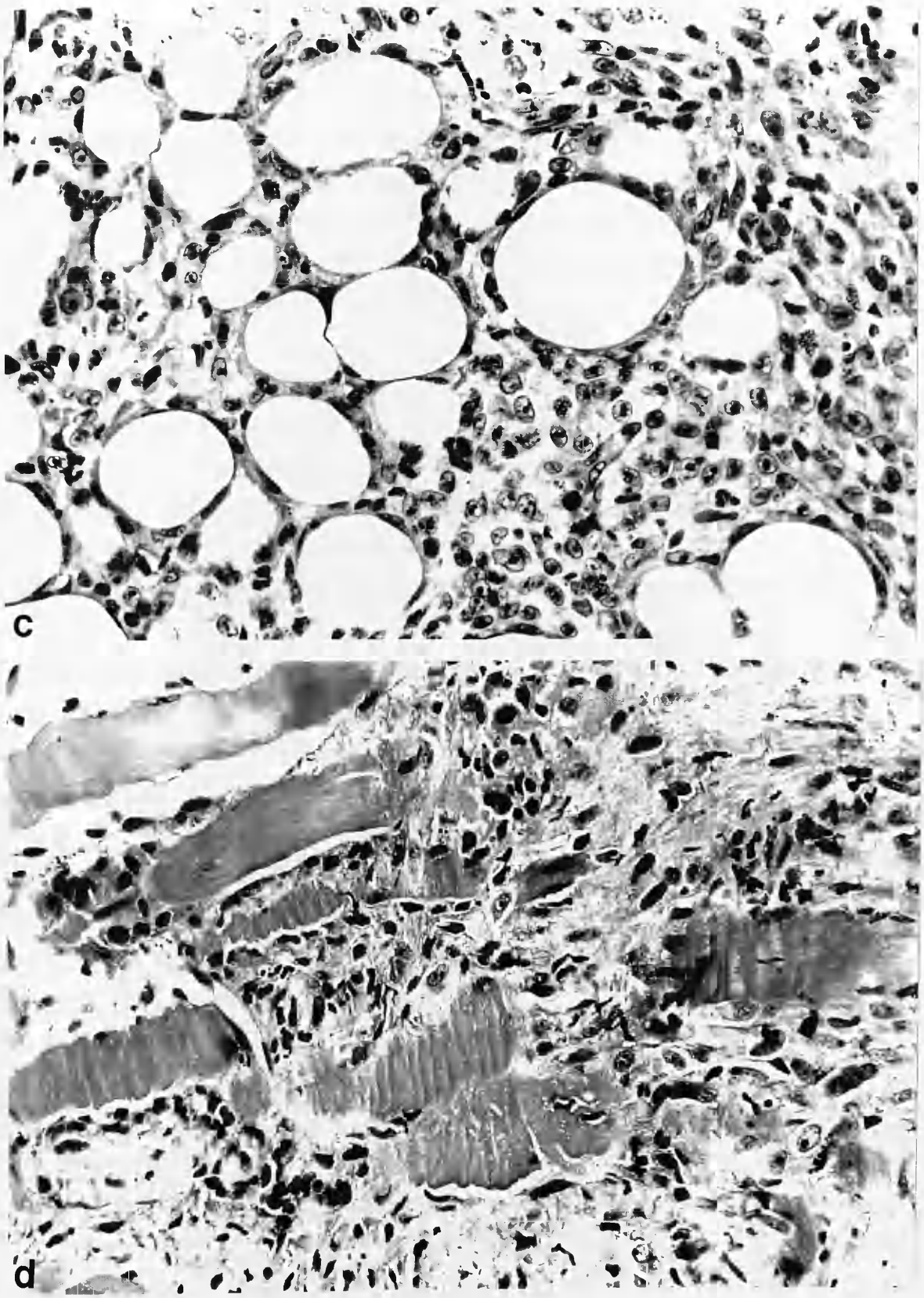



**Plate 5.25:- Histology of tumour invasion into surrounding host tissues.**

Primary tumours were sampled at the interface between tumour and surrounding host tissue, and processed as outlined in General Methods.

(a). Mvl tumour showing well defined edge with no sign of invasion into normal surrounding tissue (x 205, H&E).

(b). T1 tumour showing invasion and destruction of skin and appendages with penetration as far as epidermis (x 395, H&E).




**T1 Tumours**

(c). Tumour cells are seen infiltrating adipose tissue (x 502, H&E).

(d). Tumour cells are seen infiltrating muscle, with obvious damage (x 439, H&E).

## 5.4 DISCUSSION

The objectives of studies carried out in this chapter were to establish a laboratory model for the study of the phenotypic effects of oncogenesis. Mink lung epithelial cells transfected with human c-myc, normal Ha-ras, and activated Ha-ras oncogenes by individual transfections were investigated for different parameters associated with malignant transformation, both *in vitro* and *in vivo*. It was observed that the oncogene transfected cells had enhanced expression of malignancy related properties.

### 5.4.1 Confirmation of Transfection

The Southern blot analysis of normal Ha-ras transfected line showed a 6.6 Kd human ras specific fragment with multiple copies of the gene incorporated into host DNA during transfection. The activated ras transfected line showed a higher molecular weight Ha-ras specific fragment, resulting from a probable integration of endogenous ras sequence, confirming that the transfection had been successful.

Detection of the Ha-ras gene product by immunostaining techniques was not as evident as from the southern blotting. The positive staining was only focal, even after overnight incubation with primary antibody, and with some background staining. Different methods have been used for the optimal tissue fixation, preservation, and immunocytochemical localization of p21 Ha-ras oncogene product. Previously Ha-ras oncogene product has been demonstrated using cryostat sections fixed in acetone, but with poor morphological localization [Kerr *et al.*, 1985; Williams *et al.*, 1985; Candlish *et al.*, 1986]. Robinson *et al.*, [1986] have shown that p21 was not optimally preserved when formalin fixed material was used for its immunohistochemical localization. However, more recently, specific immunostaining of p21 Ha-ras protein has been reported [Going *et al*,. 1988], using Y13-259 on paraffin sections of tissue fixed in PLPD with good preservation of both intensity of staining as well as morphological details of staining. In the present study, this gave similar results to those in cryostat sections, but with a superior tissue morphology, cytoplasmic staining was noticed in controls accompanied by some loss of membrane staining. It is possible that a similar loss might have occurred in the present studies due to membrane destabilization during tissue processing and this loss could have been to the outside of the membrane; mild cytoplasmic staining was also observed. Therefore, poor ras staining might have resulted from its membrane association

and its poor preservation.

A well characterized rat monoclonal pan-ras antibody Y13-259 [Furth *et al.*, 1982; Lacal *et al.*, 1986] was used. It reacts with an epitope most probably amino acid sequence 70 to 80 [Lacal and Aaronsen, 1986] shared by 21 kD protein product of mutationally activated and non-mutated Harvey (Ha), Kirsten (Ki), and N-ras oncogene. Staining was not observed in the control cell line (Mv1), suggesting that either the antibody did not cross react with endogenous p21 or cells were not expressing the endogenous gene product. Therefore, this antibody has been used to confirm p21 localization at the membrane of Ha-ras oncogene transformed cells, as reported in other studies [Hsu *et al.*, 1982]. The use of FHO5T1 activated Ha-ras gene expressing cell line [Spandidos and Wilkie, 1984] as a positive control has been reported by others [Williams *et al.*, 1985; Going *et al.*, 1988] when using the monoclonal antibody Y13-259 to demonstrate the ras gene product p21. It gives a membrane specific staining; however a faint cytoplasmic staining with a concomitant loss of membrane staining has also been reported [Going *et al*,. 1988].

The Southern blot analysis of the c-myc transfected line showed a faint 8 Kd human-specific c-myc fragment which was absent from the parental line, confirming that transfection was successful.

The monoclonal anti-c-myc protein antibody (myc1-9E10) was raised and described by Evan *et al.* [1985]. The antibody is a mouse IgG raised against a synthetic peptide immunogen (peptide G) corresponding to residue 408 through 439, from the open reading frame sequence in exon 2 and 3 of human p62 c-myc. The antibody binds specifically to human p62 c-myc and does not cross react with mouse p64.p66 c-myc [Evan *et al.*, 1985] or endogenous mink c-myc as tested in control Mv1 cells. The study of c-myc expression by immunocytochemical methods using myc1-9E10 showed focal positivity in the M1 tumours or cell cultures. The positive staining increased when primary antibody was applied overnight at higher concentration, but only at the expense of background staining. Nevertheless, this observation lends further weight to the idea that c-myc is not expressed in sufficient quantities to be detected by these two techniques.

### 5.4.2 Confirmation of Species and Lineage

Chromosome and isoenzyme analyses confirmed the origin of the lines as mink. Before morphological and *in vitro* growth characterization the oncogenic transfection was also confirmed using the geneticin (G418) selection system,

where the parental cell line was sensitive, while the transfectants having the aph gene remained stably resistant to the drug which was used for selection of these lines, implying that the selection system was intact and effective. The integrity of the selection system was tested at regular intervals to make sure that the population was homogeneous, since heterogeneity following transfection has been reported in other studies [Spandidos *et al.*, 1985].

Having confirmed their species of origin, the cell lines were characterized also for their lineage. Mv1Lu cells have been described as normal and epithelial-like, in previous studies [Henderson *et al.*, 1974; Barbacid *et al.*, 1978] but they have never been characterized for their lineage or tissue of origin.

All cell lines stained positive for cytokeratin intermediate filament proteins (IFP) with the antibodies AE3, and CAM5.2. Cytokeratins have been used as lineage markers in other studies [Ramaeckers *et al.*, 1983]. These are generally used to differentiate the epithelial origin of the cells, however, mesothelial cell derivatives will also stain positive for these proteins. Vimentin, another member of intermediate filament proteins is generally expressed by cells of mesodermal origin. In this study all cell lines also stained positive for vimentin. However, vimentin co-expression with cytokeratin is a common finding, especially in continuous cell lines in culture [Franke *et al.*, 1978; Virtanen *et al.*, 1981] as well as in many of malignant cells both *in vivo* and *in vitro* [Ramaeckers *et al.*, 1983].

Both the parental as well as transfectants stained positively for membrane proteins or cell surface antigen e.g EMA, and HMFG1&2. These proteins, expressed by epithelia, have been used to differentiate epithelium from stroma. Specific antibodies have been used to elucidate these markers immunohistochemically such as anti-HMFG1&2 [Burchell *et al.*, 1983], and anti-EMA [Heyderman *et al.*, 1979].

In addition, ultrastructural study of the cell lines revealed moderately developed adhesion specializations such as desmosome-like structures, and intermediate filaments. Hence, culture morphology, expression of epithelial markers, together with ultrastructural evidence have suggested an epithelial origin of both the parental and oncogene transfected cell lines.

### 5.4.3 Morphological Characterization *In Vitro*

A number of morphological changes were observed in transfectants including a decreased spreading on the substrate in sparse cultures, deficient formation of monolayer epithelial sheets, and decreased attachment to the substrate, decreased spreading in the dense cultures, formation of various

abnormal multicellular patterns and foci, and invasive behaviour in the mixed cultures with normal fibroblasts. Most of the transformation signs listed above are manifestations of decreased cell-cell and cell-substrate attachments in the transformed cell cultures, with high saturation density, and loss of density dependent growth inhibition. Similar results have been shown in previous studies. Spandidos and Wilkie [1984] demonstrated a morphologically transformed phenotype in early passage rodent cells transfected with activated Ha-ras oncogene. Cells transfected with activated ras oncogene showed spindle shaped cells in contrast to normal polygonal epithelial cells, suggesting an effect of oncogenic transfection on cell morphology, either due to alterations in cytoskeletal elements of the transfectants, or cell surface alterations secondary to receptors or ligands changes which are commonly the products of oncogenes.

Oncogenes can alter the cell morphology by a number of proposed mechanisms, such as production of growth factors, loss of or altered synthesis of surface receptors, or altered signal transduction. Genetic change in the cells, e.g oncogenic insertion into the host genome, may also lead to altered cell-cell interactions or loss of cell receptors necessary for its attachment to substrate, or interaction with stroma. Normal cells attach to substrate and stroma by receptor for fibronectin or laminin, therefore, loss or denaturation of these receptors or matrix proteins would lead to decreased cell-cell or cell-substrate interactions, which is characteristic of malignant cells. Morphological change can result from abnormal growth factor expression. It has been shown that EGF controls cell surface glycoproteins expression in normal cells [Chen *et al.*, 1977].

### 5.4.4 Growth Characterization *In Vitro*

Serum had variable effects on cell growth properties depending upon its concentration, type of oncogene, and status of culture, and substrate. All cell lines were capable of growing, following serum removal, once the cells had entered into the exponential growth phase. However, they did not grow for more than a few passages once serum free, except the activated ras transfected line which was able to grow in serum free conditions for 8-10 passages, when plated at relatively high seeding cell density. The parental cells, on the other hand, grew only for a few passages under similar conditions. The growth of c-myc transfected cells remained intermediate in serum free conditions. Growth stimulation was noticed at 0.5%, 10%, and 20% serum in the parental line. There was no inhibition of growth, though growth stimulation was maximum between 10-20% serum concentration. The c-myc transfected line also showed

stimulation of growth in 0.5% and also at 10% serum concentration, but there was no significant stimulation of growth at 20% serum. Transfection with normal Ha-ras had similar effects as c-myc, where 20% serum concentration was no more stimulatory than 10%. However, activated Ha-ras transfection had slightly different effects, growth stimulation at 0.5% serum was evident but comparatively less than in other lines. Stimulation at 10% serum was slightly higher, but no further growth stimulation was observed in 20% serum.

The effects of serum concentration on the saturation density of the cell lines were also studied. Generally the saturation density of all the cell lines increased in a concentration dependent fashion, when the serum concentration was raised gradually up to 20%. The effects of serum on saturation density of different lines can be analyzed in two ways. Firstly the comparison of saturation density in relation to different serum concentrations for an individual cell line, and secondly, a comparison of saturation density among different cell lines. Cell concentrations at saturation density were different for each cell line, however, consistently higher values for ras transfected cells were found, suggesting an increase in saturation density following ras transfection. High saturation density of the transformed cells could be due to a high rate of proliferation, and altered morphology of cells, where rounded and crowded piled up cells were found in ras transfectants, with loss of contact inhibition and multi-layering of the cells in a disordered manner. The cell number at saturation density was much less than expected from the morphology of the transformed cells, and perhaps this could be due to the loss of cells on the top layer, due to the rounded cell shape, resulting from both high mitotic activity and transformation, and poorer attachment to the substrate.

Plating efficiency of transfectants was significantly higher in all different serum concentrations compared with the parental cell line. A generalized increase in colony size was found in monolayer, in 20% serum, regardless of whether the colony number had increased or decreased. The same effect was found when cells were grown in medium with 10% serum, containing 1 $\mu$M dexamethasone, but the colony size was much larger in dexamethasone stimulated cells. It has been found that transfected cells were able to survive and grow in culture conditions with either low serum, and high seeding cell density or high serum concentration with low seeding density.

Agar cloning has been used in almost all transfection assays as a criterion for the transformed phenotype following oncogene insertion into the cells. The serum effects on cloning efficiencies in agar of the cell lines were also studied.

The parental lines cloned poorly while transfected cells cloned efficiently in agar. There was no colony formation in serum free or 0.5% serum concentration. Significant differences were found in 10% serum where growth of parental cells was negligible compared with a very high cloning efficiency in activated ras transfected cells, normal ras transfected cells also showed high colony formation, but the size of colony was very small compared with other cell lines. The c-myc transfectants had a sightly better cloning efficiency in agar compared with parental, but very low compared with the Ha-ras transfectants. The results were different in 20% serum concentration, where a stimulation was noticed in colony formation both in the parental and the c-myc lines, but a decrease in colony formation was noticed in both the ras transfectants. An increase in colony size was observed in 20% serum, regardless of whether the colony number had increased or decreased. The transfected cells remained alive in agar in serum free and 0.5% serum, but there was no cell division or proliferation, while the parental cells died under similar conditions. Stimulation of parental, and the c-myc transfected lines, and inhibition of the Ha-ras transfected cell lines at 20% serum suggests that the cell response to serum is not general, but specific in nature, dependent on the oncogene, substrate, and concentration of serum in the medium.

These results show that growth of the ras transfected cells was significantly higher in serum deficient conditions, compared to parental line, suggesting serum dependence of normal cells, and its independence in transformed cells, which is characteristic of the difference between malignant and normal cells. These properties have been attributed to an autocrine growth potential in transformed cells [Eagle *et al.*, 1970]. The growth rate of the parental line was as high as those of transfected cells, under optimal growth conditions, however differences in growth of transformed and non-transformed cells were evident under sub-optimal conditions, such as decreased serum concentration, growth in agar, saturation density, and decreased cell number etc [Buehring and Williams, 1976]. Transformed cells were able to grow in serum free conditions, and grew actively at 0.5% serum compared with parental line. Dulbecco, [1970] has reported similar results. Clarke *et al.* [1970] and Pitts, [1971], have also shown similar effects of cell transformation and serum response. These observations suggest that the threshold concentration of serum needed for initiation of proliferation may be decreased to zero in transformed cells compared to non-transformed counterparts.

It has been suggested that transformed cells produce their own growth

factors. It has been shown that cells with decreased serum requirement have altered sensitivities to growth factors contained in the serum [Moses *et al.*, 1978]. The insensitivity to the exogenous growth factors may be associated with the formation of endogenous growth factors by neoplastic cells, these factors may then block corresponding receptors on the cell surface. Thus a transformed cell may lose both the ability to stop proliferation without growth factors and also the ability to initiate proliferation in presence of them. Todaro and de Larco [1978] have demonstrated that one of the manifestations of the transformed phenotype is the alteration of the cell's reaction to various components of growth medium such as serum factors or growth factors. It is possible that transformation is accompanied by the acquisition of a decreased sensitivity not only to the growth inhibitory conditions, but also to certain growth stimulatory factors. Transformation of the normal cell phenotype by growth factors has been reported. Induction of anchorage-independent growth in NRK cells by TGF-$\alpha$ [Todaro & de Larco 1978], has suggested a possible role of growth factors in transformation of cells. These data have supported the autocrine hypothesis of growth transformation in transformed cells.

In previous studies it was shown that the activated ras oncogene transfected cells did not respond to EGF or TGF-$\beta$, while c-myc transfected cells had a reduced inhibitory response to TGF-$\beta$ and an exaggerated response to EGF [Kerr *et al.*, 1990]. Transforming growth factor-$\beta$ (TGF-$\beta$) has been shown to inhibit incorporation of $^3$H-thymidine by mink epithelial cells MvlLu, in a dose dependent manner [Kerr *et al.*, 1990]. Epidermal growth factor (EGF) had the opposite effects with stimulation of $^3$H-thymidine incorporation. It was also demonstrated that $^3$H-thymidine incorporation depended upon the relative concentration of the growth factors, where TGF-$\beta$ and EGF shifted each other's dose response curves to the right, which is consistent with the phenomenon of antagonism between these growth factors. Similar results have been reported by others [Coffey *et al.*, 1988]. An altered response to growth factors was found in oncogene transfected cells. myc transfected cells had a reduced response to TGF-$\beta$, and an exaggerated response to EGF. There was no significant effect of normal ras transfection on the response of mink epithelial cells to these growth factors. However, activated ras transfected cells were refractory to the effects of exogenous growth factors, both TGF-$\beta$ and EGF.

Effects of growth factors can be mimicked by oncogenes in some instances such as abnormal production of PDGF, and expression of erb-B receptor. It has

previously been shown that the endogenous production of growth factor is one of the cellular responses to viral transformation (Kaplan & Ozanne, 1982). Such studies were extended to include transfection of cells with various proto-oncogenes and oncogenes. The ras transformed cells were shown to have lost their EGF requirement with increased growth factor production [Anzano *et al.*, 1985; Stern *et al.*, 1986; Saloman *et al.*, 1987; Leof *et al.*, 1986]. It has been found that the cells transfected with ras oncogenes circumvent the EGF requirement for their monolayer growth in culture [Stern *et al.*, 1986; Salomon *et al.*, 1987; Kelekar and Cole, 1987] perhaps as a result of growth factor production.

While growth factor regulation seems to be the possible regulatory mechanism for these effects of serum, polarity of cells may also be important. In agar cells are exposed to environmental stimuli from all sides, while in monolayer only the top surface is exposed to growth medium and lower surface to the substrate. These two different configurations in the culture systems may be important in interpretation of results. The increase in colony size in 20% serum could be due to a clonal effect of serum on the cells capable of cell division, especially when a heterogenous cell population exists in the transfected lines due to growth transformations.

### 5.4.5 Transformation and Chemosensitivity

Chemosensitivity studies showed that the normal Ha-ras transfected cell line was resistant to ara-c, compared to the parental line. However, there was no effect of other drugs investigated on any of the transfected cell lines, relative to the non-transfected parental line. It has been shown in previous studies that the activated ras transfected cell line was significantly more resistant to adriamycin compared to the parental line [Kerr *et al.*, 1990]. Sklar [1988] have shown that Ha-ras transfected NIH3T3 cells were more resistant to cisplatin compared to non-transfected control. The current data suggest that oncogene transfection can alter the sensitivity of mink lung epithelial cells to cytotoxic drugs, but only in selected cases, and the rate of proliferation does not always regulate the sensitivity to cytotoxic agents, following oncogene transfection. It has been shown by Kerr *et al.* [1990] that although TGF-$\beta$ and EGF have significant effects on DNA synthesis of the parental, c-myc, and normal Ha-ras transfected cells, their exposure did not alter the response of the lines to cytotoxic drugs adriamycin and vincristine. This also suggested that rapidly proliferating cells may not necessarily be more sensitive to cytotoxic drugs. Therefore, despite its higher rate of proliferation and shorter doubling time, the Ha-ras transfectants

were resistant to the effects of the cytotoxic drugs.

It is possible that oncogene activation may confer resistance to tumours, and therefore, though the tumours are rapidly proliferating yet they are resistant to drugs, due the mechanisms outlined below. It has been commonly found in clinical practice that drug sensitive tumours following drug treatment and remission become resistant upon clinical relapse, and it might be that oncogenes are involved in this phenomenon. Therefore, it is essential to know oncogenic status of tumours both before and after drug treatment.

The emergence of the multidrug resistant phenotype following chemotherapy in initially sensitive tumours, such as small cell lung carcinoma [Little *et al.*, 1983], and the expression of ras oncogene in inherently resistant tumours such as colorectal cancers [Thor *et al.*, 1984] also raised the possibility of oncogene involvement in the underlying molecular mechanism of resistant the phenotype. A relationship has been proposed between transformation *in vitro* and the drug resistant phenotype, following chemical, viral, or oncogenic insult to a normal cell, suggesting a probable common mechanism underlying these processes.

The mechanisms by which experimentally induced tumours develop pleiotropic drug resistance include alterations in the levels of several enzyme systems involved in detoxifying xenobiotics e.g glutathione-S-transferase [Cowan *et al.*, 1986] and membrane associated glycoproteins acting as active pumps to expel drug out of the cell e.g P-glycoprotein [Thorgeirsson *et al.*, 1987]. Both p-glycoprotein [Moscow and Cowan, 1988], and glutathione-S transferase [Wolf *et al.*, 1986] are thought to play an important role in the phenomenon of multidrug resistance (MDR) phenotype. Cellular resistance to adriamycin and vinblastine has been shown to be secondary to the increased levels of MDR gene [Moscow and Cowan, 1988] and glutathione-S-transferase [Doroshow *et al.*, 1980]. Burt *et al.* [1988] in similar studies have shown that the transformation of rat liver epithelial cells with v-Ha ras was associated with expression of MDR-1, glutathione-S-transferase-$\pi$ and an increased resistance to cytotoxic drugs including adriamycin and vinblastine.

How exactly oncogenes do induce resistance in the recipient cells is far from clear. The c-myc and Ha-ras oncogene products are not directly responsible for the drug resistance phenotype. The Ha-ras p21 is a membrane G-protein associated with signal transduction pathways, and the c-myc p62 is a nuclear protein and may be involved in the expression of other genes associated with drug resistant phenotype, but this is not known yet. Whether oncogenes can

induce drug resistance by a direct mechanism by acting directly at the genetic level is unknown.

From these findings it can be concluded that either there were no effects of oncogene transfection on the sensitivity of the cells, or where some effects have been observed, they were only marginally significant. There can be many possible explanations to these findings. Firstly it could be that the cells exposed during exponential growth phase have comparatively little differences in their mitotic rates, as the growth rate differences could only be clearly observed when compared at saturation densities of the cell lines. Therefore it would be interesting to see the effects of cytotoxic drugs at saturation density. Although it depends on the type of drug and the cell cycle, generally drugs are known to be effective on cells during their exponential growth phase, and less so in the plateau phase. Secondly, it may be possible that the oncogenes did induce the resistant phenotype in the transfected cells, but the parental cell line was already resistant so that any effect of oncogene transfection was not detectable.

### 5.4.6 Growth Characterization *In Vivo*: Correlation With *In Vitro* Growth Characteristics.

A significant correlation has been observed between growth properties of the cell lines *in vitro* and *in vivo*. A mild degree of spontaneous phenotypic transformation was observed in the parental non-transfected cells. The expression of the transformed phenotype was enhanced following tumour passage *in vivo*, and was amplified by oncogenic transfection. Mvl line is immortalized, M1 is moderately malignantly transformed, while both N1 and T1 are highly malignant, but T1 is always more aggressive where distinctions can be made. Land *et al.* [1983] have shown a correlation between expression of cellular oncogenes and multi-step carcinogenesis. Spandidos and Anderson [1987] have proposed that the process of oncogenesis is multi-step, and that each stage is probably regulated by one or a set of oncogenes.

*In vitro* growth patterns of the cell lines were reflected by tumour formation *in vivo*. Under standard culture conditions the parental cells were non-clonogenic in semi-solid medium, and were serum dependent. However, they were able to form colonies in soft agar as well as to grow in low serum conditions, but only, when a high cell concentration was seeded. The Mvl cell line did not produce tumours below a critical cell number. However, following inoculation of a high cell number, tumours were produced in only 20% of the

cases, and with a relatively long latency period and prolonged doubling time, but these produced 100% tumours during their first passage *in vivo* with a shorter latency period and doubling times. These findings are in agreement with Stiles *et al.* [1975]. They could not obtain tumours from several human cell lines transfected with SV-40 viruses, but later several of these cell lines were found tumorigenic when large numbers of cell were inoculated into the animals subcutaneously [Koprowsky and Croce, 1977], suggesting a positive correlation between cell number, cloning *in vitro*, and incidence of tumour formation *in vivo*.

A number of separate, but interrelated, issues emerge following these observations. First, in the spontaneous acquisition of transformed or oncogenic properties by the parental cells, an increase in cell number was associated with a corresponding increase in expression of oncogenic properties.

The non-transfected cells did not express a transformed phenotype under standard experimental conditions, both *in vivo* and *in vitro*. However, a milder acquisition of transformed characters have been observed, but only at the expense of very high cell number. The same characteristics were enhanced by varying degrees following transfection of the cells, by different oncogenes individually. Spontaneous acquisition of transformed phenotypes including tumorigenicity and invasion have been found in normal mouse [Aaronson & Todaro, 1968] and rat [Van Roy *et al.*, 1986] fibroblasts, suggesting that transformation can occur in apparently normal cells without transfection. Mareel *et al.* [1975] have shown spontaneous expression of tumorigenic phenotype in C3H/3T3 immortalized cells.

According to one theory, it is possible that some oncogenes, yet unknown are activated in the parental cells, either due to culture conditions, host influences, or spontaneously by some other yet unknown mechanisms. Another possibility is that perhaps a subpopulation with this genetic background is present in the original heterogeneous cell population. It has been reported that rat fibroblast cell line 208F transfected with a single human oncogene was expressing other oncogenes with both nuclear-(c-abl) and membrane- (c-fos) associated products [Wyllie *et al.*, 1987].

Passage of cells *in vitro* and tumour passage *in vivo* seems to have a selective effect for a subpopulation of cells. The observations made under standard experimental conditions have shown a significant difference between the parental and the transfected cells as far as their growth properties are concerned *in vitro* and *in vivo*. Therefore, taken together, these data suggest that oncogenes are involved in the progression, if not initiation, of malignant transformation.

The second important finding is a significant enhancement in the

malignancy associated properties by oncogenic transfection of parental cells, suggesting their role in the regulation of these phenotypes. *In vivo* growth potentials have been enhanced by separate transfections with human oncogenes. All transfectants produced tumours in 100% cases with shorter doubling times and latency periods. The growth of the activated Ha-ras transfectants was most aggressive followed by the normal ras, and the c-myc in that order. No significant effects of oncogenic transfection on expression of markers and ultrastructural features have been observed. Either there are no effects or the methods were not sensitive enough to discriminate the minor alterations in phenotypic expression. This pattern of growth has already been shown *in vitro* studies.

An interrelationship has been demonstrated between *in vivo* and *in vitro* transformed phenotypes, e.g. a highly clonogenic cell line in agar *in vitro* was also highly tumorigenic *in vivo* and a poorly clonogenic cell line exhibited a very low tumorigenicity *in vivo*. The comparison is shown in the table below.

**Table 5.16: Correlation of Growth Properties *in Vitro* and *in Vivo* .**

| Cell line | [1]Clonogenicity (%) | Tumorigenicity (%) |
|---|---|---|
| Mvl | 0.5 | 20 |
| M1 | 5.4 | 100 |
| N1 | 10.3 | 100 |
| T1 | 10.7 | 100 |

[1] 20% FBS

When cultured cells implanted into an animal formed a progressively growing tumour nodule at the site of implantation, the assessment of tumorigenicity was considered positive. However, it may be that tumours can arise from the host tissue, e.g. stromal cells under the action of inducing agents (e.g. viruses, or oncogenes) contained in the implanted material. There are other possibilities as in some cases variants of implanted cells can be tumorigenic, which might have been present initially in the implanted population or might have been induced spontaneously, or by micro-environmental influences, after implantation. Additional studies were undertaken to exclude these possibilities,

including histological examination of tumour, immunohistochemical marker expression, and ultrastructural studies. The histological examination was useful to distinguish an epithelial tumour from a reactive granuloma. Therefore these controls were taken into consideration to prove tumorigenicity of the transfectants. It is difficult to interpret a negative result of tumorigenicity, as it may be due to certain limitations of the assay methods e.g. antigenicity and immunological incompatibility of implanted cells with the host e.g by interactions with NK cells of the host [Mora *et al.*, 1977]. However, the use of immunodeficient mice reduces the possibility of negative results due to the host immune system. One of the factors affecting results of tumorigenicity in this study was the number of implanted cells especially in the parental cell line. The critical number of cells leading to a positive take varies among species, cell lines, and in different experimental conditions. These findings are in agreement with various previous studies, where a high cell number ($10^6$-$10^7$ cells per inoculum per animal) has been used to get tumorigenicity [Winterbourne & Mora, 1977].

Why is a large cell number necessary to give rise to a tumour? There are many possibilities: 1) the cells may co-operatively form a favourable micro-environment for themselves e.g. induce stromal growth, secrete growth factors, and form intercellular communications; 2) only a small number of tumorigenic cells were present in the total cell population.

### 5.4.7 Plasminogen Activator Activity.

There was no significant difference in PA activity of cells before and after transfection with human oncogenes, and no definite correlation has been found between oncogene transfection of epithelial cells and PA production. Many of the previous attempts to correlate PA activity and neoplasia in human tumours have been conflicting. Markus *et al.* [1980] have found no difference in PA activity of benign tissue and malignant tumour from human lung. Similarly, no relationship was found between PA activity and local invasion in breast cancer [Evers *et al.*, 1982]. PA production by human normal tissues has also been reported. Schumacher and Schill [1972] demonstrated higher PA activity in normal cells of human fetal lung in early passage cultures, compared to adult lung, suggesting that PA production may be the property of tissues undergoing reconstruction as during fetal life, and cancer growth also involve destruction of tissues though repair is probably overridden.

In another study a number of clinical and histopathological variables were examined separately for their possible association with PA activity. Although a

very weak association of PA activity was found with disease stage, invasion and nodal status, the vast majority of other variables did not influence PA level including histological grades, size of tumours, and metastasis. [Colombi *et al.*, 1984].

It has also been proposed that the production of an increased amount of PA is not an obligatory feature of transformed cells in culture, in particular the cell lines with long history in culture may lose this property without losing other malignancy associated phenotypes [Rifkin and Pollack, 1977], suggesting that PA production may be the property of early stage tumours, since a transient proteolytic activity is essential for invasion at primary tumour site.

Still another reason for these discrepancies could be the production of specific protease inhibitors, the net PA activity depending upon the interaction [Sprengers & Kluft, 1987].

### 5.4.8 Invasion and Metastasis

Invasion *in vitro* showed that all cell lines, including the parental, line co-cultured with chick heart fragments, were invasive with only minor differences in the pattern and rate of invasion. All transfected lines were also invasive *in vivo*, however Ha-ras transfectants were always more aggressive. The parental line was non-invasive during primary growth. Virtually all transplanted tumours were found invasive including those engendered by the non-transfected cells. Spontaneous metastases were not found in any of the cell lines.

These data have demonstrated a significant correlation between invasion *in vitro* and *in vivo*, and in agreement with previous reports of Mareel *et al.* [1987] and de Ridder *et al.* [1987], who have been able to predict in most cases invasiveness *in vivo* on the basis of assay of invasion *in vitro*, using chick heart fragments. More recently it has been shown that invasion of glioma cells in this model was consistent with their invasive behaviour *in vivo*, suggesting a potential utility of the assay for predicting invasion in patients [de Ridder and Calliauw, 1990].

The single tumour formed by the parental line at high cell number was minimally invasive *in vivo* during primary growth, transplants took with 100% efficiency, and were all locally invasive like the tumours of the transfected cell line.

All tumours produced by the myc transfectants were locally invasive, with evidence of host tissue destruction and replacement by invading cells. Similarly

transfection of Mv1 cells with Ha-ras also enhanced tumour incidence to 100%. These tumours showed the most aggressive behaviour in terms of their invasive phenotype with macroscopic as well as microscopic evidence of host tissue infiltration and destruction of surrounding host tissue. Mareel *et al.* [1987] found similar results, cells transfected with Ha-ras oncogenes were invasive and metastatic, however, the non-transfected parental cell line also showed transformation associated characteristics, both *in vitro* and *in vivo*, including agar cloning, invasion and metastasis.

From these data it can be concluded that a subpopulation of cells in the parental cell line is capable of expressing the properties of a transformed cell and that the expression of these transformed properties has been enhanced by oncogenic transfection of the parental cell line.

Taken together these data suggest that tumours produced by mink lung epithelial cells, either before or after separate transfection with human oncogenes were locally invasive with exception of primary tumours of Mvl, and expression was enhanced after tumour passage *in vivo*, with only slight differences in their pattern and rate of invasion.

Invasive phenotypes can be obtained by transfection of normal or non-invasive primary or immortalized cells in culture with plasmid containing oncogene(s). However reports from other workers have seriously challenged a sole role of oncogene(s) in conversion of the non-invasive into the invasive cell phenotype [Mareel & Van Roy, 1986].

It has been suggested that invasion can arise in a cultured cell population in an apparently spontaneous way under different conditions such as: 1) Routine passage of cells in culture has been reported to cause conversion of non-invasive to invasive phenotype, with or without transformation of growth [Mareel *et al.*, 1988]. This has been found in a variety of cell types e.g. in mouse embryo cell lines [Mareel *et al.*, 1975; Greig *et al.*, 1985]. 2) Gene transfer procedures per se such as the calcium phosphate technique of Grahm and Van der Eb [1973]. Therefore it is essential to confirm oncogene incorporation into the host cells by southern blot analysis, to exclude the possibility that carrier DNA had an effect without oncogene. Usually this is confirmed using controls including parental cell line and parental cells treated as test cells (transfected) but without oncogene. 3) Cloning cells *in vitro* has been found to cause invasive phenotype in an immortalized cell line [Mareel & Van Roy, 1986] spontaneously. This can be due to activation of an oncogene following growth in agar, but the most probable explanation is the selection of clones (transformed or stem cells) with the invasive

phenotype, which were already present in the parental cell population but at a very low number. Our results are in agreement with these findings. Mvl formed very few colonies in agar, very low tumorigenicity, and invasion *in vitro* was possibly due to a selective process. 4) An increase in the level of the *in vitro* transformed phenotype and tumorigenicity *in vivo* have been reported in many studies following tumour passage *in vivo*. Invasion has been reported to be acquired quite frequently through a single *in vivo* passage of non-invasive tumours. Van Roy *et al.* [1986] showed that rat cells were non-invasive *in vitro* assays, but produced invasive tumours when injected into syngeneic Fischer rats through subcutaneous route. Subsequently a cell line derived from such a tumour was frankly invasive, suggesting selection of an existing subpopulation of cells with invasive properties. These findings are in agreement with present results.

There could be two possible explanations to the above observations. 1) Host factors may activate the genetic mechanisms for the transformed phenotype such as activation of oncogenes and suppression of anti-oncogene(s) resulting in a transformed phenotype. 2) Transformed cells are selected by growth *in vivo*. Apparently these observations do challenge an absolute role of oncogenes in the acquisition of the malignant phenotype, because the parental line is expressing the transformed properties as well. However, the expression of endogenous oncogenes in the parental line cannot be excluded.

Collard *et al.* [1985] have suggested a possible role of endogenous oncogene activation in tumour derived cell lines, following *in vivo* passage. Cells may acquire invasiveness or a malignant phenotype through interaction with host cells or the *in vivo* environment may select a subpopulation which is already present in the parental cell line, which in this case is heterogeneous but with a predominantly normal phenotype. This stem cell subpopulation is perhaps capable of expressing malignant properties, and may represent a similar cell type *in vivo* capable of causing tumours under various conditions of environmental changes.

Heterogeneous expression of oncogene products in a given tumour cell population has further supported the idea of heterogeneity of tumour cells both *in vivo* and vitro. Moreover, heterogeneous expression of oncogene products in an invasive cell population suggests that the malignant cells are dynamic both in function and structure. Perhaps all malignant phenotypes are transient, interchangeable, and may alter due to a high cell turn over and genetic changes taking place at relatively higher rate than in normal cells which are relatively static. These changes in tumour cells may go from bad to worse or vice versa,

depending upon the circumstances resulting in expression of differentiation or malignancy associated characteristics. It represents a spectrum of intermediate phenotypes between a normal differentiated phenotype and undifferentiated malignant phenotype.

It has been shown that invasion results from selective growth of a subpopulation of cells with invasive potential, and heterogeneity may result from random genetic changes occurring in a transformed cell population [Talmadge and Fidler, 1982; Weiss & Ward, 1983]. It has been shown in the present study, where the parental cell line becomes as invasive as the transformed cells, suggesting a selective process, and heterogeneity in the Mvl cell line. It is possible that some invasive cells are present in the parental cell line population which are selected by *in vivo* passage. It may also be possible that these cells respond to oncogene transfection and expand to dominate the culture. Oncogenes may facilitate growth expansion, but do not make cells invasive per se.

Interestingly, despite the local invasiveness of the cell lines, there was no evidence of metastatic spread, either locally to regional lymph nodes or to distant sites, including liver, lungs, and brain under these assay conditions. There was no obvious correlation between metastatic and other malignancy associated phenotypes.

Although the ability of the tumour cells to cross the basement membrane is found in general to correlate with their metastatic ability *in vivo* [Liotta *et al.*, 1986], all invasive tumour cells are not necessarily metastatic. It has been proposed that invasion and metastasis are two distinct steps in tumour progression towards increasing malignancy [Mareel and Van Roy, 1986]. A gene for the invasive phenotype has been proposed [Sporn & Roberts, 1985]. Similarly specific gene(s) have been suggested in causation of metastatic phenotype [Mareel & van Roy, 1986], or the same gene acting at different levels of malignant progression may be involved. However there is no known example of such a genetic sequence responsible for metastatic conversion of a non-metastatic model either *in vitro* or *in vivo*.

In accordance with these results, it has been found that the Ha-ras transformed cells may be capable of experimental metastasis, but not spontaneous metastasis [Thorgeirsson *et al.*, 1985]. They found that the Ha-ras transfected cells were metastatic when injected directly into the blood vessels, but the same cells were non-metastatic when injected subcutaneously, suggesting some specific effects of route of injection.

A progressive reduction in metastatic ability of the cells maintained in culture has been reported [Ossowski and Reich, 1980], apparently in contrast to the increase in the invasive phenotype with passage number [Mareel *et al.*, 1988], suggesting that metastasis may require some differentiated phenotype lost *in vitro*. Invasion on the other hand may be associated with proliferation, and a more undifferentiated phenotype.

It has been suggested that metastasis can be a transient change in growing cells either due to mutations or epigenetic changes [Weiss & Ward, 1983]. Similarly it has been observed that different cell lines transfected by the same virus, exhibited different metastatic abilities suggesting that an intrinsic cellular property may be responsible for the metastatic non-metastatic behaviour.

There could be the following possible reasons for the failure of the cells to metastasize in spite of invasion: 1) related to models and methods used, 2) and related to the process of invasion and metastasis itself.

*In vivo* metastasis is mostly studied using animal models usually syngeneic or immunodeficient mice, since no *in vitro* assay is capable of modelling all of the aspects of naturally occurring metastasis. Metastatic assay performed on heterogeneous cell populations such as those obtained following transfections with oncogenes introduces an extra requirement for an immunodeficient host e.g. athymic nude mice. There has always been some caution raised about the use of nude mice in the study of metastasis due to the non-metastatic behaviour of some malignant cell lines and the potential for the natural killer cell modulations of metastatic ability [Fidler, 1986; Chambers & Tuck, 1988], and the success of the nude mouse model for studies of cancer metastasis has been limited as most malignant neoplasms tested in this model were not metastatic. Using human melanoma cell lines in this system Tveit and Pihl, [1981] showed that human xenografts grown in nude mice retained the properties of the parental tumours. Although tumours grew extremely well in nude mice metastases were rare. Because of the reports that perhaps the NK-cell system is active in nude mice [Fidler, 1986]], and that oncogenes may increase cellular sensitivity to NK cell cytolysis [Johnson, *et al.*, 1985; 1987], it may be possible that this mechanism was operating in this model. Naito *et al.* [1987] have used the NK-cell deficient beige mice model but they did not find a higher incidence of experimental lung metastasis than in the conventional nude mice model. In addition, a SCLC cell line, NCI-H69, was metastatic in the same model [see above, Chapter three-A]. Therefore, it is unlikely that NK cells or the nude mouse model were responsible for the non-metastatic phenotype of the cells in this study.

### 5.4.9 Are Oncogenes Involved in Phenotypic Transformation?

Tumour invasion, dissemination and metastases formation are considered as advanced characteristics of malignant transformation. The tumour cell population may employ different strategies to achieve the same end. Many investigators have sought to determine the phenotypic traits which distinguish metastatic cells, yet no specific characteristics have been unequivocally recognized as being constitutively required by all cells capable of metastasis [Mareel *et al.*, 1986].

All stages of the malignant processes including invasion and metastasis are now thought to be under genetic control. Specific genes are thought to be responsible for these individual steps. Normal cellular genes or proto-oncogenes control normal cell functions. Activation of the proto-oncogenes may contribute to neoplastic transformation and progression to the metastatic phenotype. Oncogenic expression has been correlated with invasion and metastasis in several different studies, both *in vivo* in human tumours [see above, Table 1.3], and *in vitro* experimental models. Little *et al.* [1983] found that expression of c-myc gene was observed commonly in cells from a variant of small cell lung carcinoma with poor prognosis. These findings suggest that amplified or activated oncogenes may produce heterogeneity in tumour tissue by increased rate of cell proliferation, and once a diversification has occurred in the tumour cell population, the subsequent changes may not be critical for progression of the malignant process to achieve a final invasive or metastatic phenotype.

It is often difficult to determine whether oncogene expression is a primary, i.e causal, or secondary, i.e induced event, in a malignant cell. Many oncogenes including myc and ras have been found during normal growth and development. This ambiguous expression of oncogenes both in normal and tumour tissues has suggested two possible explanations for these observations. It may be possible that oncogenes simply confer greater growth potentials on cells already possessing the necessary genetic elements required for metastasis. On the other hand activation or insertion of an oncogene in the host DNA may trigger a "pre-programmed" cascade of events normally undertaken by migrating cells during embryogenesis or wound healing. Therefore, a single gene such as Ha-ras apparently can influence a complex multi-step sequence of events such as invasion and metastasis, by above mechanisms. An activated oncogene such as ras may cause invasion and metastasis by: 1) Mitogenic effect due to growth factors production e.g TGF-$\alpha$ mitogenic for epithelial and stromal cells, and

endothelia, TGF-$\beta$ mitogenic for stromal cells; these events may modify cellular adhesion and cause an increased release of cells from the tumour site. 2) Production of lytic enzymes; ras transfected cells are known to produce cathepsin L, this and collagenase type IV may be involved in the degradation of basement membrane. 3) An altered expression of cell surface receptors and antigens may alter cell interactions with growth factors, altered glycosylation with increased branching, decreased cell contacts, enhanced extravasation, and sensitivity to immune system and tumour cell survival in circulation.

Abnormal expression of ras oncogene has also been described in human colonic carcinomas by different investigators. Immunohistochemical studies [Thor *et al.*, 1984] using a monoclonal antibody broadly active against the ras group, raised against a synthetic peptide showing sequence homology to the human T24 ras oncogene product, showed that the majority of the cells in colon carcinoma were positive for ras but only less than 10% cells were positive in benign tumours, and less than 1% cells were positive in normal colon. However, about 90% cells were positive in metastatic tumours. Moreover, a correlation was shown between the depth of invasion and ras p21 expression where only a very small number of positive cells was found in normal mucosa, an intermediate numbers of positive cells in superficial invasion, and a large numbers in deeply invasive carcinoma, suggesting a possible role of ras oncogenes products in invasion and metastatic processes. Spandidos & Kerr, [1984] have demonstrated higher transcripts of Ha-ras in adenomatous polyps of colon than in carcinoma. It can be concluded from these observations that ras p21 overexpression was not per se sufficient evidence for the generation of the invasive phenotype in human colonic tumours.

Cell surface changes due to oncogene products have been described in different studies. Collard *et al* [1987] have shown that ras p21 is located in the plasma membrane therefore suggesting that the mechanism of possible interaction between p21 and cell surface molecules like carbohydrates, proteins, or lipids etc., may well be involved in alteration of these structures at the cell surface, which has been proposed to be related to malignancy. Collard *et al.*, [1987] have demonstrated p21 involvement in the metastatic phenotype of transfectants in nude mice model suggesting that the cell surface changes secondary to oncogene transfection might be due to presence of oncogene product(s) which may contribute to the invasive and metastatic changes in the cells.

A substantial number of oncogene related products have an effect on cellular functions that are implicated in invasion and metastasis, such as

migration [Mareel and de Mets, 1984] and secretion of lytic enzymes [Jones and de Clerck, 1982]. Mareel *et al.* [1981] showed that stimulation of migration in a non-invasive cell was related to the development of the invasive phenotype, suggesting that the oncogene products may act by altering specific functions in the cell that may lead indirectly to the invasive phenotype. Similarly Bade and Nitzgen [1985] have found that EGF could cause migration of liver epithelial cells on a tissue culture substrate, while EGF-stimulated migration of human foreskin fibroblasts has been shown by Westermark and Blomquist [1980]. They proposed a possible role of such factors in invasion *in vivo* of tumour cells during invasion and metastasis.

Many research groups have found that abnormal expression of members of the ras oncogene family can contribute to metastasis, using either nude mice or chick embryo models and NIH 3T3 cells. Spandidos & Wilkie [1984] demonstrated transformed phenotype in ras transfected fibroblasts, suggesting that a single oncogene can induce the malignant phenotype, probably by acting at different stages of the carcinogenic and metastatic processes. Similar results have been shown by a number of other studies using fibroblasts with ras transfection [Wyllie *et al.*, 1987; Chambers & Tuck, 1988]. Working with normal human bronchial epithelial (NHBE) cells Yoakum *et al.* [1985] have shown that following transfection with v-Ha-ras these cells acquired carcinogenic potential such as tumorigenicity, invasion, and formation of colonies in soft agar. Using mouse mammary cell lines a metastatic subclone was obtained after transfection with c-Ha-ras oncogene, and after co-transfection with PSV2 neu plus c-Ha-ras oncogene a higher metastatic frequency was observed, suggesting that ras transfection alone in some cases may not be sufficient to induce metastatic potentials. Greig *et a l.*, [1985] have shown that immortalized cells may express both tumorigenic, invasive and metastatic phenotypes, and the exact role of ras in such cases is not clear. He further suggested that the majority of immortalized cell lines are far from normal and need special caution in the interpretation of transfection data.

In summary, the data presented in this chapter have shown that the transfection of mink lung epithelial cells, with human myc normal Ha-ras, and activated Ha-ras has imparted malignancy associated characteristics to the transfected lines, by varying degrees of severity, both *in vitro*, and *in vivo*, in a pattern consistent with the multi-step theory of carcinogenesis.

The parental line was already immortalized, but serum dependent, with mild growth transformation, very low clonogenicity in soft agar, and tumorigenicity in nude mice, invasion *in vitro* assay and mildly so *in vivo*. On the

other hand, transfection with activated Ha-ras exerted the most aggressive and malignant transformation to the transfectants, while the normal Ha-ras and the myc transfectants expressed properties intermediate between the parental and activated Ha-ras transfected cells.

These data therefore suggest a role of oncogenes in progressive malignant transformation of epithelial cells. While the myc may have carcinogenic potential, the role of activated Ha-ras seems to be more crucial. This model, therefore, may be useful for the study of oncogenesis in epithelial tumours in general, and lung carcinomas in particular, with special reference to oncogenic involvement.

# CHAPTER SIX

# GENERAL DISCUSSION

**As the results have already been discussed in detail in each chapter, the purpose of this chapter is to bring together the main findings of different chapters, with more general conclusions, possible implications, and the future prospects.**

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## 6.1. The Objective And Achievements.

The purpose of the studies performed in this thesis was to investigate and establish, whether induced or spontaneously arising alterations in the phenotypes of lung cancer cells affected their malignancy and chemosensitivity. Phenotypic alterations have been shown to arise spontaneously in the H69 small cell lung carcinoma cell line and to be inducible by differentiating agents. In both cases the changes in phenotype resulted in decreased malignancy, but had no consistent effect on chemosensitivity. Genetic manipulation of mink lung epithelial cells, by transfection with human oncogenes, resulted in increased malignancy but again with only minor effects on chemosensitivity. Attempts to modify the phenotype of non-small cell lung cancer cells met with only limited success.

While attempts to manipulate the phenotype to alter chemosensitivity have been unrewarding, it is clear that the expression of phenotypic characteristics associated with malignancy can be manipulated in a manner which can regulate tumour growth *in vivo*.

Spontaneous phenotypic changes have been observed in H69 cells in culture. The cells changed from typical SCLC morphology to NSCLC type morphology, accompanied by biological and biochemical changes. While the parental line expressed markers of neuroendocrine differentiation both the derivative lines either did not express or expressed only small quantities. The parental cells were invasive *in vitro*, while derivative lines were not. H69 cells were also invasive *in vivo*, and showed widespread metastases, but the derivative lines were neither invasive nor metastatic. These, with a number of other parameters studied in the present report [Chapter three-A], suggested that the derivative lines were of H69 origin, but had transformed to NSCLC phenotype, spontaneously.

Further studies [Chapter three-B] showed that the H69 cell phenotype could also be altered by experimental manipulation. The cells exposed to various potential phenotypic inducers (cyclic AMP, HMBA, Na-But) also showed morphological and biochemical changes, suggesting that the H69 cell phenotype can be altered by external influences, in addition to the changes arising spontaneously.

The effects of microenvironmental changes on a NSCLC cell line were also investigated [Chapter four]. WIL cells (lung adenocarcinoma derived cell line) grown in histotypic culture exhibited a more differentiated phenotype.

There was an increase in mucin production, accompanied by morphological change. The cells grown in the histotypic model were able to form surface microvilli, compared to smooth surfaced cells grown without histotypic culture in a conventional plastic monolayer. These changes again suggested that the cell phenotype can be modulated by microenvironmental manipulations.

In addition to microenvironmental manipulations, the effects of genetic alterations on cell phenotype have also been investigated [Chapter five]. An immortalized lung epithelial cell line transfected with human Ha-ras, and c-myc oncogenes individually showed significant phenotypic changes in cell phenotype. Transfectants acquired changes in morphology and growth both *in vitro* and *in vivo*, suggesting that a small genomic change can have extensive effects on phenotypic expression. To summarize, it has been demonstrated in different models in these studies that the tumour cell phenotype can alter spontaneously, or can be altered following microenvironmental changes, and by genetic manipulation.

Phenotypic changes either spontaneous or induced by genetic alterations were stable. Both the derivative lines of H69, H69V & H69VZ [Chapter three-A], and oncogene transfected lung epithelial cell lines [Chapter five] showed phenotypic stability. However, changes induced by chemical inducers were mostly reversible [Chapter three-B]. Cells exposed to chemical agents showed growth arrest, and loss of invasive capacity, but following drug removal cells were able to grow again, and regained invasiveness. These findings have demonstrated that phenotypic change once induced may become stable or revert back to the parental phenotype, once the inducer is removed, suggesting that the phenotypic changes following genetic alterations are more stable than the changes due to microenvironmental influences. This suggests that H69 cells may have changed their genotype to give rise to H69V and H69VZ [Chapter three-A], in an analogous fashion to the transfection of lung epithelial cells with human oncogenes [Chapter five], although with opposite effect.

## 6.2 Phenotypic Transition In SCLC: Implications For Lung Cancer Classification.

*In vitro* conversion from classic SCLC to NSCLC accompanied by the coordinate shifts in morphology, growth and neuroendocrine phenotype has been

shown in the present study [Chapter three-A]. This *in vitro* model could be useful for the study of phenotypic conversions in lung cancer *in vivo*. It may also be a useful model for studying response of SCLC to radiation and chemotherapy. This model can also be used to investigate the interrelationship of chemosensitivity and neuroendocrine differentiation and its potential therapeutic implications. Heterogeneity has been found in lung cancer both in clinical and in experimental studies, and may be the cause of treatment failure due to emergence of resistant variants in the original tumour. These mixed histology tumours have a significantly lower complete response rate to cytotoxic therapy and worse prognosis [Radice *et al.*, 1982]. Generally a change in the SCLC phenotype toward a NSCLC phenotype is noticed [Goodwin *et al.*, 1983]. It has been found difficult to quantitate tumour progression in precise terms by conventional histopathological studies of the tumour, and a lack of this knowledge hampers tumour treatment and ultimate outcome. Although, not immediately identified by conventional histopathological examination, lung cancers may show a profound heterogeneity when examined by ultrastructural or immunohistochemical techniques. It has been shown that only 34% of lung carcinomas were homogeneous, even on simple histological examination [Roggli *et al.*, 1985]. Significant heterogeneity has also been found in cytological specimens of lung cancer [Hess *et al.*, 1981] and on ultrastructural examination [Trump *et al.*, 1982]. A so called tripartite differentiation within the same tumour cell has been reported [McDowell *et al.*, 1981], who showed squamous, adenomatous and small cell differentiation in a small cell lung carcinoma at ultrastructural level, suggesting a link between these three tumour types, possibly by a common differentiation pathways originating from a stem cell. In another study, Broers *et al.* [1987] have examined 218 cases of human lung cancer, and found combined phenotype only in 6% on routine histology, however, after immunohistochemical examination heterogeneous staining for epithelial and neural antigens was observed in about 66% cases. These reports have suggested that lung cancer diagnosis, and hence treatment and prognosis may be significantly improved, by immunohistochemical and ultrastructural investigations of not only the borderline cases, but all suspected cases that may show any sign of heterogeneous cell types on histological examination. The present report confirms the heterogeneity of some lung tumours and shown that alterations in phenotype can influence outcome.

Changes in histological features are usually associated with the biological,

biochemical and pathological behaviour, and with changes in the sensitivity of the tumour to radiation and chemotherapy. It is unknown whether this change represents the evolution of two separate lung cancers present from the outset, transformation of SCLC to other lung cancer types, development of a second tumour following cytotoxic therapy, or in fact it reflects a common origin for all lung cancers with different levels and pathways of differentiation. The present study favours the last of these.

It is possible that multiple oncogenic events may simultaneously transform different cells in close proximity to each other. The transformed cells of more than one histological type then grow together to form a composite tumour, but this concept has not been supported by enough evidence. On the contrary, it has been shown that the lung cancer tumours and tumour derived cell lines had a single aneuploid peak [Gazdar *et al.* [1981], suggesting that the concept of multiple oncogenic events occurring in normal cells to give transformed cells within a heterogeneous tumour may be unlikely. Another explanation could be that a single transforming event may give rise to morphologically different cell types. Tumours of one morphology may then convert to tumours having different morphologies. There is evidence that SCLC may undergo squamous metaplasia, and both SCLC and squamous cell carcinoma have a common etiology, and are commonly found in clinical specimens as mixed SCLC-squamous histology [Sidhu, 1979], suggesting some common features between small cell and squamous cell carcinomas of lung. Similarly, in *vivo* studies have shown that nitrosamine induced pulmonary neoplasia in the hamster is preceded by bronchial endocrine cell hyperplasia and subsequent squamous metaplasia, suggesting a link between NE-cell related tumours and squamous cell carcinoma.

The development of a second tumour following therapy is an other possibility, perhaps by the overgrowth of a mutant subpopulation derived from the initial clone [Abeloff *et al.*, 1979]. However, most of the morphological, biochemical, and biological data now clearly suggest that SCLC has the pluripotential ability to differentiate along more than one pathway of differentiation [McDowell *et al.*, 1981; Nomori *et al.*, 1986; Bepler *et al.*, 1987; Gazdar *et al.*, 1987], thus it seems more than likely that a stem cell population may exist in the H69 cells, which is capable of generating cells with SCLC and NSCLC phenotype, suggesting a common origin at least for these two types of lung cancer. One could argue that a heterogeneous cell population existed in the parental cell line H69, because the sublines were not derived from clones of H69.

However, this seems unlikely as preliminary studies on H69 clones showed morphological heterogeneity similar to the parental line, suggesting a stem cell nature of H69 clones. Furthermore, karyotypic investigation confirmed a common origin of both H69 and the derivative cell lines H69V & H69VZ, suggesting that the transition from H69 to H69V & H69VZ was an *in vitro* conversion. These data in agreement with previous observations imply that lung tumours may change slowly their whole biological behaviour *in vivo* from one characteristic of the classic cell types to another characteristic of the variant cell type, and ultimately to NSCLC, and that this is also reflected *in vitro*, where a link can be established between SCLC and NSCLC through various transitional types, having characteristics of both cell types.

In light of these observations, it appears that the existing lung cancer classification should be revised, in line with histogenetic, biological, histological and clinical data. The first official WHO lung cancer classification [Kreyberg, 1967] included four subtypes of SCLC based on the original morphologic description of Barnard [1926]; fusiform, polygonal, lymphocyte-like and others (SCLC containing small foci of squamous cell nests, tubules, or giant cells). The WHO lung cancer classification was subsequently revised and published in 1981. The lymphocyte-like subtype was segregated and the term oat cell replaced the lymphocyte-like designation. The remaining three subtypes of the WHO classification were included in an intermediate category, implying a continuum between small and non-small cell lung carcinoma. However, no attention was paid to these morphological variants i.e by separating them into a subgroup, however, it could be justified, because at that time data were not available to demonstrate whether patients with large cell features behaved in a clinically similar way to patients with pure SCLC.

The morphological subclassification of the WHO subtypes of SCLC into the oat cell and intermediate cell were subjected to intense clinical and experimental studies to evaluate clinical course, ultrastructure, immunocytochemical and biological characteristics. Clinical studies have shown that SCLC tumours whether viewed as oat cell or intermediate in subtype, did not have any significant clinical applications, as no correlation was shown between the subtypes and the clinical course reflected by survival, response rate, sensitivity to chemotherapy and radiotherapy [Hansen *et al.*, 1978; Burdon *et al.*, 1979; Carney *et al.*, 1981; Gazdar & Linnoila, 1988]. Others [Radice *et al.*, 1982; Hirsch *et al.*, 1983] have shown that patients with mixed small cell-large cell

tumours have a poorer response to chemotherapy and a decreased median survival compared to pure SCLC, suggesting a lack of correlation between the WHO subclassification and clinical behaviour of SCLC.

With respect to the biology of the disease, some significant advances have been made in the understanding of human lung cancer that may have clinical implications. Most of the experimental work has been done on tumour cell lines. These cell lines are usually able to retain the histological and biochemical properties of the parental tumours in culture. Carney *et al.* [1985] and Gazdar *et al.* [1985] have proposed a new subclassification of SCLC based on extensive morphological and biochemical analyses of more than 50 cultured SCLC cell lines. In this classification, SCLC cell lines have been divided into two major groups, classic (approximately 70% of cell lines) and variant (approximately 30% of cell lines) cell lines. Classic cell lines express high levels of NE-cell markers (DDC, BLI, NSE, CK-BB & DCG), have a characteristic culture morphology, relatively long doubling time, low cloning efficiency, higher radiation sensitivity, and an intermediate cell type of SCLC [see above, Table 1.1]. Variant SCLC lines have a low or undetectable levels of DDC and BLI, but continue to express elevated levels of NSE and CK-BB, have a looser morphology in culture, a higher cloning efficiency, and faster doubling time and are radiation resistant *in vitro* [Goodwin & Baylin, 1982; Carney *et al.*, 1983]. In addition these variant lines have up to 60 fold DNA amplification of c-myc, and also show an increased expression of the gene [Little *et al.*, 1982; Gazdar *et al.*, 1985], suggesting a more malignant behaviour for the variant SCLC lines, which may in part be related to c-myc amplification and expression in these lines. In many of their features the variant cell lines correspond to and in fact may represent mixed small cell-large cell carcinomas *in vivo*. In addition to classic and variant lines of SCLC, the multipotent cell lines capable of growing towards both SCLC and NSCLC lines have been observed [Carney *et al.*, unpublished data]. Data from this thesis [Table 6.1] suggest that these multipotent cell lines may correspond to, or at least contain, the stem cell population *in vivo*, and provide evidence for a common origin of all bronchial carcinomas.

The original lung cancer classification was entirely based on histological criteria. This type of classification takes into account the morphological similarities between the tumour cells and their assumed progenitor or normal counterparts in the particular site in which the tumour arises. In identifying an unknown tumour, one is actually looking for morphological features which bear

**Table 6.1:- Properties of the parental and its derivative cell lines.**

| **Characteristics** | **H69** | **H69V** | **H69VZ** |
|---|---|---|---|
| Morphology | Aggregates | epithelioid | epithelioid |
| Doubling time | long | short | short |
| CFE | low | high | high |
| NE properties | present | abs/dec | abs/dec |
| Karyotype | SCLC | SCLC | unknown |
| Tumorigenicity | present | present | present |
| Invasion | present | absent | absent |
| Metastasis | present | absent | absent |
| c-myc expression | low | high | high |

FFA: free floating aggregates. CSF: colony forming efficiency. NE: neuroendocrine. SCLC: small cell lung carcinoma. abs/dec: absent and/or decreased.

resemblance to the appearance of the putative cell of origin and in this way placing the tumour in a particular category. This approach is based on the histotypic hypothesis that tumour cells retain at least some of the characteristic features of the normal cells. This histogenetic approach is not entirely justified for lung tumours in which the morphology is not a reliable criterion of the origin of the tumour [Bolen & Thorning, 1982], and in which multidirectional differentiation is often reported [Gould *et al.*, 1981] and shown also in the present studies, suggesting a pluripotent stem cell subpopulation in the tumour capable of generating tumours cells with different phenotypes.

However, with the accumulation of more information both from clinics and laboratory research, a number of facts have emerged. First, there are no significant clinical or biological differences between the subtypes of SCLC (the oat cell and intermediate cell). Second, there are significant clinical, diagnostic and therapeutic differences between classic SCLC and SCLC combined with other lung cancer types, as patients with mixed histology have significantly worse prognosis. Third, a significant proportion (approximately 20%) of NSCLC express neuroendocrine, and cytogenetic characteristics of SCLC, suggesting a link between two types of lung cancer, and therefore a need for common histogenetic classification. Finally, NSCLC with NE-cell marker expression are sensitive to cytotoxic therapy, unlike other NSCLC which are resistant to radiation and chemotherapy, suggesting that NE-cell phenotype is more sensitive to cytotoxic therapy like SCLC and these NSCLC should be treated as SCLC.

On the basis of *in vitro* SCLC studies, a new histological classification of SCLC was proposed by the Second Workshop of the International Association for the Study of Lung Cancer at Gleneagles in 1984. It has been proposed that SCLC should include tumours with no NSCLC element, and it has been shown that more than 90% of untreated SCLC fall within this category [Hirsch *et al.*, 1988]. Small cell-large cell carcinoma should be grouped separately. It is estimated that 4% to 6% of untreated SCLC fall into this category, and these tumours have significantly worse prognosis compared to pure SCLC [Radice *et al.*, 1982; Hirsch *et al.*, 1983]. Combined small cell carcinoma is SCLC with squamous or glandular neoplastic components, and it is estimated to be less than 1% of untreated SCLC. However, once again no proper attention was paid to the growing concept of a common origin of different lung cancer types. It is very important to consider the biological aspects of the disease in its classification. This will make histopathological classification of lung cancer simpler and

reproducible among pathologists, and therefore better biological, clinical, and therapeutic comparisons would be made to understand changing clinico-pathological behaviour of the disease.

## 6.3 Signification Of Phenotypic Changes In Lung Cancer.

A number of phenotypic changes can be found in lung cancer during differentiation *in vitro* and *in vivo*. Although, induction of a differentiation associated marker in lung cancer cells may not necessarily imply a reduction in malignancy, there is growing evidence in other systems that induction of the differentiated phenotype is inversely correlated with malignancy associated properties [Roblin & Young, 1980; Freshney, 1985; 1987]. It has been shown [Sidell, 1981] that when a human neuroblastoma cell line, LA-N-1, was induced with retinoic acid to form neurite extension this was accompanied with a decrease in colony forming capacity in soft agar. Others [Rudland *et al.*, 1982] have shown that rat mammary carcinoma, RAMA 25 cells, synthesised casein, and formed domes, had reduced DNA synthesis and were less tumorigenic in nude mice following induction with prostaglandin-E and DMSO. Induction of differentiation with suppression of malignancy in leukaemia has also been shown [Sachs, 1978; Lotem & Sachs, 1984]. An inverse relationship has also been shown in human glioma cells treated with dexamethasone [Frame *et al.*, 1984], and glia maturation factor. Induction of differentiation associated properties was accompanied by a reduction in PA activity and endothelial cell mitogenesis. Similarly, it has been shown that B16 mouse melanoma cells treated with anthracycline antibiotics showed morphological differentiation with simultaneous inhibition of proliferation and tumorigenicity [Raz, 1982]. These are the few of the examples where an inverse correlation between differentiation and malignancy could be established.

This type of interrelationship between malignancy and differentiation has significant therapeutic implications. This means that the malignant cells could be treated not only with cytotoxic agents, but also with agents that may modify the biological behaviour of the tumour cells, e.g. differentiation inducers. Differentiation therapy can be used to inhibit malignancy phenotype. Inhibition of cell proliferation or invasion by chemical agents (e.g. HMBA) would provide an alternative treatment of cancer cells, by reversal of abnormal phenotypic expression.

An inverse correlation between differentiation and malignancy has been demonstrated in some lung cancer models. Tralka & Robson, [1976] have demonstrated cilia formation and reduced tumorigenicity of human lung carcinoma cell line ChaGo treated with DMSO, suggesting that the malignant phenotype was reduced with induction of differentiation. It has been shown that normal human bronchial epithelial cells transfected with v-Ha-ras oncogene had decreased sensitivity to differentiation inducing agents as TGF-$\beta$, TPA and serum [Yoakum *et al.*, 1985; Masui *et al.*, 1986] suggesting an inverse relationship between differentiation and malignancy in this model. A549, an alveolar cell carcinoma derived cell line, expressed surfactant production when grown in a filter well system in the presence of dexamethasone and embryonic fibroblasts [Speirs & Freshney, 1990], and induction of surfactant was correlated with suppression of the malignancy associated phenotype e.g., PA production, tumorigenicity in nude mice and cloning in agar. It has been demonstrated in these studies that a phenotype can be represented by a change in expression of either some specific markers of differentiation (e.g., mucin in adenocarcinoma, surfactant synthesis in alveolar cell carcinoma) or by changes in non-specific cellular activities (e.g., morphology, growth or cytostasis, anchorage dependence, serum dependence, contact inhibition, density limitation of growth, proteolytic activity, angiogenesis, apoptosis, invasion, and metastasis).

An inverse correlation between malignancy and differentiation has been demonstrated in the studies presented in this thesis. Apart from specific markers of differentiation, various other functional properties of cells have also be used to determine whether the shift in cell phenotype is towards a differentiated phenotype or towards a more malignant phenotype.

It is difficult to establish an inverse relationship between malignancy and differentiation in H69 and its two derivative cell lines [Chapter three-A], because of lack of differentiation markers in SCLC. However, it could be easier in NSCLC, where specific markers can be used to represent change in malignancy and differentiation e.g. mucin in adenocarcinoma, surfactant in alveolar cell carcinoma. However, it has been shown in this study that both the derivative lines exhibited benign properties, compared to the parental line. These data have suggested that SCLC cell line H69 was more malignant, while both NSCLC-like derivative lines were relatively benign. It implies that NSCLC in fact may represent a more differentiated phenotype, characterised by expression of specific differentiation markers (e.g. mucin in adenocarcinoma), loss of neuroendocrine

properties, loss of invasive and metastatic potential, induction of resistance to radiation and chemotherapy, perhaps due to genetic alterations e.g. overexpression of c-myc oncogene. More studies are required to establish whether NSCLC are differentiated compared to SCLC, by establishing and investigating more lung cancer models, for specific markers, oncogene expression and response to therapy.

Generally, malignant cells are highly proliferative, and arrest of cell proliferation is usually associated with differentiation in malignant cells, following treatment with differentiating agents. H69 cells treated with phenotypic inducers showed arrested growth of cells as long as the cells were in the drug. Most of the previous studies have shown G1 arrest following treatment with phenotypic inducers [Terada *et al.*, 1977; Wintersberger *et al.*, 1983], but this remains to be investigated with HMBA, Na-But, or dibutyryl cyclic AMP treated H69 cells. Loss of invasiveness in tumour cells following induction with differentiating agents is also a good criterion to indicate loss of malignant phenotype. Invasion was delayed in H69 following treatment with the phenotypic inducers, HMBA, Na-But and cyclic AMP, and this was correlated well with decrease in tumour size *in vivo*, when H69 tumour bearing mice were treated with these agents for 14 days. Whether the effect was due to cytostasis or differentiation is not known, and it would be worth pursuing in future studies, by treating H69 cells with inducers and testing for tumorigenicity, invasion and metastasis in nude mice and clonogenicity in agar. However, there is no specific marker of differentiation in SCLC, and it may be that other cell lines such as A549 with surfactant production may be used. In this study it has been observed that treatment of SCLC with differentiation inducers was associated with suppression of malignancy, but whether it was related to induction of differentiation *in vivo* remains to be investigated. Preliminary results have suggested induction of morphological features of differentiation in treated tumours, and this could be pursued with other markers, and an attempt made to determine the effects on metastasis.

Mucin induction was also observed in WIL cells grown in histotypic culture in filter wells, with morphological evidence of differentiation e.g., formation of surface microvilli [Chapter four]. Mucin expression being a property of normal cells of the respiratory epithelium, has been used in previous studies as a marker of differentiation in normal and malignant lung tissues *in vitro* and *in vivo* [Spicer *et al.*, 1980; Hall *et al.*, 1980; Boat, 1982]. Although no attempt was made to analyze malignancy associated properties of WIL in these experiments, this

remains a potential model for investigating the relationship of differentiation and malignancy as invasion could be measured in the filter well system. Whether mucin expression and formation of surface microvilli was related to reduced malignancy associated properties remains to be tested. It would be also interesting to test the cells following induction in histotypic culture, for cloning in soft agar and tumorigenicity, invasion, and metastasis in nude mice.

Mink lung epithelial cells transfected with human oncogenes also demonstrated an inverse correlation between malignancy and differentiation [Chapter five]. Changes in cellular morphology are the important criteria to differentiate a normal from transformed cell *in vitro* following oncogene transfection. Transfection of Ha-ras oncogene changed the flat morphology of mink lung epithelial cells to a spindle cell type, and this was the most significant effect of transfection that could be identified as an early sign of successful transfection. The transfected cells also showed an increase in the rate of cell proliferation, and increased clonogenicity in soft agar, loss of contact inhibition, serum independence, high labelling indices at saturation density and tumorigenicity in nude mice, all properties of the malignant phenotype. There was a good correlation between oncogene transfection and increased in malignancy-related characteristics of the transfected cells, with the exception of invasion. The parental line was found to be invasive in the *in vitro* invasion assay, leaving some doubts on validity of the assay with this cell line. It is possible, however, that a subpopulation of the parental line was transformed, as the line was mildly tumorigenic as well. This could be examined by characterization of clones of the parental cell line.

So far, phenotypic changes in lung cancer have been studied, for the most part, only at the extreme ends of cancer development. An ideal experimental strategy would be to identify specific markers (morphological, biochemical or functional) at various stages in the development of the differentiated or malignant phenotype in the respiratory epithelium. Then a phenotypic shift may be detected in its early phase, allowing stage-specific correlation between malignancy and differentiation. This may involve *in vitro* experimental models of both organ cultures and cell cultures, and *in vivo* experimental systems of both normal and malignant cells. Such an ideal model would be useful for the study of differentiation and carcinogenesis. So far only one model has been developed on these lines by Harris *et al.* [1985], using normal respiratory epithelium from both animal and human tissues for the study of differentiation and malignancy. It

would be interesting to grow normal epithelial cells in histotypic tissue culture, to see whether differentiation could be induced, and then progressively more malignant cells from lung or other systems could be examined as discussed in Chapters three & four i.e. by regulation of the microenvironment and use of chemical inducers.

## 6.4 Phenotypic Changes In Lung Cancer: Effects On Radiation & Chemotherapy.

It has been suggested that the cellular phenotype may be correlated with sensitivity to therapy and this would clearly be important in any attempt to combine differentiating agents and cytotoxic drugs. It is a common notion that more rapidly proliferating cells are generally more sensitive to radiation or chemosensitivity, compared to normal differentiated cells. To evaluate this further, the sensitivity of cells representing different degrees of differentiation and malignancy to cytotoxic drugs and radiation was tested. It was asked whether phenotypic change could influence chemosensitivity and what could be the implications? Do drug resistant mechanisms correlate with phenotypic change and how could they be modulated?

A SCLC cell line H69 and two of its NSCLC-like derivative lines were tested for radiation sensitivity *in vitro*. In this study, the parental H69 cell line was significantly more sensitive to radiation treatment compared to both the derivative lines and a NSCLC cell line WIL. This was in agreement with the previous clinical [Salazar *et al.*, 1776; Owens & Abeloff, 1985] and experimental [Carney *et al.*, 1983; Duchesne *et al.*, 1986] studies on classic SCLC. H69V & H69VZ cell lines showed significant change in phenotype from classic SCLC to NSCLC-like phenotype, morphologically, biochemically and for growth properties *in vitro* and *in vivo*, and it correlated well with resistance to radiation. These data correlate with clinical response to radiotherapy, where SCLC is generally sensitive while many of these tumours on relapse are unresponsive. Cell lines derived from these relapsed tumours often exhibit the so called variant phenotype, which is associated with loss of neuroendocrine differentiation, more rapid growth *in vitro*, and increased expression of certain cellular oncogenes [Gazdar *et al.*, 1985; Carney *et al.*, 1985]. Carmichael *et al.* [1989] have reported radiation sensitivity of a panel of lung cancer cell lines from both SCLC and NSCLC origin. It was

found in their studies that SCLC cell lines were generally more sensitive, except the variant lines, and most of the NSCLC cell lines were resistant, except the cell lines that express neuroendocrine markers, suggesting that changes in lung cancer phenotype may alter response to radiation therapy, and this may have significant clinical implications. Therefore, *in vitro* predictive testing may be of clinical value for the detection of lung cancer patients with sensitive tumours.

The SCLC H69 cell line and the derivative lines, H69V & H69VZ, were investigated for their sensitivity to cytotoxic drugs vincristine and adriamycin *in vitro*. In this study H69 was more sensitive to vincristine, but resistant to adriamycin, while both the derivative lines were significantly more resistant to vincristine, but sensitive to adriamycin [Chapter three-A]. Vincristine sensitivity of the cell lines was consistent with previous observations [Carney *et al.*, 1983; Duchesne *et al.*, 1986]. However, resistance of SCLC and sensitivity of NSCLC to adriamycin was inconsistent with other phenotypic changes observed in this model. It was expected that the adriamycin sensitivity pattern should be similar to that of vincristine in this model, because resistance to these drugs have been shown to implicate the MDR-phenotype. However, this does not seems to be the case with adriamycin in this model at least. It was not entirely surprising, since adriamycin may act through a number of different mechanisms. In addition to inhibition of DNA synthesis, it can also form free radicels which can combine and inactivate or denature protein, carbohydrate, or lipid molecules resulting in wide spread effects on cellular functions. It can also have damaging effects on cell membrane. Though adriamycin has different mechanisms of action, it does not however explain different effects seen in H69 and its derivative lines. Whether it is related to penetration, neuroendocrine differentiation or alterations in growth control due to activation of some oncogenes, may be investigated in future studies.

It has been shown previously that H69 cells treated with different phenotypic inducers reduced some of the neuroendocrine markers (e.g. DDC), but this change did not influence the sensitivity of the cells to cytotoxic agents [Murray, 1989], suggesting that modifying the neuroendocrine phenotype may not always be associated with a change in chemosensitivity of the cells *in vitro*. It may be that the sensitivity of the cells may only change with induction of terminal, and irreversible differentiation. Hence, changes in H69V & H69VZ may be due to a stable phenotypic shift, while phenotypic change in H69 following treatment with inducers was adaptive, since the effects were reversible,

following removal of the inducer from the cell culture. It would be interesting to investigate phenotypic changes following more prolonged treatment with inducers, to determine whether more permanent changes could occur and chemosensitivity is altered.

The NSCLC cell line, WIL, co-cultured with fetal fibroblasts showed some changes in culture morphology [Chapter four], but there was no significant effect on sensitivity of the WIL cells to adriamycin and/or vincristine, suggesting that stromal interaction may not be important for the sensitivity of tumour cells *in vitro*. Similarly when the NSCLC cell line A549 was induced to differentiate in a filter well system in stromal co-culture, significant changes were observed in cell phenotype following stromal interaction including increased secretion of surfactant, reduced PA production and increased anchorage dependence, but there was no effect on chemosensitivity [Speirs & Freshney, 1990]. Again it may be that the phenotypic changes were reversible with the cells regaining original tumour phenotype once out of the inductive microenvironment. Further studies are required to compare these findings in models with stable phenotypic changes as shown in H69 and its derivative lines; this may then answer whether permanent changes in cell phenotype can alter sensitivity to cytotoxic agents.

Sensitivity of mink lung epithelial cells transfected with Ha-ras oncogene showed significant resistance *in vitro* to cytosine arabinoside compared with the non-transfected cell line [Chapter five], suggesting a role of Ha-ras oncogene in drug resistance, at least in some cases. Similarly in an other study, activated Ha-ras transfected cells of mink lung epithelium showed significant resistance to adriamycin, compared to the untransfected cells [Kerr *et al.*, 1990], suggesting again that oncogene expression may be related to drug resistance. Mink lung epithelial cells transfected with H-ras, activated Ha-ras or c-myc oncogene did not have any significant effects on the sensitivity of transfectants to vincristine [Kerr *et al.*, 1989; 1990], or cis-platin [Chapter five], suggesting that the effects of oncogene transfection may be specific to certain drugs. Further studies could examine whether drug resistance is correlated with oncogene transfection.

The multidrug resistant phenotype (MDR) in human cancer cell lines has been associated with overexpression of a plasma membrane glycoprotein (P-glycoprotein) of approximately 170,000 dalton [Kartner *et al.*, 1983; Riordan & Ling, 1985; Gerlach *et al.*, 1986]. The level of P-glycoprotein expression correlates with the degree of drug resistance, in many cell lines increased expression is accompanied by MDR-gene amplification [Riordan *et al.*, 1985].

However, in other studies MDR was not correlated with P-glycoprotein overexpression [Mirski *et al.*, 1987]. These data have suggested that there may be factors other than P-glycoprotein which may account for the development of MDR-phenotype in lung cancer.

However, ara-C does not fall within the MDR group, and adriamycin and vincristine which changed their sensitivities in opposite directions, do. This suggests MDR was not the significant mechanism in these studies, although it has previously been shown to have increased expression in differentiated cells [Meyers, 1990; Biedler *et al.*, 1990].

Increased phosphorylation of a 20 kD protein has also been correlated with drug resistant phenotype in SCLC [Fine *et al.*, 1985]. A possible role of detoxification enzymes in drug resistance has also been suggested [Doroshow *et al.*, 1980; Cowan *et al.*, 1986]. Elevated glutathione-S-transferase enzyme has been found in resistant cell lines [Wolf *et al.*, 1986]. However, no significant difference were found in the levels of a number of detoxification enzymes including glutathione-S-transferase, between drug sensitive classic SCLC and drug resistant variant cell line *in vitro* [Carmichael *et al.*, 1988]. Drug resistance may also be related to efficient damage repair mechanisms in cells. It has been shown by Zijlstra [1987] that increasing intracellular drug concentration did not alter the sensitivity of adriamycin resistant human SCLC cell line $GLC_4$/ADR, suggesting that resistance may be multifactorial. These potential mechanisms of drug resistance could be investigated in H69 and the derivative cell lines.

The development of drug resistance has also been associated with specific chromosomal alterations such as homogeneously staining regions (HSRs) and double minute chromosomes [Robertson *et al.*, 1984]. A number of resistant lung cancer cell lines have increased expression of one or more oncogenes [Gazdar *et al.*, 1985; Johnson *et al.*, 1987]. The c-myc oncogene overexpression has been found in radiation and drug resistant variant cell line of SCLC *in vitro* [Gazdar *et al.*, 1985]. In preliminary studies using immunohistochemical staining techniques it has been found in these studies [Chapter three-A] that both the derivative lines H69V & H69VZ had increased expression of c-myc oncogene, compared to the parental line. An increased oncogene expression in the derivative lines may be responsible for resistance to radiation and some of the cytotoxic drugs. It may also be related to changes in growth rate and clonogenicity of the lines. However, there seems to be no direct causal relationship between c-myc oncogene expression to radiation or chemosensitivity,

either between classic and variant lines of SCLC or between SCLC and NSCLC.

Further studies are needed to investigate this in more detail, perhaps by oncogene transfection studies. It may be reasonable to assess radiation and chemosensitivity of different lung cancer cell lines with and without overexpression of an oncogene to answer whether oncogenes have a role in sensitivities of cells to therapy. Effects of oncogene transfection on phenotypic expressions, radiation and chemosensitivity of H69 and its derivative adherent cell lines is a potential model for future studies. Working on a similar approach it has been shown in this thesis [Chapter five] that transfection of immortalized mink lung epithelial cells with H-ras oncogene has increased resistance of the transfectants to cytosine arabinoside, and in an other study activated Ha-ras oncogene transfection in same cell line imparted resistance to adriamycin [Kerr *et al.*, 1990] and cis-platin [Sklar, 1988], suggesting that oncogene may have a role to play in drug resistant phenotype, and it may have clinical implications, as a significant proportion of human tumours have been found overexpressing oncogenes [Field & Spandidos, 1990]. Sklar [1988] has shown that Ha-ras transfection in NIH 3T3 cells was associated with radiation resistance. However, others have found no correlation between *in vitro* radiation sensitivity testing and clinical responsiveness to radiation [Deacon *et al.*, 1984; Malaisse *et al.*, 1986]. Radiation sensitivity of the oncogene transfected cell lines (M1, N1, & T1) was not investigated in present study, however, it remains an interesting area to investigate the effects of radiation on oncogene transfected lines relative to the untransfected parental line.

As more and more efforts are being put into various aspects of lung cancer research to elucidate biology of the disease, at the same time a number of potential therapeutic strategies are developing, taking advantage of these phenomena. The use of drug resistance modulators such as verapamil which overcome multidrug resistance associated with P-glycoprotein expression in some lung cancer cell lines *in vitro* is an attractive area of future research, however, it shows no success *in vivo*, mainly because the concentrations required are not achievable in patients [Rogan *et al.*, 1984]. Furthermore, there was no indication of MDR involvement in the present studies.

It has been suggested that differentiating agents may be more effective as an adjuvant therapy after debulking of the main tumour mass with surgery or radiotherapy, or in combination with cytoreductive drugs. Significant effects of retinoids [Lotan *et al.*, 1981], and NMF [Gescher & Gibson, 1982] have been

seen in small tumours grown in mice. Combination of cytotoxic drugs and cytostatic agents have also been suggested as a potentially useful therapy. Retinoids have been shown to exert additive antitumour effects in combination with tamoxifen [Wetherall & Taylor, 1986] and synergistic effects in combination with cytosine arabinoside [Findley *et al.*, 1985], or dexamethasone [Bregman *et al.*, 1983]. *In vivo* studies with experimental animals have shown that retinoids can enhance the antitumour effects of cyclophosphamide [Cohn & Carbone, 1972], or 5-fluorouracil [Tomita *et al.*, 1982]. Similarly a combination of retinoid with tamoxifen was effective against mammary cancer [McCormick & Moon, 1986]. It has been shown that cytotoxic effects of 5-fluorouracil were modulated by N-methylformamide on a human colon carcinoma cell line [Zupi *et al.*, 1988]. In the present studies [Chapter three-B] HMBA has shown significant cytostatic effects on growth of H69 cells both *in vitro* and *in vivo*. A combination of cytotoxic drug such as cis-platin with HMBA could be worth investigating in H69 cell line. These observations have suggested a future role of differentiation therapy in cancer treatment in conjunction with conventional cytotoxic chemotherapy. However, more studies are required to identify new agents with higher specific activity, more effective combinations, and to design appropriate experimental models for evaluation of these agents. Inducer agents which are effective *in vitro* and *in vivo* in experimental models could then be presented, with appropriate recommendations for scheduling with cytotoxic drugs, for clinical trials.

## 6.5 Model Of Cancer Cell Invasiveness *In Vitro*: Chick Heart Invasion Assay.

A large variety of heterologous model systems of *in vitro* invasion have been employed, using different natural substrates. These include: 1) Complete mono- or multilayers of normal cells e.g. endothelial cells [Nicolson, 1982; Yee & Shiu, 1986], fibroblasts [Benke *et al.*, 1984]. 2) Biological membranes such as chick chorioallantoic membrane (CAM) [Hart & Fidler, 1978; Poste *et al.*, 1980; Poste, 1982], human amniotic membrane [Liotta *et al.*, 1980] and reconstituted acellular membranes [Terranova *et al.*, 1986]. The biological membranes have often been used, because of their simpler structure, consisting of two layers of epithelium with an intervening layer of mesenchyme. Penetration and crossing of membrane by tumour cells constitutes invasion in this system. 3) Whole

organ pieces e.g. from aorta, veins, bladder and thyroid [De Ridder, 1979; Poste *et al.*, 1980]; fragments of organs from various tissues e.g. lung [Lohmann *et al.*, 1980; heart [Mareel *et al.*, 1979], cartilage [Pauli *et al.*, 1980] and brain [Wang *et al.*, 1983]. 4) A filter well system currently in use in this laboratory was described in Chapter four. In this system a synthetic membrane covered with a complete monolayer of normal cells is used as a barrier for invasive cells. Penetration of the normal cell layer by tumour cells, and recovery of tumour cells from the under surface of the filter membrane and the bottom of the well confirms invasion. Though potentially useful, the efficacy of this system is yet to be proven.

The measurement and investigation of tumour cell invasion *in vitro* has been the subject of many research studies as discussed above, all of them aiming to fulfil two main criteria, namely, the *in vitro* invasion assay must resemble as closely as possible the situation *in vivo*, and at the same time must be reproducible, quantifiable, and amenable to experimental manipulation. However, despite the plethora of *in vitro* model systems, few of them actually satisfy the above criteria.

In the present studies, a three dimensional histotypic *in vitro* invasion model [Mareel *et al.*, 1979] has been used to quantitate the ability of malignant/transformed cells to invade PHF. The assay is both qualitative and semi-quantitative. This, *in vitro* invasion model, mimics the natural situation more closely than other available systems, as no artificial substrates are involved and the geometry is closer to the *in vivo* situation. It has been demonstrated from the studies presented in this thesis that this three dimensional model system does provide a standard method to evaluate the invasive behaviour of transformed cells *in vitro*. The three-dimensional confrontations can be analyzed by the same methods as natural tumours. The test cells can readily be identified in the heart fragment on simple H & E staining because of their characteristic histological appearance in the background of chick fibroblasts and myoblasts. Immunohistochemistry can be used for identification of tumour cells in the heart fragment or heart tissue can be identified using specific chick mesodermal antibodies. Electronmicroscopy can also be performed for ultrastructural analysis of the invasion process [Mareel, 1982]. The assay can also be used for metabolic and biochemical investigations [Nicolson, 1982: Laug *et al.*, 1983; Starkey *et al.*, 1984], and to quantify radiolabelled cells in the heart fragment [Waller *et al.*, 1986]. Computer-assisted image analysis can be used for quantitative analysis of

invasion of tumour cells in the chick heart assay [De Neve *et al.*, 1985]. The staging of natural tumours can be performed for prognostic and therapeutic purposes, and the grading system used in this assay [Bracke *et al.*, 1984] may be useful for various other purposes such as search for oncogenes involved in invasion [Mareel & Van Roy, 1986; Van Roy *et al.*, 1986], and the development of anti-invasive means of cancer therapy [Mareel & De Mets, 1984]. The assay allows scoring and comparison of different variations of invasiveness between cells of different systems [Mareel *et al.*, 1987], and the possibility of manipulating the process of invasion [Bracke *et al.*, 1986]. The assay has a number of technical advantages over the other available invasion assays. It is reproducible, and consistent results have been obtained in the present and previous reports. It provides a three-dimensional tissue structure comparable to *in vivo* tumour-host relationship, but has an extra advantage that the host immune influences are no longer effective. The culture conditions can be monitored by morphological appearance and the beating property of the heart fragment.

Although, a good correlation between invasiveness *in vitro* and invasive behaviour of tumours *in vivo* has been demonstrated in this assay system in various studies [Mareel *et al.*, 1987; De Ridder & Calliauw, 1990], there are some basic differences between *in vitro* and *in vivo* invasion in natural tumours that are also found in this system. The assay does not model the pre-invasive (carcinoma in situ) state of tumour cells, and may be more relevant to secondary invasion rather than primary invasion *in vivo*. Some of the important components of invasion *in vivo* are missing e.g. functional blood vessels and lymphatics (intravasation/extravasation), and the basement membrane with epithelium. Cellular reactions (e.g. inflammation) are not present during invasion *in vitro*. The host tissue used *in vitro* assay is not ideal, as heart is rarely seen as a site of invasion. It may be worth trying in future studies to use normal lung tissue instead of heart tissue for the study of invasion in lung cancer models.

Different patterns of invasion, colonization, and degradation of normal heart tissue have been observed in the studies presented in this thesis. The SCLC cell line H69 was invasive in the present assay system [Chapter three-A]. The pattern of invasion was very interesting, the tumour cells invaded the heart fragment singly at several fronts, and were able to reach the centre of the heart fragment. This type of invasion is not what is generally reported in the standard grading system [Bracke *et al.*, 1984; Mareel *et al.*, 1987]. Solitary invasion may reflect early metastasis in SCLC *in vivo*, as single cells could invade and colonize

different organs in the body.

The derivative cell lines expressed NSCLC-like properties, and were non-invasive, compared to the parental SCLC cell line which expressed classic small cell properties and was invasive. This correlated well with *in vivo* invasion and metastatic studies [see below, section 6.6], suggesting that the model may be valid and reflect *in vivo* characteristics of invasion. This could have significant clinical implications, where the assay system could be used to predict tumour invasiveness and metastatic potential in SCLC. Furthermore, H69 with its sublines may be a useful *in vitro* model to study cellular properties associated with early metastatic spread in small cell lung cancer.

The invasive capacity of the H69 cells treated with HMBA or Na-But was significantly retarded [Chapter three-B]. This was in agreement with the other effects of the phenotypic inducers found in the present study. A decreased invasion was correlated with suppression of growth *in vitro*, a reduced colony forming efficiency in soft agar and reduced tumorigenicity in nude mice, following treatment with phenotypic modulators. It has been shown previously that invasion may be stopped by certain chemical agents such as catechin [Bracke *et al.*, 1986], suggesting that the *in vitro* chick heart invasion assay may be used to identify anti-invasive agents. The correlation between *in vitro* and *in vivo* activity of HMBA & Na-But in these studies further validates the assay in its capacity to identify anti-invasive activity. This may have significant clinical implications as the possibility of manipulating the process of invasion can be used as a potential mode of cancer treatment in future. More investigations could be carried out to screen chemical agents with anti-invasive activity using this assay, and with parallel *in vivo* studies, to determine the potential use of anti-invasive treatment in clinical situations, such as in tumours with high invasive potential e.g. SCLC.

The chick heart invasion assay was also employed to investigate the effects of human oncogene transfection on invasiveness *in vitro* of mink lung epithelial cells [Chapter five]. It was interesting to find that all the transfected cell lines were highly invasive, but, it was surprising to find that the parental non-transfected cell line was also invasive. Spontaneous acquisition of transformed phenotypic characteristics (tumorigenicity, invasion, metastasis) have also been observed previously in non-transfected 3T3 cells [Mareel et al., 1975; 1986] and also in various other cell lines [Van Roy et al., 1986; Mareel et al., 1988], in agreement with the present report. It was concluded from further studies of the parental line, that perhaps an invasive subpopulation of cells may exist in the

parental line, as it was also found to be mildly tumorigenic. The invasive cells could be retrieved from the heart fragment and characterised to establish whether all cells or only tumorigenic subpopulation of the parental line was invasive. The mechanism of spontaneous transformation in these cell lines could be investigated by genetic analysis for oncogenic sequences in cell DNA e.g. myc and ras overexpression or loss of suppressor genes and also by cell surface expressions e.g. N-CAM.

The molecular mechanisms of invasion *in vitro* models are being explored. Different steps of invasion in the chick heart assay have been described such as adhesion, motility, penetration, degradation and progressive replacement of host tissue by tumour cells in time and space [Mareel *et al.*, 1979; Mareel, 1983; Mandeville *et al.*, 1987]. Adhesion was found in H69 and its derivative lines, but only the H69 cells were invasive, suggesting that although adhesion is a step towards successful invasion, adhesiveness per se may not be sufficient to cause invasion, therefore, all adhesive cells may not be invasive, though all invasive cells must adhere with the host tissue first before invasion. H69 cells treated with the chemical inducers (HMBA, Na-But) showed reduced adhesion, and therefore delayed invasion, again suggesting that although adhesion is not a guarantee for invasiveness, yet it is important step for invasion to take place. It has been shown that homotypic adhesion is decreased in malignant cells, compared to their normal counterpart [Coman & Anderson, 1955: Coman, 1961]. Others have shown that both spontaneously transformed cells and cells transformed by viruses had greater heterotypic adhesiveness compared to the normal cells [Dorsey & Roth, 1973]. The present studies have shown that all invasive (H69, mink lung lines) or non-invasive (H68V & H69VZ) cell lines investigated did attach to the heart fragment, irrespective of their invasive and neoplastic potential, but invasion was found only in some of them, implying that adhesion may not be used as a criterion of invasiveness.

Penetration of normal host tissue by tumour cells is the next step for progressive invasion in this assay. It involves motility of invading cells [Strauli, 1979] and may be accompanied by destruction of host tissue, and its progressive replacement by the invading cells [Mareel, 1983]. In the present studies, the pattern of penetration, and progression of invasion differed considerably between the cell lines. In the H69 cells [Chapter three] invasion was focal or solitary where single cells were found in the heart fragment at different areas, usually embedded in, and surrounded by, apparently healthy normal host tissue in which

the histological structure of myoblasts was well preserved, and no traces of the path of infiltration could be detected. This behaviour was in sharp contrast to that of mink lung epithelial cell lines [Chapter five], both non-transfected (Mvl) and transfected with human oncogene (M1, N1, & T1). These lines showed aggressive behaviour, with rapid invasion of PHF, forming a continuous sheet of invading cells, and replacing the whole front of the invaded heart fragment. This was accompanied by rapid proliferation of invaders at the expense of normal heart tissue, resulting in disintegration of PHF, within 10 days after co-culture.

The precise mechanism of these differences in the pattern of invasion of malignant or transformed cells in heart fragment is yet to be investigated. One might suppose that different growth rates of cell lines may be responsible for their different invasive potential, and patterns. However, it has been shown that proliferation is not an absolute requirement for invasion to occur [Thorgeirsson, 1984]. Mandeville *et al.* [1987] have shown that the invasive capacity of the cells was independent of cell proliferation as determined by their relative mitotic index and invasive potential. In the present studies, it has been shown that though the derivative lines, H69V & H69VZ, had a more rapid growth rate, yet they were non-invasive, compared to the parental H69 cell line which had a slow growth rate *in vitro*, but was invasive [Chapter three-A], suggesting that the cell proliferation and invasion are not correlated. In agreement with above observations, it has been shown that the human oncogene transfected lines (M1, N1 & T1) were highly proliferative and invasive, but the parental line (Mvl) though it had a low mitotic index, was almost equally invasive [Chapter five]. These data are in agreement with previous studies suggesting that cell proliferation and invasion may be related but not interdependent.

It might be useful in future studies to investigate cell surface glycoprotein expression and correlate it with invasiveness, in H69 and its non-invasive sublines (H69V & H69VZ), and in the mink lung epithelial cell line (Mvl) and its transfected and non-transfected clones. This may give insight into the properties of the invasive phenotype and experiments could then be designed to target these properties specifically.

## 6.6 Model Of Cancer Cell Invasion And Metastasis *In Vivo*: Nude Mouse Assay.

Clinical and experimental studies of lung cancer have shown that SCLC has an early metastatic potential, whereas NSCLC may become metastatic, but during later stages [Matthews *et al.*, 1973; Hansen *et al.*, 1978]. The present data suggest that the H69 and its derivative cell lines may reflect clinical behaviour of lung cancer, and may be used as a valid model system for studying metastasis. A number of phenotypic changes *in vitro* have already suggested a NSCLC-like phenotype of both H69V & H69VZ. What makes an invasive and metastatic cell (H69) become non-invasive, and non-metastatic (H69V & H69VZ)? To answer this question further studies are required, first to establish and confirm the validity of the nude mouse model, and by growing tumours for relatively long periods. In the present study tumours were removed after approximately six weeks, and it may be possible that they could form metastases, if grown for a longer period, as NSCLC do metastasize, but in late stages. Common sites of metastases formation other than lungs and liver such as brain, and kidney could also be examined. Once established it would be interesting to manipulate this model, both genetically, and microenvironmentally in conjunction with the *in vitro* assay, to delineate the biological mechanisms responsible for these changes in cell behaviour.

The phenotypic inducers showed significant reduction in H69 tumour growth in nude mice [Chapter three-B], but whether they had an anti-metastatic effect was not determined, so it may be useful in future studies to see effects of inducers on metastasis of H69 in this model. It may be worth investigating the cell surface expressions of the parental and the derivative lines for glycoproteins involved in cellular adhesion and locomotion such as N-CAM and integrins. Also the expression or deletion of certain oncogenic sequences may be responsible for these phenotypic differences between the parental and the derivative lines. The cell lines may be screened for the differences in oncogene expression e.g. myc family, and cross-transfections attempted. In preliminary studies differences in c-myc expression in the parental and the derivative lines have been observed in this model, and a role of myc oncogene family in lung cancer has been reported in different studies [Little *et al.*, 1982; Carney *et al.*, 1985; Gazdar *et al.*, 1985; Field & Spandidos, 1990].

The studies of human oncogene transfected cell lines of mink lung origin have shown that they were non-metastatic, either to regional lymph nodes or

distant organs including brain, lungs, and liver, in spite of their invasive behaviour both *in vitro* and *in vivo* [Chapter five], suggesting that there is no apparent correlation between invasiveness and metastasis, or oncogenes and metastasis, at least in this model.

The results of transfection studies, on the role of oncogenes in metastasis are still controversial. The present study agrees with some of the previous results where oncogene transfection and invasiveness were not interrelated. Waller *et al.* [1986] have shown that a mouse lymphoma cell line was highly invasive *in vitro*, but had very low metastatic potential, suggesting that invasion and metastasis are two separate phenotypes. Thorgeirsson *et al.* [1985] showed that ras transfected cells were non-metastatic when implanted subcutaneously into the nude mouse, in agreement with the present results, but that the cells were able to form metastases when injected directly into the circulation, suggesting that the lack of invasive capacity at the primary tumour site may be important. However, this may not be the case in the present study, because the cells were invasive locally, suggesting that lack of metastatic growth was not due to the lack of invasiveness at the primary tumour site, in the present case. It has been suggested that though the ability of tumour cells to cross basement membrane *in vivo* may correlate with their metastatic ability, but all invasive tumour cells are not necessarily metastatic [Liotta *et al.*, 1986]. It has been proposed that invasion and metastasis may be two distinct phenotypes in tumour progression [Mareel & Van Roy, 1986]. Others have suggested that tumour cell invasion and metastasis may be under separate genetic control mechanisms [Mareel *et al.*, 1988]. It has been reported that rapid growth in tumours may also be responsible for a lack of metastasis in experimental tumours [Mareel *et al.* [1987], suggesting that it may be the case in the present study, as all the transfected cell lines were highly tumorigenic with rapid growth rate. H69 tumours grew relatively slower than the tumours of mink lung lines and were able to metastasize, but under the similar conditions, tumours of both the derivative lines were non-metastatic, making it more difficult to interpret the results.

In some reports it has been cautioned that failure of metastatic growth in nude mice model could be due to activity of natural killer (NK) cells [Fidler, 1986], and others have proposed that oncogene transfection may stimulate cellular sensitivity to NK-cell cytolysis [Johnson *et al.*, 1985; 1987]. However, it is not definite whether NK-cells are active in nude mice. Naito *et al.* [1987] have shown, using NK-cell deficient beige nude mice model, that there was no

difference in metastatic behaviour of tumours grown either in nude mice or in beige nude mice, suggesting that NK-cells apparently had no effects on metastatic behaviour of tumours. In agreement with these observations, in the present study, H69 cells were metastatic in the same nude mice model under similar experimental conditions, where mink lines or the derivative lines of H69 were non-metastatic, suggesting that NK-cells may not be the cause of non-metastatic behaviour, rather it may be a cell specific property. H69 was metastatic, while its NSCLC-like cell lines and the parental and human oncogene transfected mink lung cell lines were non-metastatic, suggesting that there may be some common phenotypic characteristics in all non-metastatic lines, different from the metastatic H69 cell line. Whether, metastatic phenotype is under specific genetic control remains to be investigated.

The apparent differences between metastatic and invasive phenotypes are consistent with previous observations, suggesting that the two are separate, but, closely related phenotypes. The differences between metastatic and non-metastatic cells should be investigated in future studies, as suggested above, at both cell surface and genetic level, and this may have significant clinical implications in the future. Therefore, it is concluded from these observations that the nude mouse model is a suitable model for the study of invasion and metastatic behaviour of human lung cancer *in vivo*.

## 6.7 Clinical Significance Of Myc And Ras Oncogenes Expression In Human Lung Cancer.

The majority of previous investigations have been carried out on SCLC [Little *et al.*, 1983; Nau *et al.*, 1986; Johnson *et al.*, 1987]. It has been shown that expression of L-myc and c-myc is coordinately regulated [Alt *et al.*, 1986; Watanabe *et al.*, 1988], and may be related to the biological behaviour of the lung cancer. A panel of SCLC cell lines has been established from treated and untreated patients, and investigated for c-myc oncogene amplification [Johnson *et al.*, 1987]. C-myc amplification was seen only in cell lines established from treated cases, and the patients with c-myc amplification have significantly shorter survival times, compared to the patients without c-myc amplification. More recently [Field & Spandidos, 1990], it has been observed that c-myc is overexpressed in various NSCLC specimens, by immunohistochemical staining

with c-myc specific monoclonal antibody (1-9E10). Elevated c-myc expression was found in 43% (16/37) squamous carcinomas, 29% (4/10) adenocarcinomas, 42% (3/7) large cell carcinomas, and 19% (4/21) in SCLC. In the present studies, the c-myc overexpression in H69V & H69VZ was in agreement with these observations, suggesting that c-myc may be involved in tumour progression from SCLC to NSCLC-like phenotype, and hence with altered response to therapy.

Amplification and overexpression of the c-myc oncogene has been reported in NSCLC-like morphological variant SCLC cell lines while L-myc was overexpressed in classic SCLC cell line [Gazdar *et al.*, 1985]. Similarly, Johnson *et al.* [1986] have demonstrated that transfection of the c-myc oncogene into a classic SCLC cell line resulted in altered morphology and growth of SCLC towards a variant phenotype, suggesting a role of c-myc in phenotypic conversions in SCLC towards NSCLC-like phenotype. In the present study, it has been shown that the derivative lines stained more uniformly for the c-myc oncogene expression, compared to the parental line, again suggesting that phenotypic alterations in the derivative lines may be due to overexpression of c-myc oncogene.

It has been shown in the studies presented in this thesis [Chapter five] that the transfection of an immortalized mink lung epithelial cell line MvlLu with human c-myc oncogene increased growth and tumorigenic potential in the transfected cells (M1 cell line), suggesting that enhanced growth stimulation by c-myc oncogene overexpression may contribute to abnormal growth regulation leading to neoplastic transformation. It has been proposed that the regulation of c-myc oncogene is involved in the control of proliferation and differentiation of cells [Klein & Klein, 1985], and the overexpression of c-myc in H69V and H69VZ may be related to the differentiated, NSCLC-like phenotype, suggesting that the modulation in myc gene expression may be the underlying cause of phenotypic shifts in human lung cancer. Therefore, the c-myc overexpression may affect the histopathological diagnosis, and therapy of lung cancer. It would be interesting to investigate the expression of L-myc and c-myc in both the derivative and the parental cell lines, and the effects of transfection of L-myc gene into the H69V and H69VZ, and c-myc into SCLC H69. It may be possible that H69 would change to H69V & H69VZ-like phenotype, while the derivative lines may revert to parental cells.

Kiefer *et al.* [1987] have investigated 12 SCLC cell lines for myc

and ras family oncogenes. The c-myc amplification was found in 7/12, N-myc amplification in 3/12, and both c-myc and N-myc amplification in 1/12 cell lines. All lines has similar expression of Ha-ras, K-ras, and N-ras, there was no overexpression of these genes, suggesting that myc rather than ras overexpression may be more crucial in SCLC. Co-expression of c-myc amplification and Ka-ras point mutation has been found in a large giant cell lung carcinoma [Taya *et al.*, 1984], suggesting that oncogene co-operation may be essential for this type of tumour phenotype. Ka-ras mutation and overexpression has been demonstrated in NSCLC. Rodenhuis *et al.* [1988] have shown that 9/35 adenocarcinomas of lung had K-ras mutation, and were able to establish a correlation between smoking and Ka-ras mutation in these cases, suggesting that mutational events in Ka-ras may be related to the carcinogenic agents in tobacco smoke. In another study ras p21 overexpression was found in the majority of squamous cell carcinoma (9/12) compared to 1 out of 12 non-squamous cell carcinomas [Kurzrock *et al.*, 1986], suggesting that ras oncogene overexpression may be involved in the evolution of squamous cell carcinoma.

Overlap in features of different main lung cancer subtypes have been well documented in previous [Gazdar *et al.*, 1987; Bepler *et al.*, 1987; Watanabe *et al.*, 1988] and in the present studies [Chapter three-A], suggesting that the tumours in transitional stages may coexpress features of more than one histological cell types. The c-myc overexpression was observed both in H69 and its derivative lines, though the expression in latter was quantitatively more than the former, suggesting that H69V & H69VZ may represent transient phenotype between SCLC and NSCLC, however, ras expression has not been investigated in these lines in the present study. It may be interesting to screen these lines for all members of the myc and ras oncogene families, to establish whether any correlation could be made, between oncogene overexpression and phenotypic shifts, and resistance to therapy. Transfection of mink lung epithelial cell line with human normal Ha-ras and activated Ha-ras oncogenes, individually, induced malignancy related properties, in agreement with the previous observations. The transfectants showed increased growth *in vitro*, anchorage independence, and tumorigenicity in nude mice [Chapter five], suggesting that ras oncogene activation may be important in lung cancer. The fact that the effects of activated ras were more pronounced compared to normal ras suggested that activation of ras oncogene may be more crucial in malignant progression. However, although these preliminary observations have suggested a role of myc and ras oncogenes

in lung cancer, no clear pattern has yet emerged. The c-myc oncogene family has a role in the genesis or progression of SCLC, but there is no general consensus on which member is important. A significant involvement of the ras gene family has also been implicated in lung cancer. It may well be that ras oncogene activation is related to the lung cancer development and progression. Clearly, more studies are required to establish the role of oncogenes in lung cancer, by correlating their overexpression with phenotypic changes, both in clinical specimens and in lung cancer cell line. Once established it may have significant implication in human lung cancer in future. Oncogenes may be used as molecular probes for diagnostic and eventually, prognostic purposes. Oncogenes and their products may prove ideal candidates for novel therapeutic targets selective against cancer cells, and tumour progression may be prevented by antagonizing their functions. Effects of oncogene transfection in SCLC and in NSCLC may provide an opportunity to correlate oncogene overexpression and phenotypic changes in future studies.

### 6.8 Model Of Carcinogenesis *In Vitro*: Mink Lung Epithelium.

Developing a model of truly equivalent cultures of normal and malignant cells from the same lineage is often difficult [DiPaolo *et al.*, 1983] and *in vitro* transformation has often been used as an alternative. Since the discovery that transfection with oncogenes can induce a malignant change in some cell lines, many investigators have tried to develop *in vitro* model systems of normal and transformed cells from the same lineage. In the present study [Chapter five], successful transfection of a normal (immortalized) mink lung epithelial cell line MvlLu with human c-myc, Ha-ras and activated Ha-ras oncogene, individually, presented the possibility of investigating transformed lung epithelium and comparing it with a normal (immotalised) equivalent.

Transfection with each oncogene increased growth both *in vitro* and *in vivo*. A progressive rise in expression of malignancy associated phenotypes was observed, where c-myc exhibited slight differences from the parental line, while transfection with Ha-ras and activated Ha-ras produced more pronounced differences from the parental line, and was found generally more aggressive. These data in agreement with previous studies [Yoakum *et al.*, 1985; Spandidos & Anderson, 1987; Barbacid, 1986], suggest that c-myc and Ha-ras human

oncogenes can induce malignancy associated properties in the transfected cells, implying their possible role in human lung carcinogenesis.

Whether these differences represent intrinsic differences in transfection capacity or a lower frequency of transfection to lower copy number of c-myc is not clear from these results. It may be possible that during expansion of the M1 clone, some other mutational events took place and caused the alterations in growth characteristics. However, there is no evidence of this and staining with the anti-c-myc antibody did demonstrate a significant difference from the parental line. The effects of normal Ha-ras transfection were, in most cases, similar to transfection with activated Ha-ras, but, where the differences were apparent, activated Ha-ras was always the more aggressive phenotype. This is more likely to be an intrinsic difference in the mutated ras gene as the transfected normal ras gene appeared to be present at a higher copy number. The c-myc immunostaining was patchy, in agreement with the possibility that the gene is not uniformly distributed in the population, while the ras immunostaining was uniformly distributed to the plasma membranes.

Though the overexpression of endogenous genes cannot be ruled out, it seems more likely that this is exogenous gene product, particularly in view of minor, but distinct differences between the normal and mutated Ha-ras transfection. Furthermore, no staining was observed for either ras or myc gene products in the untransfected controls, suggesting some degree of species specificity in the antibodies used. The antibody to c-myc protein, p62 (9E10) has been shown to be human specific in previous studies [Evan *et al.*, 1985], so interaction with endogenous mink p62 is unlikely, in agreement with negative staining of the untransfected controls. However, it seems likely from the southern blot results that only a proportion of the M1 cells actually contains the exogenous myc gene. Hence the phenotypic effect may have become less than would be expected from more uniform distribution of the transfected genes.

It may be argued that the calcium phosphate transfection technique [Graham & Van der Eb., 1973] itself may contribute in the transformation of cells by carrier DNA, but this possibility was ruled out by using appropriate controls. Clones of the parental cell line, MvlLu, selected by G418 selection and although not characterized extensively, showed similar growth factor response, and morphological properties, suggesting that the altered phenotypic expressions in the transfected lines were not coincidental. Also the parental cells transfected with plasmid containing aph gene, but without oncogene, showed no sign of

transformation, suggesting that the phenotypic transformations in the oncogene transfected clones were due to the oncogenes and not the plasmids, or carrier DNA in transfection technique.

Although the oncogene transfection produced malignancy changes in the transfectants, the parental line itself was found mildly transformed, as it was invasive in chick heart invasion assay and tumorigenic (albeit at a very low level) in athymic nude mice. It would have been useful to include at least one randomly selected clone of the parental line in order to determine whether the altered growth properties of the clone are due to the selection *in vitro* from heterogeneous cell population rather than oncogene transfection. However, it can be justified in presence of other controls (i.e. presence of oncogene in the transfected cells and its absence in the parental non-transfected cells by southern blot analysis, gene expression in the transfected lines as detected by immunocytochemical techniques, no effects of transfection without plasmid or aph gene on growth or morphology of transfectants, and significant phenotypic changes seen in the transfected cells, compared to the non-transfected parental line) that the altered phenotypic effects in the transfectants were due to the transfected oncogenes, though it can not be ruled out that the parental line was heterogeneous, perhaps containing a subpopulation of transformed cells. Therefore, the validity of the parental line as a model of normal epithelium may be controversial, but this does not invalidate the effects of oncogene transfection investigated in the present studies.

Though, the parental cell line was immortalized, it maintained the growth control and physiological properties of normal epithelial cells *in vitro*. It maintained relatively flat morphology in culture suitable for transfection. It exhibited the same stringent requirements of serum factors for growth in culture as primary epithelial cells. The cell line did not spontaneously produce transformed foci at a detectable frequency, and did not produce tumours in animals at a significant frequency. The parental line was density limited in culture, and anchorage dependent for growth *in vitro*. It showed normal response to growth factor stimuli *in vitro*. The cell line has been used in a number of transfection studies, because of its relatively flat morphology, and many of the properties of normal cells as described in previous studies [Henderson *et al.*, 1974; Barbacid *et al.*, 1978; Evans & Cloyd, 1984; Kerr *et al.*, 1990].

It would have been ideal to use normal lung epithelial cells for

transfection studies. However, as mentioned earlier, such attempts have not been successful in previous studies [DiPaolo, 1983] and most of the transfection studies have been performed on non-epithelial cells, such as NIH 3T3 and other fibroblast cell lines [Spandidos & Wilkie, 1984; Barbacid, 1985; 1987; Spandidos, 1985; Kelekar *et al.*, 1986; Wyllie *et al.*, 1987]. The mechanism of this resistance in human cells, particularly of epithelial origin, is not clear. However, it has been proposed that host factors such as chromosomal stability [Harris, 1987], suppressor genes [Anderson & Spandidos, 1988; Spandidos & Anderson, 1989], and efficient DNA repair system [Topal, 1988] may be responsible.

A more appropriate, *in vitro* model of normal human bronchial epithelial (NHBE) cells has been developed by Harris *et al.* [1985]. NHBE cells have been transfected by v-Ha-ras oncogene by protoplast fusion [Yoakum, 1984] and selected for loss of differentiation control in presence of serum, based on hypothesis that malignant cells have defects in their differentiation pathways, and that they can grow in conditions that induce terminal differentiation of the normal parent epithelial cells. It has been shown that v-Ha-ras transfected NHBE cells expressed malignancy related properties such an immortalization, aneuploidy, and tumorigenicity in nude mice, suggesting that v-Ha-ras oncogene may be involved in multistep carcinogenesis [Harris *et al.*, 1985; Yoakum *et al.*, 1985]. However, it has not been reported which cell types of the normal bronchial epithelium have been selected in culture or used in transfection studies. Terminally differentiated cells do not divide, and are incapable of self renewal. Stem cells (basal cells) might have been selected in these studies, but the possibilities of other cells being selected has not been ruled out. As it has been shown that basal, intermediate and mucus cells are capable of proliferation and self renewal [McDowell *et al.*, 1978]. It would be interesting to determine whether different lung cancer phenotypes can be induced in the basal cells, using different carcinogenic agents and whether basal cells can be induced to differentiate to various cells of normal respiratory epithelium.

This may prove an ideal model for the study of differentiation and carcinogenesis in human lung epithelial cells. Preliminary studies have shown that Ha-ras p21 blocks differentiation in NHBE cells [Yoakum *et al.*, 1985], possibly by regulating adenyl cyclase activity [Gilman, 1984; Toda *et al.*, 1985; Manne *et al.*, 1985]. It has also been shown that transfected NHBE cells express a decrease sensitivity to differentiation inducing stimuli, such as serum, TPA, and TBF-$\beta$ [Yoakum *et al.*, 1985; Masui *et al.*, 1986], suggesting an inverse

relationship between differentiation and malignancy. These studies suggest that Ha-ras p21 induced transformation can be linked to GTP-binding protein pathways, where p21 may have a role to deregulate the differentiation pathways. It would be interesting to investigate the effects of human myc and ras oncogene transfection on NHBE cells phenotype, and how this may be reversed by using appropriate inducer of differentiation, such as HMBA, Na-But, cyclic AMP etc. These studies would delineate the mechanisms of normal differentiation control, and how these mechanisms become aberrant in carcinoma cells. The role of oncogenes in human lung epithelial carcinogenesis, and reversal by different induction methods may have significant clinical implications, both in diagnosis and therapy of lung cancer in future.

## 6.9 Further Experiments And Future Prospects.

**1).** Adherent lines of H69 clones could be established and characterized *in vitro* and *in vivo* for growth, neuroendocrine phenotype, cytogenetic analysis for chromosomal markers, ploidy by flow cytometry, activation of oncogenes or loss of suppressor genes, invasion and metastasis. The results could be compared with the H69 and its derivative lines in the present study, to determine whether the adherent lines represent mono- or polyclonal origin.

**2).** H69 and its derivative lines (H69V & H69VZ) may be used as a model of phenotypic changes in lung cancer. These lines could be further characterized for karyotypic analyses to see any differences between the parental and the derivative lines. The lines could also be screened for oncogene expression such as c-myc and Ha-ras. Transfection of lines with these oncogenes may be interesting, as there is some evidence that different members of the myc family may be associated with phenotypic conversions in lung cancer; SCLC overexpress L-myc, while variant lines with NSCLC-like phenotype overexpress c-myc. Therefore, transfection of H69 with c-myc and transfection of the the derivative lines, H69V & H69VZ, with L-myc may change the phenotypic expression of the transfectants, as reflected by altered morphology, neuroendocrine marker expression, resistance to radiation and cytotoxic drugs. These studies may have significant clinical implication, in both diagnosis, and therapy of lung cancer.

**3).** Phenotypic conversion in human SCLC cell line H69 was due to a

common stem cell subpopulation in the parental line, however, it is not known whether transformation of one malignant cell phenotype into another malignant cell phenotype is possible. Cloning a homogeneous cell population such as H69V or H69VZ may provide a good model to answer this question. Heterogeneity, or appearance of cells with H69 phenotype in the clones of the derivative lines would confirm whether inter-conversions between different lung cancer types is reversible i.e. can NSCLC clones give rise to SCLC phenotype?

**4).** Cytostatic effects of phenotypic inducers have been observed on H69 cells both *in vitro* and *in vivo*, in the present study, this study may be followed further to establish whether anti-tumour activity of HMBA or Na-But could inhibits metastatic growth in H69 xenografts. This may have significant therapeutic implications in SCLC. The cytostatic effects of inducers were reversible *in vitro*, but apparently the effects were not reversible within 16 days *in vivo*. This needs further experiments to see reversibility *in vivo* after a prolonged recovery period, following induction therapy of the tumour bearing animal. As the cloning efficiency of H69 in soft agar was significantly reduced, by induction treatment, it would be interesting to determine whether pre-treatment of H69 cells with inducers, in culture, before inoculation, shows a correlation between clonogenicity in soft agar and tumorigenicity in nude mice.

**5).** As it has been shown in previous studies that short term (72 hours) induction of H69 cells with the inducers did not significantly alter chemosensitivity, it may be worth trying a combination of cytostatic and cytotoxic agents. Alternatively, the H69 cells may be induced for 7 days before cytotoxic drug exposure, because maximum cytostatic effects have been observed after prolonged treatment with these inducers in the present studies, and preliminary data from this laboratory and others [Zupi *et al.*, 1988] suggest that sensitivity to some drugs may be enhanced by prior exposure to differentiating agents. This may have significant clinical implications, the cytostatic agent may turn a heterogeneous cell population into a homogeneous one, and the use of a specific cytotoxic agent may be able to eradicate the tumour cells altogether.

**6).** The histotypic tissue culture model described in Chapter four may be useful to establish the differentiated growth of normal respiratory epithelial cells in culture for the study of differentiation and carcinogenesis. It may also provide with the opportunity to carry out the comparative studies of normal and transformed cells, and to investigate whether microenvironmental changes could induce similar phenotypic expression in normal and transformed cells. This may

have both biological and clinical significance. There was only mild effect of histotypic culture on WIL cells, but this model of histotypic culture may prove useful in malignant cells from other systems e.g. thyroid, breast, and gut etc.

**7).** It has been shown that the parental mink lung epithelial cell line Mvl was mildly transformed, as it formed colonies in agar (0.1%), tumours in nude mice (20%), and showed spontaneous invasion in chick heart fragment, suggesting heterogeneity in the parental line. It would be interesting to isolate the clonogenic cells from clones in agar, invasive cells from the invaded heart fragments and tumorigenic cells from xenografts in nude mice, and compare these with Mvl cell clones, to determine whether all cells or only one or perhaps more than one cell subpopulations exist in the parental line with different transformed phenotypes. The clones and other lines (clonogenic, invasive & tumorigenic) would provide the opportunity to characterize these cell lines, originating from one parental line, for a wide range of phenotypic properties. Heterogeneity in clones would indicate the stem cell nature of the parental line, as has been shown in H69 cell line.

**8).** Transfection studies could be performed using normal human bronchial epithelial model as established by Harris and coworkers [Harris *et al.*, 1985; Yoakum *et al.*, 1985]. Transfection of normal human bronchial epithelium with human oncogenes, and induction of differentiation in the transformed cells could be the ideal model for the study of differentiation and carcinogenesis in human lung.

**9).** Future prospects of lung cancer treatment will be improved by using probes for specific DNA sequences that would help early recognition of lesions at the molecular level, in limited disease. As malignant phenotypic properties are due to alterations in gene expression, these genes and their protein products could be eradicated or reversed or blocked by differentiation inducers. Manifest disease with complete expression of the malignant phenotype might require synergistic cytoreductive therapy, consisting of cytotoxic and differentiation chemotherapy.

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