ABSTRACT

Recent research has indicated that growth factors play an important role in normal and malignant cell growth. Previous work in this laboratory established that RPMI-2650, a cell line derived from a human carcinoma, produces growth factors including TGF-α, TGF-β and an autocrine factor. The purpose of this thesis was to investigate whether growth factor production is a property of all cells, or of a sub-population of cells. If high-producer clones were available it would greatly facilitate production of material for purification and characterisation.

In order to answer this question it was necessary to isolate clones of RPMI-2650. A number of experimental variables were examined, with the objective of improving cloning efficiency. Initially, basic parameters related to the environment of the cells were examined : The use of a basal medium consisting of DMEM:HamsF12 (1:1) gave a higher cloning efficiency then MEM or DME. Choice of a suitable batch of serum was very important, and serum concentration was important in determining the size of the colonies. It was also found that trypsinizing cells at 4^{o}C gave better cloning efficiencies than trypsinization at 37^{o}C. On the other hand, use of gas atmosphere with reduced oxygen tension caused a decrease in cloning efficiency.

Feeder cells proved to be of considerable significance. The use of mitomycin C-treated feeder cells consistently improved the cloning efficiency of RPMI-2650 cells; Mouse 3T3 or human RPMI-2650 cells were both effective as feeders. Use of RPMI-2650 conditioned medium and pre-coating of culture dishes with DEAE dextran gave a marginal improvement in cloning efficiency.

Transfer of cells from the initial clone to a larger growth area proved difficult. This problem was largely overcome by a procedure involving transfer of cells to a small volume of medium (in 96-well microplates) following trypsinization using a cloning ring.

Using this improved procedure, twenty independent clones of RPMI-2650 were isolated and frozen in liquid nitrogen. Many of the clones appeared morphologically distinct from one another. Fourteen of the clones were examined for cloning efficiency in monolayer and agar, and for production of TGF-α, TGF-β and autocrine factors. Considerable variation between clones was observed in all of these properties. All clones examined, however, produced significant amounts of growth factors. It was concluded that growth factor production is not a property confined to a small sub-population of RPMI-2650 cells in culture.

The techniques developed in this thesis should facilitate future work in preparing clonal populations of human cancer cells. The finding that TGF-α and TGF-β production is a general property of the carcinoma cells studied, rather than a property of a sub-population, suggests that production of these growth factors may be related in some way to the process of carcinogenesis.

Clonal variation in Growth Factor Production by Human Carcinoma Cells

by

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SECTION 1

INTRODUCTION

1. INTRODUCTION

The work in this thesis concentrates on clonal variation in the human nasal carcinoma line RPMI 2650.

Previous work in this laboratory has shown that this cell line produces TGF-α, TGF-β and an autostimulator (McManus and Clynes, 1984; Dooley and Clynes, 1986). A major goal of the work described here was to develop a system for isolating clones and testing both cells and conditioned medium in various assays in order to investigate clonal variations related to these growth factors. Thioguanine resistant variants of RPMI 2650 were also isolated as another variant cell type.

Having developed these methods, a number of parameters were investigated for clonal variation. These are parameters which may be related to the TGF system and include morphology, colony forming efficiency in monolayer and soft agar, TGF-α, TGF-β and RPMI autostimulator production.

Colony forming efficiency in agar may show variation. Todaro et al. (1980), originally reported that human tumour cell lines that clone well in soft agar, released greater quantities of TGFs then did normal cells or tumour cells that grow poorly in agar. Hamburger et al. (1985) examined the hypothesis that these tumour cells that grow well in soft agar are more efficient producers of transforming peptides than those that grow poorly in soft agar. They examined primary human tumour cells both for their ability to clone in soft agar and to produce diffusible factors that induce anchorage independent growth of NRK cells. Todaro et al., 1980, suggested that the ability of primary human tumour cells to clone in soft agar was not related to their ability to produce NRK colony stimulating factors.

TGF-α and TGF-β production from each clone was evaluated, since the production observed in bulk cultures may be due to uniform production by all cells or a mixture of high and low TGF producers.

In view of the current widespread interest in TGFs, it is surprising that work on clonal variation of TGF production has not been reported.

Investigation of clonal variation in TGF production in RPMI 2650 carcinoma cells represented two potential challenges :

a) much previous cloning work has been done with long established cell lines well adapted to in vitro conditions, whereas RPMI 2650 cells are a low passage quasi-diploid line and so perhaps more representative of human tumours in vivo; but also more difficult to clone.

b) since no previous work has been reported on clonal variation of TGF production, the possibility existed that information relevant to cancer and growth factor biology might emerge from these studies.

The term 'autocrine secretion' is used to describe self-stimulation whereby a cell secretes a hormone-like substance for which the cell itself has functional external receptors (Sporn and Todaro, 1980). This model may explain the fact that malignant cells have a lesser requirement for exogenous growth factors. Transforming growth factors (TGFs) have been isolated from the conditioned medium of cultures of a variety of human tumour cell lines, including a bronchogenic carcinoma, a rhabdomyosarcoma and a melanoma cell line (Todaro, Fryling, & DeLarco; 1980).

TGFs can be defined operationally by their ability to stimulate the anchorage independent growth in soft agar of cells which are otherwise anchorage-dependent (Todaro et al., 1980). TGF-α and TGF-β are two very different molecular entities. TGF-α alone in serum-containing medium only weakly stimulates soft agar colony formation (Anzano et al., 1983).

TGF-α competes for binding to the EGF receptor (Massague et al. 1984; Todaro et al. 1980) and thus far its biological activity in vitro appears to be virtually identical to that of EGF (Moses et al., 1986). TGF-β on the other hand, stimulates the growth in soft agar of AKR-2B (Moses et al., 1981) and NRK cells (Roberts et al., 1981).

Although TGF-β was active in the soft agar assay on AKR-2B (Clone 84A) cells alone, the soft agar response of NRK (Clone 49F) cells to TGF-β required the presence of EGF (Roberts et al., 1981). TGF-β does not bind to the EGF receptor. The biological effects of TGF-β depend on the indicator system used. Thus far TGF-β appears to stimulate mesenchymal cells and to inhibit normal and neoplastic epithelial cells. No epithelial cell line has been demonstrated to be stimulated by TGF-β thus far (Moses et al., 1985). The method used for detection of TGFs in this report uses normal rat kidney (NRK) and NRK clone 49F as indicator cells.

In the remainder of this introduction, previous work done on cloning (including methods and applications) is reviewed.

Whether a tumour develops from one cell or many and how changes in the biological characteristics of a neoplastic population occur over time, are questions of theoretical and practical interest (Nowell, 1976). A concept gaining increasing acceptance is that

neoplasms frequently develop as a clone from a single cell (Nowell, 1976). "Clone" as used throughout this text, simply implies a population of cells descendant from a single cell Most neoplasms have a unicellular origin and clonal growth pattern.

This implication that tumours originate from a single cell is not intended to deny that carcinogens can simultaneously affect many cells in a tissue. It does suggest, however, that even though a large number of cells may be affected by a carcinogen, the macroscopic tumour that ultimately develops usually represents the progeny of a single cell or at most, very few cells. Presumably other neoplastic or pre-neoplastic cells in the exposed tissue never successfully proliferate or they are destroyed before progressing to a fully developed tumour (Teebor and Becker, 1971). For the vast majority of neoplasms, both cytogenetic evidence and biochemical evidence supports the validity of this unicellular concept (Linder and Gartler, 1965; Sandberg and Hossfeld, 1970 and Failkow, 1974).

The cells of a given neoplasm, derived from a single progenitor, should therefore, share certain common characteristics. Perhaps most likely would be a common membrane-related metabolic alteration or antigen acquired at the time of the initial neoplastic change. One could hope in theory to eradicate the clone and effect a cure through exploitation of this knowledge.

Only in the relatively few tumours that appear multicentric reflecting either an inherited gene defect in all of the patients cells or perhaps infection of adjacent cells by an oncogenic virus, might recurrence be likely after elimination of the original clone

(Linder and Gartler, 1965; Failkow, 1974 and Friedman and Failkow, 1976).

Although most tumours are thought to arise from single cells, a relatively high degree of heterogeneity may be found in cells from the same tumour (Fidler et al., 1978). The heterogeneity occurs in such diverse traits as antigenic makeup, hormone sensitivity, karyotype, tumour histology, growth characteristics in culture, transplantability and drug sensitivity. Particular attention has focused on heterogeneity of the capacity to metastasize because this property is thought to be the hallmark of malignancy. Cells arising within a clonal metastasis may differ from one another in metastatic capacity, particularly those cells isolated from long standing metastases (Fidler et al., 1978). The possibility exists that neoplasms are heterogeneous and contain subpopulations of cells with differing metastatic capabilities (Fidler et al., 1978). They suggest that a neoplasm should no longer be considered a uniform entity and efforts to design effective therapeutic agents should be directed toward the few but fatal, metastatic subpopulations of cells.

The question of whether these unique metastatic cells pre-exist in the tumour population or whether they arise during metastasis by a process of adaptation to local environmental conditions remains unanswered. Fidler attempted to answer this question with a series of experiments. In one experiment, a cell suspension of the B16 melanoma parent line was divided into two parts. One portion was used for intravenous injection into syngeneic C57BL/6 mice. The other portion was used to produce seventeen clones which were then also injected intravenously into groups of C57BL/6 mice. The number of lung metastases in each recipient was counted, eighteen

days after the tumour cells were injected. The cloned sublines gave rise to widely different numbers of lung colonies, suggesting that the parent tumour was heterogeneous and that cells of both high and low metastatic potential pre-existed in the parent population. This clearly demonstrated that the B16 melanoma was heterogeneous. This was not surprising considering that the tumour arose in 1954 and was transplanted for approximately ten times the life span of a mouse. Repeated passage, both in animals and in cell culture provided ample opportunity for variant cell types to arise. However, with a tumour of more recent origin, similar results were found (Kripke et al., 1978).

In order to understand carcinogenesis and tumour progression more completely, the heterogeneity so characteristic of this system must be studied (Dexter, 1978).

The existence of sub-populations of tumour cells differing in sensitivity to therapeutic agents, within a single neoplasm presents a challenge to development of assays capable of predicting drug response and to the selection of combination therapies (Heppner et al., 1978). Further exploration is needed on the consequences of therapeutic heterogeneity to the clinical management of neoplastic disease. It may be that sub-populations in a heterogeneous neoplasm interact in such a way as to make the response of the entire tumour unpredictable, even if the responses of the isolated clones are known (Leith et al., 1982). The possession of a series of different sub-populations of a tumour allows the search for a correlation between a particular biological characteristic and a graded series of invasive and metastatic behaviours (Sweeney et al., 1982).

An important area of research with direct clinical significance is the ability to detect, characterise and manipulate sub-populations of tumour cells. Cloned cell lines provide a useful research tool for the investigation of tumour biology and for the trial of new or established anti-cancer agents. It may be possible to use these cell lines to search for characteristics which correlate with the different levels of tumorigenicity.

The first attempts to clone cells were made around 1948 by Sanford, Earle and Likely and were described in a paper 'The Growth in vitro of Single Isolated Tissue Cells'. They wrote that a major obstacle found in tissue culture was the inability or difficulty in growing a single isolated tissue cell into a clone or pure culture. Even cultures growing in vitro for many years do not carry assurance of purity of cell strain, even though they appear to be homogenous. Most cultures have originated from a mixed population of cells in a tissue explant (Sanford et al., 1948).
Puck and Marcus in 1955 reported on clone production with He La cells, with the use of X-irradiated cells to supply conditioning factors. They wrote "studies of many aspects of the genetics and metabolism of animal cells are seriously handicapped by a lack of a simple effective technique for large-scale colony production from single cells".

Earle et al., (1954) emphasised the necessity for "conditioning" by the living cells of the nutrient media currently employed in tissue culture. They were able to produce clones from a variety of cells by a fairly exacting technique involving sealing of individual cells in capillary tubes, where diffusible metabolic products essential for multiplication remained in association with the cells. However, only a few per cent of the single cells

isolated by this method could be carried to the point of self-sustaining colony development. Earle et al., (1954), emphasised the need to conserve diffusible cellular products, and they directed their efforts toward furnishing isolated cells with maximal amounts of these substances.

Sanford et al., (1948) suggested that single cells may fail to grow because they may be unable to adjust the fluid sufficiently for survival and proliferation. For these reasons, the surface area of vessel and volume of medium used are very important.

It may be important to :

a) reduce the amount of culture medium bathing the single cell to that volume which the cell can adjust;

b) supply the cell with a culture medium already adjusted by the growth of large cultures of living cells.

Puck and Marcus (1955), made an attempt to provide a continuing supply of conditioned factors by a large number of metabolising cells which were themselves incapable of multiplication (feeder cells). If such growth-inhibited cells were arranged in close proximity to a single multiplying cell, they could supply the cell with the necessary growth factors which should allow multiplication into a colony.

Three basic methods are commonly used for the production of clones in cell culture. These are the single-cell plating technique, a method involving use of sterile cloning rings and the soft agar method.

The first most commonly reported method involves disaggregating cultures into single-cell suspensions, and dispensing cell suspension into the wells of a 96-well micro-test plate adding on the average one cell per well. Wells containing only one cell may be identified by microscopic inspection and identified with a mark on the overlying lid. Colonies formed in these wells may be transferred subsequently to vessels of increasing size. To assist in minimizing contamination, only wells located at the central area of the plate may be employed.

This method has been reported by Biedler et al., 1978; Kripke et al., 1978; Dulbecco et al., 1981; Sweeney et al., 1982; Wang et al., 1982; Poste et al., 1982; Horan Hand et al., 1983; Machead et al., 1984; Augeran and Laboisse, 1984; Engvall et al., 1984; Layton and Franks, 1984; Schmidt et al., 1984; Geradts et al., 1986; Shirasuna et al., 1986; Potworowski et al., 1986; Takaki et al., 1986; Korach et al., 1986 and Azuma et al., 1986, amongst others.

The second method, involving use of sterile cloning rings has been used by Biedler et al., 1978; Shapiro et al., 1981; Augeron and Laboisse, 1984; Gazdar et al., 1980; Tsao et al., 1985 and Nister et al., 1986.

This method involves initial seeding of 60-100mm plastic tissue culture dishes with concentrations of adherent cells between 50-1000 per dish. To permit isolation of individual colonies, they must be sufficiently separated from each other and for this some work was needed on the initial seeding concentration. After 14-30 days, dishes containing colonies were rinsed with sterile phosphate buffered saline and sterile cloning rings secured with sterile grease around each selected colony. The grease effectively

isolates each colony, minimizing cross-contamination with other cells. The colony could be removed with enzymes and seeded in a separate vessel and cultures expanded.

The final method involved use of soft agar or agarose for cloning. Reports on this have been made by Courtenay, 1976; Hamburger et al., 1978; Dexter et al., 1978; Von Hoff et al., 1980; Suzuki et al., 1980; Bradley et al., 1980; Sweeney et al., 1982; Thomson et al., 1982; Romerdahl and Rubin, 1984; Hays et al., 1985; Shirasuna et al., 1986; Nagashima et al., 1986 and Sugimoto et al., 1986.

The soft agar method basically involved an underlayer of hard agar in growth medium above which cells are suspended in soft agar medium (See Materials and Methods, Section 2.20). Colonies that grew could be removed after 2-3 weeks and grown separately and cultures expanded.

Von Hoff et al., (1986) have also reported on use of capillary tubes for cloning but this appears to be a difficult and rarely used method.

The three basic methods described are the same for different cell types. The methods can however, be adjusted to give variations in media formulations, serum percentage and type, agar/agarose percentage, presence/absence of feeder layers, pre-coating of dishes, gaseous atmosphere and subcultivation technique.

Examples of the basic techniques and variations on them are given below.

In 1982, Sweeney et al., working with a rhabdomyosarcoma cell line used Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal calf serum and pencillin-streptomycin as standard growth medium. Cultures were grown at 37°C in a humidified atmosphere containing 10% CO_2. For cloning in liquid medium, a one cell/ml suspension was distributed at 0.25ml/well in a microtest culture plate. Wells were observed microscopically and those where two or more colonies appeared to be forming were eliminated. Selected colonies were replated for one passage and then re-cloned to ensure their monocellular origin.

Azuma et al., (1986), working with a human salivary adenocarcinoma cell line, inoculated 50µl of a disaggregated cell suspension to 96-well microtiter plates adding on the average 1.5 cells/well. Cells were grown in Eagle's Minimal Essential Medium with 10% newborn calf serum and for cloning, 20% newborn calf serum.

Wang et al., (1982) maintained fibrosarcoma cells in Rosewell Park Memorial Institute medium with 10% foetal calf serum with penicillin and streptomycin. For cloning, cells were diluted to a final concentration averaging 3 cells/ml. An aliquot of 0.3ml was then placed in each well of a microtiter plate.

Macleod et al., (1984), attempted to clone thymoma cells by limiting dilution in Dulbecco's MEM with 20% foetal calf serum.

Geradts et al., (1986), used conditioned medium for cloning a rat mammary tumour. A cell suspension was diluted to one cell per well in 96-well microtiter plates and colonies were isolated in a medium composed of equal parts of DMEM : Hams F12 (1:1) and this same medium that was conditioned for 24 hours by established cultures of the same cells. They also cloned by seeding single cells into collagen and isolating resultant colonies.

Engvall et al., (1984), promoted growth of single mouse

teratocarcinoma derived endodermal cells by adding mouse thymocytes. Augeron and Laboisse (1984), added insulin (10μg/ml) for cloning human colonic adenocarcinoma cell line. The cell suspension was diluted in DMEM with 20% foetal bovine serum and insulin (10μg/ml) to yield a final concentration of 30 cells/ml. Each well of a 96-well microtest plate was inoculated with 50μl of cell suspension, adding on the average 1.5 cells/well.

Takaki et al., (1986) used a slightly different cloning procedure. They maintained insulinoma cells in RPMI 1640 medium with 5% foetal bovine serum. Cell suspensions were diluted to the concentration of 100 cells/ml with a mixture of fresh medium and a conditioned medium (1:1). Conditioned medium was prepared by harvesting the 24 hour spent medium of the same cells during the exponential growth phase. Two and a half milliliter of cell suspension was placed into a 60mm petri dish and a single cell was sucked up using a peaked pasteur pipette under observation with an inverted microscope and inoculated to a well of a microtest II plate together with a drop of mixed medium. Two drops of the medium, which consisted of equal amounts of fresh medium and the conditioned medium were added to each well every 2 or 3 days.

Layton and Franks (1984) used a 1μl pederson pipette to select single mouse lung carcinoma cells from a dilute cell suspension and transfer to wells of a Titertek plate. A similar method was used by Dulbecco et al., in 1981, but with the use of a mitomycin C treated feeder layer of LAT cells for cloning a mammary tumour.

Poste et al., (1982), found that the use of 5% O_2 significantly enhanced the recovery and growth of cells of the B16 melanoma cell line from small inocula. Cells were grown in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 and DMEM plus 20% foetal calf serum was used. Culture vessels containing cells were placed inside air-tight transparent plastic chambers equipped with input and

output valves. The chambers were gassed at 2 litres/min for 10 minutes, sealed and re-flushed with the gas mixture any time culture vessels were removed and/or replaced.

A low temperature trypsinization technique was used by Agy et al., (1981), for routine subculturing and preparation of clonal inocula. This was modification of the technique used by McKeehan (1977). A culture flask containing a nearly confluent cell layer was chilled on ice. Medium was removed and cells washed gently twice with 5ml cold solution. Two millilitres of cold trypsin (0.01%) were added and allowed to remain in contact with the cells for 2 minutes. The trypsin solution was removed as completely as possible and the cells were left on ice for an additional 4 minutes. Cold serum free medium (5.0ml) was added to the flask. The resulting cell suspension was pipetted several times to disperse clumps and ensure a uniform single cell suspension. This trypsinization procedure minimizes the amount of trypsin introduced into stock cultures and clonal growth experiments.

An unusual method was used by Kripke et al., (1978) for cloning fibrosarcoma cells. They placed 0.1ml of a cell suspension containing 1×10^3 cells/ml in medium containing 20% foetal bovine serum on a polyacrylamide gel containing 49 wells. The polyacrylamide gels were conditioned by incubation for 1 week in medium. After the cells settled, the gels and surrounding dish were flooded with medium. Wells containing single cells were marked and after sufficient growth, the contents of these wells were transferred to the wells of a microtest plate.

The second widely used method for cloning, with the use of cloning rings has been used by Augeron and Laboisse in 1984 to isolate morphologically altered colonic cancer cells after treatment with sodium butyrate. Tsao et al., also used this technique in 1985 to

isolate rat liver tumorigenic epithelial cells treated with glutamyl transpeptidase staining mixture. Three authors have reported on this method for isolation of clones from brain tumour cells. The evidence for heterogeneity of malignant brain tumours has so far only been suggested by their pathological appearance (Shapiro et al., 1981).

Biedler et al., in 1978, maintained a neuroblastoma cell line in Eagle's MEM with non-essential amino acids, 15% foetal bovine serum and penicillin (100 Iu/ml) and streptomycin (100µg/ml). Cells were seeded into 100mm plastic tissue culture dishes at a concentration of 200 cells/dish and colonies isolated with metal cylinders.

Shapiro et al., (1981) working with human malignant gliomas seeded 60mm petri dishes with a single cell suspension. After 12 to 18 hours of incubation, each petri dish was marked for single cell attachment and then returned to the incubator for 4 days. Following this incubation period, clusters of 3 to 5 cells were seen within the marks. On the seventh day, 0.5ml of conditioned medium was added. When a clone of 12 to 30 cells was evident, the clone was isolated with a 6mm glass cloning ring using conditioned medium and transferred to a 96-well dish. Slower growing clones remaining in the dish continued to grow and were collected as late as 28 to 30 days. Glass cloning rings were also used by Nister et al., (1986), for isolation of clones from a human malignant glioma cell line. Cells were seeded at a concentration of 1×10^3 cells per dish into 100mm culture dishes. Dishes were either untreated or containing a confluent feeder layer of normal human glia cells (Westermark, 1973, a,b.). Cultures were incubated for 2-5 weeks and well-demarcated colonies isolated.

The third method described is the soft agar method. The term "anchorage independence" (A.I.) was first used by Stoker in 1968 to describe the ability of virus-transformed (but not normal) cells to grow in vitro without attaching to the solid substratum of a culture flask. Anchorage independent growth of single cells into colonies in semi-solid medium is often used as a marker for cellular transformation of both fibroblasts and epithelial cells (MacPherson and Montagnier, 1964; San et al., 1979 and Stoker, 1968). The capacity for anchorage independent growth is also a good indication of the ability of the cells to produce tumours in vivo (Freedman et al., 1974; Shin et al., 1975 and Barrett et al., 1979) and is not confined to virus-transformed cells. The growth of single cells in a semi-solid matrix may be used as a test for clonal expansion of a single cell type responsive to certain growth or differentiation factors that are added to the medium (Metcalf et al., 1973; DeLarco et al., 1978) as well as for an assay of tumour stem cells (Hamburger and Salmon, 1977).

This system is based on the observations that nutrients and growth factors can diffuse through the medium to reach the test cells and that the medium is of a consistency to immobilize single cells and allow their progeny to remain in close proximity. The cell number in the inoculum must be low so that the cells are widely dispersed and colony formation occurs by cell division and not by cell migration or aggregation (Hays et al., 1985).

Sweeney et al., (1982), cloned cells with a modification of the soft agar technique of MacPherson and Montagnier in 1964. Cells were plated at a concentration of 100 cells per plate and plated in standard growth medium with 0.5% agarose. Isolated colonies were removed from the gel and replated in liquid medium. Nagashima et

al., (1986), cloned fibrosarcoma cells by this method. A base layer, 5ml of the culture medium containing 0.5% Difco Nobel agar was set in 60mm plastic culture dishes and overlaid with 1.5ml of a second layer of 0.33% agar containing a suspension of 200 single cells. Dishes were examined under an inverted microscope to mark the position of a single cell sufficiently apart from other cells. After 12 days incubation, colonies were individually selected and maintained in culture medium. A neoplastic epithelial duct cell was cloned by Shirosuna et al., 1986, in semi-solid agar. 10^2 or 10^3 cells were mixed with 1ml of growth medium containing 0.3% agar and then overlaid onto a 2ml basal layer of the growth medium containing 0.6% agar in a 30mm petri dish. After 2 weeks incubation, a clone was isolated with a pasteur pipette from a single cell in semi-solid agar.

Courtenay in 1976, working with Lewis lung tumour and B16 melanoma used a gas phase with 5% O_2 instead of agar for a soft agar colony assay. By using a gas phase with 5% O_2, the O_2 tension at the surface of the medium is reduced to a level comparable to that in the tissues in vivo (Jamieson and Van den Brenk, 1964).

Applications of cloning are manifold, some of these have already been discussed. In addition, a few more applications will be discussed. Clonal variations in various properties may be investigated. To illustrate this, I have chosen a paper by Nister et al., 1986. They found clonal variation in the production of a platelet-derived growth factor like protein and expression of corresponding receptors in a human malignant glioma.

In recent years, much attention has been focused on the putative role of tumour cell-derived growth factors as effectors in neoplastic transformation. Strong support for this notion was obtained from the amino acid sequence analysis of PDGF which revealed a virtual identity between the β-chain of PDGF and part of the protein product of the v-sis oncogene, that is, the transforming gene of simian sarcoma virus (Waterfield et al., 1983).

Nister et al., wanted to investigate whether the wild type population could be heterogeneous with regard to production of glioma PDGF. Clones with varying growth factor synthesis and PDGF binding should be valuable tools for further investigations on the role of PDGF production in human tumour cells.
They derived several clones from the U-343 MGa line using cultures of low and high passage levels as well as formerly cloned cells, as starting material. A high degree of glioma PDGF production was found predominantly in clones derived from cells of high passage levels. This may indicate that growth factor production is of selective advantage for glioma cells. A possibility which has to be taken into account is that the extent of glioma PDGF production is related to the functional or developmental state of the cell. This notion was suggested by the finding of a morphological correlation with glioma PDGF production, most of the high-producing clones were found among "immature" looking, tightly packed cells, whereas low producing clones had a more astrocytic or glia-like morphology. They also found that the extent of glioma PDGF production correlated with the growth role in serum-free medium.

These results also have bearing on the model of autocrine growth stimulation of tumour cells (Sporn and Todaro, 1980). Autocrine

growth stimulation, however, presupposes a concomitant synthesis of growth factor and the corresponding receptor. The presence of clones within a population with varying expressions of PDGF and PDGF receptors, suggests a more complex situation involving both autocrine and paracrine growth stimulation. The glioma clones described by Nister et al., (1986) will have value for further studies on the role of PDGF and the PDGF receptor in human glioma.

In addition to the cloning work, some preliminary work on SW1088 Astrocytoma cells was done, including effects of feeder layers on colony forming efficiency in agar.

Also, some primary samples of brain tumour were set up as primary explants for the purpose of growing cell lines. Preliminary work on attempted primary culture of human glial cells is described. It is hoped that this work will form a basis for future cloning of glial cells.. Other aims of primary culture are described. Glial cultures have already yielded much valuable information on the control of cell proliferation and cultures of glioma and normal glia may provide much more information on specific differences in glioma and normal glia (R.I. Freshney, Brain Tumours, Thomas and Graham, Chapter 3). The establishment of permanent cell lines from human malignant gliomas has been described (Ponten and Macintyre, 1968; Westermark et al., 1973). Production of established cultures with an infinite life span has been found in about 30-50% of gliomas (Freshney, 1980). Repeated subculture over a period of six months or so enables the isolation of established lines from a proportion of glioma cultures. It may not, however, be convenient to wait this time or appropriate to risk the possibility of further transformation. Separation of individual components of a cultured cell population is generally achieved by cloning (Freshney, 1980).

Freshney et al., (1980) report that cloning efficiency's in glioma cultures can often be increased by the use of a glucocorticoid. As glucocorticoids repress clonal growth of cells from normal glia, this technique may select for malignant cells as well as enabling the isolation of individual clones.

If pure cultures of tumour cells can be maintained from glioma and can be shown to proliferate, they become a potentially valuable tool in demonstrating differences in chemosensitivity between tumours. This is an attractive idea although it still remains to be demonstrated whether there is any correlation between drug sensitivity in vitro and response to chemotherapy in vivo (Freshney, 1980).

Assays of chemosensitivity may be made on cultures derived from biopsy material. The effect of the drug may be followed by a variety of metabolic parameters, for example, respiration, glycolysis and DNA, RNA or protein synthesis. However, direct measurement of either cell proliferation or cell survival following drug treatment and removal may be more reliable.

Clonal growth assays are limited by the low cloning efficiency usually experienced with early passage cultures. Work done by Guner et al., (1977) and Mealey, Chen and Schanz (1971) on cloning brain tumour cells, shows that glucocorticoids stimulate survival and proliferation of glioma cells when the cell density is very low but limit growth as the size of the colony increases (and consequently cell density).

High density cultures may be more analogous to the solid tumour in vivo, therefore, the cytostatic effect demonstrated with glucocorticoids may be the more relevant. The possibility,

however, remains that the survival and proliferation of isolated cells may be enhanced by glucocorticoids.

The relatively high cloning efficiency of glial cultures when treated with glucocorticoids and grown on feeder layers (Westermark, 1973 a, b) make them a particularly useful *in vitro* tool.

Much still remains to be learned about the heterogeneity of the tumour cell population *in vivo* with respect to drug sensitivity and whether resistance to chemotherapy is the product of :

a) the intrinsic resistance of all the cells of the tumour;

b) phenotypic diversity or

c) permeability barriers peculiar to the site of the tumour (Freshney *et al.*, 1980).

Further exploration with these cultures would seem well worthwhile.

SECTION 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Water

High purity water is critical for cell culture work. For preparation of all media and reagents, water used is passed through a Millipore Milli-Q ultrapure water system. This system consists of a reverse osmosis system with two prefilters removing ionic and non-ionic solutes and a further purification stage consisting of two ion-exchange filters, a carbon filter and a 0.22μm cellulose acetate filter. Monitoring of water was achieved by an on-line conductivity metre, an acceptable resistivity being 10-18 megohms/cm.

2.2 Glassware

All glassware used in tissue culture was reserved for this purpose solely. Also, glassware for cell culture work was washed separately under fairly stringent conditions, involving soaking and multiple rinses. Glassware was soaked for 1-2 hours in hot water containing detergent RBS-25. The glassware was then scrubbed and the detergent rinsed out with ordinary tap water. The next stage was three rinses in reverse osmosis water and the final stage involved rinsing in Millipore Milli-Q ultrapure water. The glassware was dried in an oven along with the metal lids which were treated similarly but kept separate from glassware.

2.3 Maintenance of cells in culture

Cells were cultured routinely in $25cm^2$ sterile flasks growing attached to the flask surface. Different basal media was used according to cell line being cultured (see Table 2.1). Media was supplemented with foetal calf serum, unless otherwise stated, at a concentration of 5% v/v.

Every 3-6 days, depending on cell type, cells were subcultured by a trypsinization procedure. A 0.25% trypsin + 0.02% EDTA solutions (trypsin-versene) was used to disaggregate cell layers. This solution was incubated at 37°C for 20 minutes before use. Medium was then removed from the cells and any residual medium in the flask washed out with trypsin- versene. 2ml of trypsin-versene was then added and the flask incubated at 37°C for 5-20 minutes until all the cells had detached. This could be observed microscopically. To stop the action of trypsin, medium containing FCS was then added and the contents spun at 1000rpm for 5 minutes. The supernatant was discarded and the cells resuspended in 5ml of medium (unless otherwise stated).

A haemocytometer was used to estimate cell numbers and flasks/assays set up at required cell concentrations.

Trypan blue dye (0.4%) was used on occasion to estimate viability. 250µl of cell suspension was added to 50µl of trypan blue, mixed and allowed to stand for 4-12 minutes. Cells were then counted, dead cells absorbing the blue dye.

2.4 Incubation

Incubators were maintained at 37°C and cells were cultured in sealed flasks, multi-well plates or petri dishes, allowing pH equilibration with the external environment. For certain assays, it was necessary for gaseous exchange with the external environment. In these cases, trays containing plates were placed in incubators filled with 5% CO_2 (balance air). Trays of sterile water were placed on the base of the incubator to maintain a high humidity level. incubators were cleaned regularly as the risk of contamination was increased due to the high temperature and moist atmosphere.

2.5 Sterility

2.5.1 A major problem in tissue culture is contamination by microorganisms. These can be introduced to a culture via the operator, atmosphere, reagents, equipment or surfaces. Antibiotics may be used but sparingly due to cytotoxic effects.

A laminar flow cabinet with a vertical air-flow, was used for all work with cells or reagents. Before use, all surfaces were swabbed with alcohol and all bottles, instruments, etc., swabbed before being placed in the laminar flow. Also, either sterile gloves or hands swabbed with alcohol were used. Aseptic techniques were used throughout (with the help of a bunsen flame in the Laminar Flow Cabinet).

To sterilise bottles and water for media preparation, an autoclave was used at 15 p.s.i. for 20 minutes. Autoclave

tape was used to indicate sterility., The autoclave was also used for waste to assist in disposal.

All plastic materials, including flasks, pipettes, universlas, plates, etc., were purchased sterile.

2.5.2 Sterility testing

Medium, after preparation, was tested for sterility by incubating a small aliquot at 37°C for 48 hours. If the medium was cloudy, contamination was present and the batch had to be discarded.

Nutrient agar plates were also used to test medium. Fresh medium was streaked on nutrient agar plates. Nutrient agar was prepared by suspending 25g in 1 litre of distilled water and allowed to soak for 15 minutes before heating in a boiling waterbath until dissolved. This was then autoclaved for 15 minutes at 100 KPa and poured into bacteriological grade petri dishes before setting. Colony formation on plates indicated contamination.

2.5.3 Sterile filtration

To sterilise solutions, a number of methods were used. One method involved passing a solution through a Millipore Millex-GV 0.22μm filter using a syringe. For larger volumes, sterivex filters with a 0.22μm membrane were used for sterilising. The filter is enclosed in a sterile plastic case and the end protected by a bell-shaped shield. This method was used to sterilise large volumes of powdered media especially Hams F12. A peristaltic pump was used to pump the medium across the membrane.

2.5.4 Mycoplasma detection

Mycoplasma contamination is a major problem in tissue culture causing a gradual deterioration of cultures, Infection cannot be detected by the naked eye or by usual laboratory microscopes and therefore, special tests are necessary to detect them. Routine periodic testing of all cell cultures for mycoplasma contamination is an essential part of cell culture.

The main method used was a fluorescent staining technique (Chen, 1977). This method involved use of a fluorescent stain, Hoechst 33258*, which binds to DNA and mycoplasma, could be detected by observation of extra-nuclear fluorescence.

*(Hoechst (2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)-benzimidazol-trihydrochloride) 33258).

The method used was an adaptation of that used by Chen, (1977). If mycoplasmas are present in a culture, they will also be found in the medium which was in contact with the cells. A normal rat kidney cell line (NRK) was used as the indicator cell line. NRK cells were set up on sterile coverslips at a concentration of 1×10^4 - 1×10^5 cells/ml and these were incubated at 37°C and 5% CO_2 for 24-48 hours. Medium was removed from cultures of all the cell lines to be tested and control medium consisted of medium which had not come in contact with cells. Medium (between 500µl - 1000µl) was added to the NRK cells and these were incubated for a further 48-72 hours.

After this period, excess medium was poured off the coverslips and the cells fixed in pre-cooled methanol for 8 minutes and acetone for 2 minutes at -20°C. The coverslips were then rinsed for 5 minutes in PBS and 2 minutes in distilled water.

A few drops of 0.05µg/ml Hoechst stain (diluted in a balanced salt solution) were added to the cells and incubated for 10 minutes in the dark. The cells were washed three times in distilled water and mounted in McIlvaine's buffer, pH 5.5.

A Nikon fluorescent microscope with a wavelength of 405nm was used to examine for fluorescent staining of DNA. Oil immersion was required to detect low level mycoplasma infection.

2.6 Culture media

2.6.1 Cells were maintained in culture in a sterile nutrient medium. This medium, without the addition of foetal calf serum, was chemically defined. Foetal Calf Serum, a necessary addition containing undefined components was used to supplement medium. A lot of work is being done to attempt to replace foetal calf serum in medium with more defined components for different cell types, but in this work, foetal calf serum was used.

The basal medium which was chemically defined, consisted of a balanced salt solution, vitamins and energy source, glucose or other and essential or non-essential amino acids.

Liquid media was obtained as sterile 10 x concentrates and diluted before use with sterile ultrapure water. Other concentrates were added to give final concentrations, 20mM HEPES buffer (4-(2-hydroxyethyl)-piperazine ethane sulphonic acid), 2mM glutamine, sodium bicarbonate (7.5%), 5% foetal calf serum and if required, non-essential amino acids and penicillin G-100 International units (I.u.)/ml and streptomycin - 100 mg/ml (added in combination).

The pH was adjusted to 7.4-7.5 with the addition of 1.5N NaOH or HCl. The medium also contained a phenol red indicator for monitoring pH. To confirm exact pH, a small aliquot of medium was removed aseptically and tested with a pH meter.

Powdered media was made from a lyophilized basal medium. Powdered Hams F12 was made by dissolving one package of

Gibco's powdered medium in 985 ml of Milli-Q ultrapure water. Sodium bicarbonate (1.176g/l) was added and the pH adjusted to 7.4-7.6. The medium was passed through a Millipore Sterivex unit and collected in sterile glass bottles and stored for up to 4 weeks at 4°C.

2.6.2 Buffered 2 x medium

This medium was required in the clonogenic assay and the transforming growth factor assay.

76.0ml of ultrapure water was autoclaved and 20ml of the 10 x concentrate added. Additions included 2.2ml of sodium bicarbonate and 4.0ml of hepes and the pH was adjusted to 7.4-7.5 with NaOH.

Table 2.1

2.6.3 Media used for different cell types

MEDIUM	CELL TYPES GROWN
Modified Eagles (MEM)	RPMI 2650
Dulbecco's Modified Eagles Medium (DMEM)	3T3 NRK
Hams F12	RPMI 2650
DMEM : Hams F12 (1:1)	RPMI 2650
BME	SW1088 Brain Samples

MEM non-essential amino acids were added to modified Eagles medium.

2.6.4 Agar/Agarose Medium

This was also required in the clonogenic assay and the transforming growth factor assay.

Table 2.2

Constituents	Volume/ml
1.4% agar(ose)	40
Buffered 2x medium	41.6
Foetal Calf Serum	16.0
Penicillin-streptomycin	0.8
Glutamine	0.8
Fungizone	0.8
Growth Medium or 10x additives	11.2
Growth medium or non-essential amino acids (n.e.a.a.)	0.8

2.7 Cell Lines

2.7.1 The cell lines used in this work were RPMI 2650, NRK, normal rat kidney, 3T3 fibroblasts and SW1088.

RPMI 2650 (Rosewell Park Memorial Institute) was established in 1962 from the pleural effusion of a patient with a malignant tumour of the nasal septum.

The 3T3 cell line is a mouse embryo fibroblast cell line isolated initially from a mouse embryo (Todaro and Green, 1963).

SW1088 is an astrocytoma cell line.

Cell Freezing and Long-term Storage

2.7.2 Freezing cells

Cultured cells can be preserved by storage in liquid nitrogen. A high concentration of cells (2×10^6 cells per ml) were preserved in a 10% solution of dimethylsulphoxide (in medium with F.C.S.).

This 10% DMSO solution was added dropwise to the cell suspension in a 1:1 (v/v) ratio. The mixture of DMSO/cell suspension was then dispensed into sterile cryotubes, approximately 2ml per cryotube. The cryotubes were allowed to stand in the vapour phase above liquid nitrogen for 3 hours.

After this, they were placed into liquid nitrogen proper. Cells could be stored on a long term basis in this way.

2.7.3 Thawing Cells

To thaw cells when required, a cryotube was removed from liquid nitrogen and thawed at 37°C. The contents were mixed with 5ml of medium with serum in a laminar flow and

centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in medium with foetal calf serum and reseeded in a $25cm^2$ flask.

2.8 Pre-treatment of test cells and feeder layer preparation

Cells used in agar/agarose, clonogenic and monolayer assays were pre-treated by passaging into fresh medium 48 hours before use, in an attempt to standardise assay conditions.

For preparation of feeder layers, two types of feeder layer were possible :

i) Live feeder layers were prepared at the required concentrations and incubated in plates at 37°C in 5% CO_2 for 24-48 hours;

ii) Mitomycin-C treated feeders were prepared by incubating sub-confluent cultures for 3 hours in medium with 4µg/ml of mitomycin C, prepared from a stock solution of 200µg/ml. After incubation, the cells were trypsinized and reseeded at required densities in fresh medium and incubated for 24-48 hours at 37°C in a 5% CO_2 atmosphere (MacPherson and Bryden, 1971).

2.9 Collection of conditioned medium

Cells were grown to between 50 and 90% confluence and medium changed, generally unless stated to serum-free medium and this medium collected after 48 hours and 96 hours. This was centrifuged at 5000g for 15 minutes and filtered through a 0.2µm sterilising filter.

2.10 RPMI Monolayer Assay System

This assay was generally carried out in 60mm dishes placed on trays in a 5% CO_2 humid atmosphere at 37°C. RPMI 2650 were grown routinely in MEM + 5% foetal calf serum, unless otherwise stated.

Dishes were set up with a final volume of 6ml per dish. The method used involved adding 1ml of cell suspension in MEM +5% foetal calf serum and 5ml of medium to be tested with foetal calf serum.

Medium was dispensed into the dishes and allowed to equilibrate in a 5% CO_2 atmosphere for 30 minutes. During this period, cells which had been growing were trypsinized and the required concentration made up. 1ml of cell suspension was added to each dish and the dishes mixed.

The trays on which the dishes were placed were covered with aluminium foil to reduce the risk of contamination and were placed at 37°C in a 5% CO_2 humid atmosphere.

This method could be modified for different size plates. To test possible cloning aids, costar tissue cluster dishes (16mm well diameter) were used. These variables included pre-coating with DEAE dextran, media variations, feeder layers, both 3T3 amd RPMI, conditioned media and different gas atmospheres.

Cell concentrations seeded in 24-well plates varied between 10 and 100 cells per well. Common concentrations used were 20,40 and 50 cells per well, as these concentrations generally gave consistently good growth.

2.11 Staining procedure

After the required growth period, generally 10-14 days, trays were removed from the incubator and medium discarded from the dishes. The cells were then rinsed with PBS and 1.5-3.0ml of Leishmann's stain (0.3% in methanol), added to each dish for 10 minutes.

Distilled water was then added for 10 minutes and the mixture poured off and the dishes rinsed twice in distilled water and inverted to dry. Cell colonies stained a dark blue.

Colonies were counted with an AMS 40-10 Image Analyser. The image of the darkly stained colonies against a bright background was observed with a camera lens. This image was transferred to a television screen linked to a computer memory. The computer counted the number of colonies and calculated the total area.

2.12 Pre-coating of plates with DEAE dextran

DEAE dextran was made up at a stock concentration in ultra-pure water and sterilized by passing through a 0.22μm millex-GV filter. This was diluted down in sterile ultrapure water to the required concentration and added to the dish for pre-coating to cover the base of the dish. After the required time for pre-coating, DEAE dextran was removed from the dishes and the dishes washed first with PBS and then medium. The assay was then set up on the pre-coated dishes.

2.13 Gas Chambers

Cells were cultured on a routine basis in a 5% CO_2/95% air atmosphere at 37°C for assays. The gas atmosphere could be altered experimentally with the use of a Flow Laboratories Modular Incubator Chamber. The chamber was swabbed with 95% Industrial methylated spirits before use. To maintain humidity, petri dishes containing sterile water were included with test petri dishes. Gas was flushed through the chamber for 15 minutes, at less than 2 psi, to avoid rupturing the chamber, before the inlet and outlet ports were closed. The chamber was then incubated at 37°C in a constant temperature room for 10-14 days with regassing at 5-7 days. The main gas mixture in these experiments consisted of 5% CO_2/3% O_2/N_2 balance.

2.14 Clonogenic Assay

Some cells, particularly virally transformed fibroblasts, will clone readily in suspension. To hold the colony together and prevent mixing the cells were suspended in agar or agarose and plated out over an agar underlay. Medium was prepared and cells pre-treated 2 days prior to use by trypsinizing and feeding them. Cell concentrations to be used were determined and petri dishes labelled accordingly. If required, a feeder cell layer was prepared. Agar(ose) was melted by placing the bottle in a boiling water bath. The bottle was air-cooled briefly and transferred to a 45°C waterbath. Growth medium was prepared and buffered 2x medium at pH 7.4-7.5. Agar(ose) medium was prepared without agar(ose) and left at 45°C for 15-20 minutes. The agar(ose) medium was then mixed with agar(ose) at the required proportions and incubated at 41°C for 30 minutes. Cells were trypsinized and cell suspensions prepared at three times the required concentration.

If no feeder cell layer was present, the base layer containing 0.5% agar(ose) could be poured at any temperature between 41°C and 45°C and placed in the fridge for 5-10 minutes to set.

If a feeder layer was present, the base layer could not be poured until the agar(ose) medium had been fully equilibrated to 41°C, otherwise cell death due to scalding would occur and the feeder effect would be absent. The base layer had a volume of 2ml per 35mm petri dish.

When the agar(ose) medium had equilibrated to 41°C it was mixed with the 3x cell suspension to the ratio of 1ml of 3x

cell suspension : 2ml agar(ose) medium.

1ml of 1x cells in agar(ose) was poured onto each plate.

After all the dishes had been poured they were incubated at $37^{o}C$ in a humid CO_2 incubator for approximately 2 weeks and colonies counted with the use of a graticule.

2.15 Serum Batch Testing

There may be considerable variation in serum batches obtained, therefore, an assay was used to test and compare serum batch variations.

Costar tissue cluster dishes, (16mm well diameter) were used with a 1-2ml per well capacity.

Two concentrations of foetal calf serum were tested, 1% and 5%. Cell dilutions were made up in serum free medium. When serum and cells were ready, 500µl of serum containing medium and 500µl of medium containing cells were dispensed into each well. The final volume per well was 1ml.
A range of cell concentrations was tested. 1×10^2 cells per well, 10^3 and 10^4 cells per well. Wells were stained after 1-2 weeks and colonies counted with the Image Analyser.

2.16 Primary Culture

The procedure used was a primary explant technique used by Freshney et al., 1980. The biopsy was collected in medium with foetal calf serum and antibiotics. This was transferred to the laminar flow and rinsed in phosphate buffered saline (PBS) 2-3 times.

The biopsy was then chopped as small as possible with a sterile scalpel and pieces, $1mm^3$, transferrred to a culture flask and distributed evenly over the growth surface in 0.5ml growth medium.

Explants were left overnight at 37°C to attach and 1.5ml growth medium added gently and flasks returned to 37°C. Medium was gradually built up to 5ml over about 1 week. After 1-2 weeks, cells may be seen to migrate out of the explants. When outgrowths were about 1cm in diameter, explants were picked off and transferred to a fresh culture vessel and cells in first flask refed until the outgrowth had spread to cover at least 50% of the growth surface. At this stage, cells could be passaged, and cultures maintained.

2.17 Mutagenesis

A mutagen chosen was Ethylmethane Sulphonic acid (EMS) (See Results, Section 3). As a safety precaution, all plastic culture ware and medium which came in contact with mutagen were treated with 50% hypochlorite.

EMS Kill-Curve

$75cm^2$ flasks were seeded with 1 x 10^6 cells in MEM + 10% foetal calf serum and incubated at 37°C for 24 hours. Cells had attached during this time and undergone approximately one cell division.

EMS was added at 10 x the desired concentration and incubated with cells for sixteen hours at 37°C. EMS was made up in serum-free medium. The stock solution was filter sterilized. After treatment, EMS-medium was removed and the cells washed twice with PBS (10ml) and once with 5ml of medium. Cells were trypsinized and counted.

Hypoxanthine-aminopterin-thymidine (HAT) medium

100 x HT stock Solution	1000 x A Stock solution
0.1361g H 0.0388g T 100ml ultra-pure water	17.6mg A 80ml ultra-pure water
Heat to 70-80°C to dissolve. Sterilize by filtration. Store at - 20°C	Add several ml of 0.1M NaOH to dissolve. Bring to a final volume of 100ml using sterile ultra-pure water. Sterilize by filtration. Store at -20°C.

H = Hypoxanthine

A = Aminopterin

T = Thymidine

50 x HAT
100ml of 100 x HT stock 10ml of 1000 x A stock 90ml sterile ultra-pure water
Sterilize by filtration. Store at -20°C in 2ml aliquots
Use 2ml of HAT solution per 100ml of culture solution

2.18 Transforming Growth Factor Assays

Normal cells require anchorage for growth. However, cells which are transformed can be recognised by their ability to grow in a semi-solid support (anchorage independent). Producers are those cells which produce these transforming growth factors which give the cells the ability to grow without attachment. Addition of transforming growth factors to normal cells will also give them the ability to behave as transformed cells.

Two methods were used, a Transforming growth factor (TGF) assay and a specific assay for TGF-β, called for convenience the TGF and TGF-β assay respectively.

The basic method used was similar to that of the clonogenic assay. A base layer of 1.5ml of this mixture was poured quickly onto each plate and allowed to set. The agar(ose) medium was returned to the waterbath now at 41°C.

An indicator cell line, NRK or NRK 49F was trypsinized gently and required dilutions prepared. A stock solution of 3×10^4 NRK cells/ml was prepared for the TGF assay. When added to the assay, this gave a final concentration of 6×10^3 cells per plate.

For the TGF-β assay, a stock solution of 1.44×10^5 NRK 49F cells/ml was prepared, giving a final working concentration of 2×10^4 cells per plate.

In the TGF-β assay, a known concentration of Epidermal Growth Factor (EGF) was added (2ng ml^{-1}.

Concentrated medium, spun at 3000 rpm for 15 minutes, collected from known transformed cell lines, was used to test for the presence of a TGF.

The total capacity for each plate was 3ml. A mixture of

conditioned media, cells and agarose medium was prepared and quickly added onto each plate. Plates were incubated for 2 weeks at 37°C in a humid 5% CO_2 atmosphere and colonies counted.

2.19 Colony Counting

For both the clonogenic and transforming growth factor assays, clones greater than or equal to 40μm were considered to be colonies as this size was estimated to represent 30 cells after 5 cell divisions from a single 'parent' cell and is in accordance with current colony sizing procedures (Endressen et al., 1985). Also, gridded plates were generally used which assisted counting colonies.

2.20 Isolation of clones

For the isolation of clones, three basic methods were attempted - the single cell plating technique, the cloning ring method and the soft agar method.

The single cell method involved disaggregating a cell culture into a single cell suspension and dispensing cell suspension into the wells of a 96-well microtest plate adding on the average one cell per well. Colonies formed in these wells were transferred subsequently to vessels of increasing size (Fig. 1).

The second method which was found to be more successful was the cloning ring method. At the initial stages of cloning, antibiotics were used, as contamination was found to be a major problem.

The cloning ring method involved initial seeding of 60-100mm plastic tissue culture dishes with concentrations of cells between 50-100 per dish with 5-6ml of MEM + 5% FCS. Dishes were incubated at 37°C for a minimum of 14 days. Dishes containing colonies were marked, that is, colonies were circled on the underlid of the dish for cloning, under a Nikon microscope.

After 2-4 weeks, colonies were large enough and sufficiently separate from each other for cloning. Dishes were rinsed with sterile phosphate buffered saline and sterile metal cloning rings attached around each colony with sterile silicone high vacuum grease. The grease effectively isolated each colony, permitting the colony to be removed with trypsin-EDTA. 100µl 0.02% trypsin-EDTA was added to each ring and left approximately 1 minute, after which time approximately 80µl was removed, leaving a thin film of trypsin-EDTA. After approximately 10-20 minutes, MEM + FCS was added and clone containing medium removed with a micropipette and dispensed into a well of a 96-well microtiter plate. When the cells were confluent in the wells, they were trypsinized and seeded into a 24 well multi-well plate (16mm well diameter). Cultures were expanded in this way from 16mm well diameter to 30mm well diameter and finally into 25cm^2 flasks, from where they could be frozen in liquid nitrogen.

The third and final method attempted was cloning cells in suspension, that is agar. This was found to be both a time consuming and difficult method, so was not pursued but it may be worth attempting this again in the future. The

method involved suspending cells in agar above an agar underlay. The basic method used was the same as the clonogenic assay (See Section 2.14). When colonies had formed, they could be picked from agar with a pasteur pipette or syringe (See Fig. 3). This was carried out in a Laminer Flow Cabinet under microscope observation.

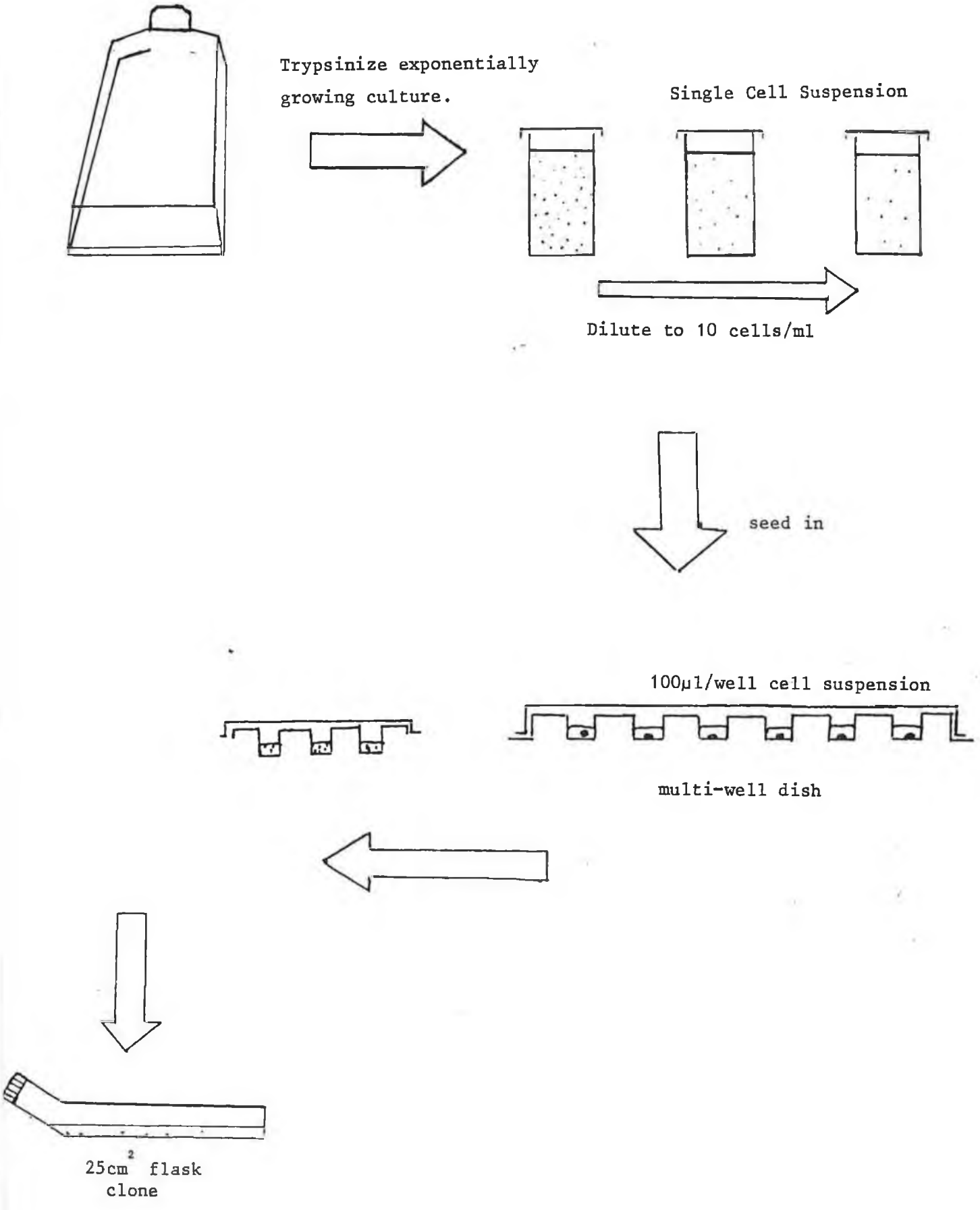


Fig. 1 : The Single Cell plating technique

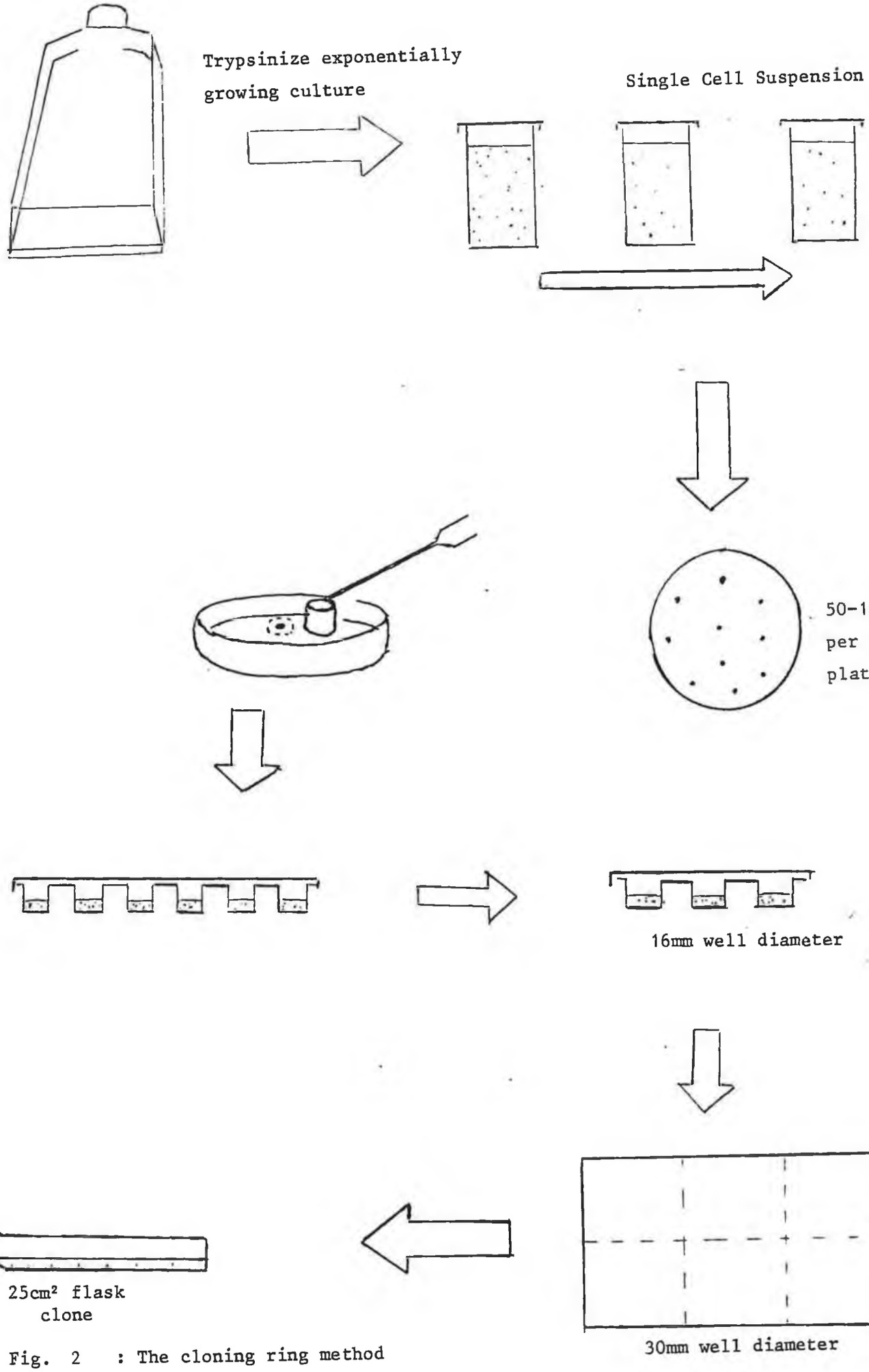


Fig. 2 : The cloning ring method

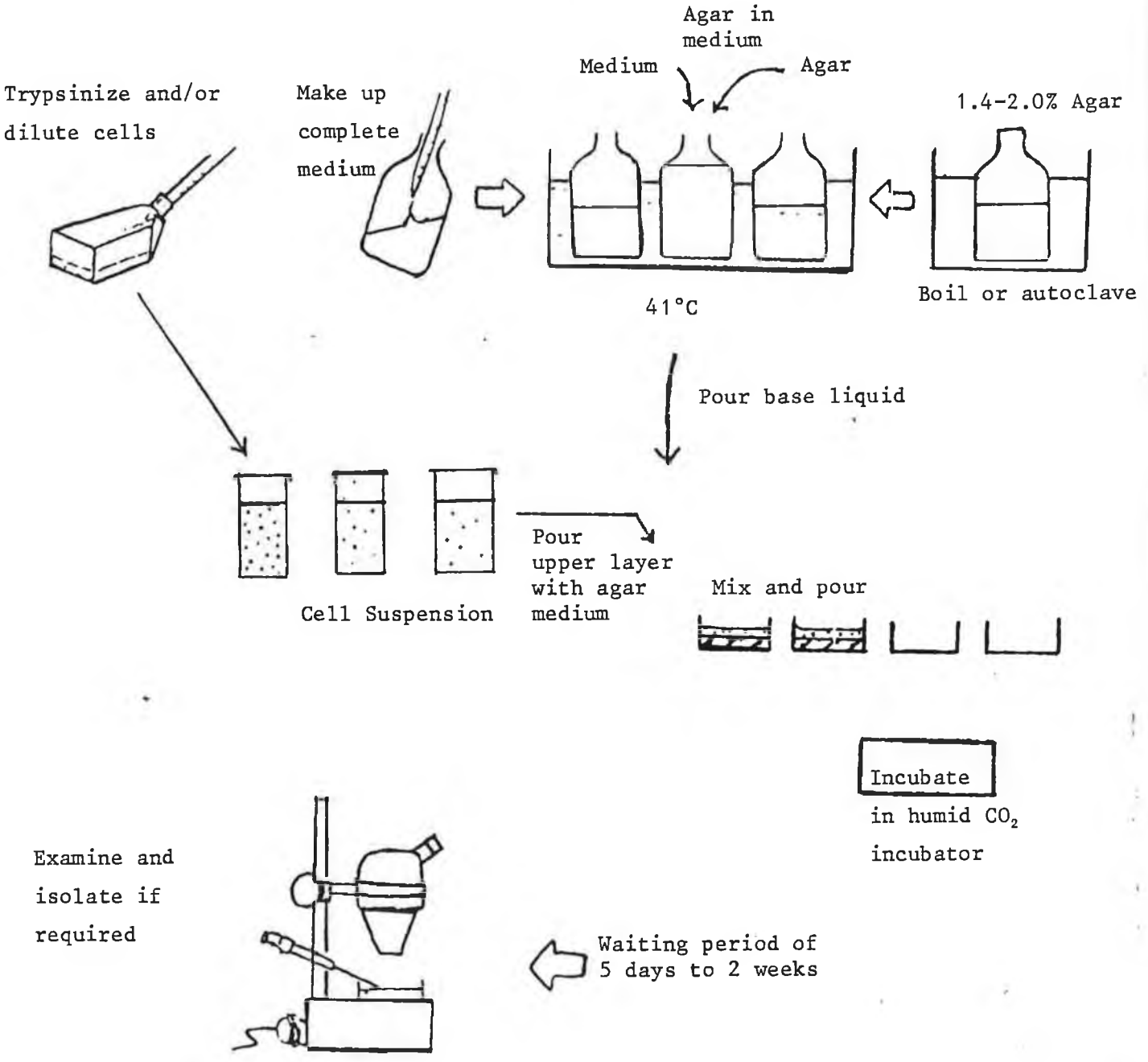


Fig. 3 : Cloning cells in suspension in agar.

SECTION 3

RESULTS

SECTION 3

RESULTS

3.1.1. Clonal Growth of RPMI-2650

RPMI-2650 is an epithelial cell line, grown routinely in MEM + 5% foetal calf serum. There have been no reports in the literature of RPMI-2650 clones. This cell line is difficult to clone compared with other cell lines, for example 3T3 fibroblasts, which have been successfully cloned in this laboratory. RPMI-2650 have a slower growth rate and are smaller in size than 3T3; this adds to the difficulties in cloning RPMI-2650 cells.

To establish initial seeding concentrations of RPMI-2650 for cloning, cells were set up over a concentration range of 50 to 6 x 10^5 cells per 60mm plate and plates examined after 2 weeks.

It was found that concentrations of RPMI-2650 suitable for cloning were between 50 and 200 cells per 60mm plate, the lower concentration being preferable as colonies were sufficiently far apart for separation.

A number of parameters were tested for improving cloning efficiencies in monolayer. These included effects of serum concentration and batch number, effects of feeder layers (both 3T3 and RPMI-2650), variation of media, addition of conditioned media, effect of temperature of trypsinization, effects of DEAE dextran precoating and effects of different gas atmospheres.

Some work was also done on cloning RPMI-2650 in agar. To establish initial seeding concentrations of cells in the upper layer, cells

were set up over a concentration range of 5×10^3 to 1×10^2 cells per 60mm plate. At a concentration of cells above 5×10^3 per plate, it was very difficult to clone, therefore this concentration range was appropriate. One clone was successfully established from agarose and frozen down.

Some work was also started with SW1088, an astrocytoma cell line. This work included clonogenic assays, with and without feeder layers. The results of these experiments are included with RPMI-2650 results.

3.1.2 Foetal Calf Serum batch testing for growth of RPMI-2650 in monolayer.

A major problem with working with foetal calf serum is found to be batch to batch variation. It was necessary to screen a number of different batches of foetal calf serum to identify batches for consistently supporting growth of RPMI-2650 in monolayer and in agar.

In Table 3.1, Experiment 1, growth was measured at 5% foetal calf serum. At this foetal calf serum concentration, growth of RPMI-2650 was not limited, that is all the necessary components for growth were present. Batch number 20F gave a higher colony forming efficiency than 10Q. Batch number 20F was used until stocks were depleted and another batch was selected.

In Table 3.1, Experiment 2, an experiment was carried out to test four batches of foetal calf serum at 5% serum concentration. It appeared that 10F gave optimum growth of the four batches tested.

In Table 3.1, Experiment 3, batch numbers 116 and 017 gave higher colony forming efficiencies than 30Q at both a limiting concentration of foetal calf serum and at a concentration of foetal calf serum appropriate for growth (4%).

As there was a limited supply of 017 foetal calf serum, it was necessary to use 116 foetal calf serum. From these three experiments, foetal calf serum batch numbers chosen for use, depending on availability were 20F, 10F and 116 FCS.

TABLE 3.1 : Growth of RPMI-2650 in two batches foetal calf serum at 5% serum concentration.

Experiment 1

Initial Cell Density per 60mm plate	Batch Number	Mean Colony Area	CFE
50	20F5256S	0.305	71
100		0.23	86
150		0.16	65.5
50	10Q	0.02	9
100		0.014	7
150		0.054	2.1

n = 3
± S.E.M.

Experiment 2

Initial Cell Density per 16mm well	Batch Number	Mean Colony Area	CFE
10^3	30Q	0.08 ± 0.021	7.77 ± 1.42
10^4		0.11 ± 0.02	7.26 ± 0.9
10^3	30F	0.07 ± 0.005	7.55 ± 1.53
10^4		0.12 ± 0.01	8.16 ± 0.15
10^3	10F	0.08 ± 0.012	11.5 ± 1.5
10^4		0.17 ± 0.013	8.27 ± 0.32
10^3	30G	0.08 ± 0.022	10.07 ± 1.44
10^4		0.14 ± 0.021	8.26 ± 0.56

Note : In experiment 1, no statistics were available.

± S.E.M.

TABLE 3.1 (cont'd) : Growth of RPMI-2650 in three batches foetal calf serum at two concentrations of foetal calf serum

Experiment 3

	Percentage f.c.s.	1%		4%	
Batch Number	Initial cell density per 16mm well	Mean colony area	CFE %	Mean colony area	CFE %
30Q	10^2	0.12 ± 0.048	3.75 ± 2.2	0.12 ± 0.013	4.75 ± 0.5
	10^3	0.11 ± 0.022	0.475 ± 0.096	0.094 ± 0.006	2.37 ± 0.275
	10^4	0.10 ± 0.013	1.95 ± 0.49	0.19 ± 0.03	4.19 ± 0.085
116	10^2	0.26 ± 0.034	5.5 ± 1.9	0.215 ± 0.09	5.0 ± 2.45
	10^3	0.19 ± 0.016	0.65 ± 0.1	0.1 ± 0.02	2.47 ± 0.92
	10^4	0.10 ± 0.018	0.87 ± 0.18	0.22 ± 0.042	4.9 ± 0.27
017	10^2	0.23 ± 0.098	7.0 ± 6.05	0.21 ± 0.061	6.0 ± 2.16
	10^3	0.30 ± 0.067	0.27 ± 0.096	0.11 ± 0.011	2.42 ± 0.096
	10^4	0.08 ± 0.006	0.75 ± 0.124	0.24 ± 0.02	5.22 ± 0.205

n = 4

3.1.3 Parameters tested for improving cloning efficiencies of RPMI-2650

A number of parameters were investigated with a view to improving cloning efficiencies of RPMI-2650.

Varying Serum concentration

The first parameter tested was the variation of serum concentration (see Table 3.2). An attempt was made to find optimum serum concentrations for growth. Cells were set up at 20 and 40 cells per 16mm well and percentage serum concentrations tested were 1, 5, 10 and 15% foetal calf serum.

TABLE 3.2 : Growth of RPMI-2650 cells in varying serum concentrations

Test layer conc. cells per 16mm well	20 cells		40 cells	
Percentage serum	Mean colony area	CFE	Mean colony area	CFE
1	0.22 ± 0.13	50.0 ± 35.5	0.19 ± 0.19	37.3 ± 33.5
5	0.3 ± 0.1	56.6 ± 23.3	0.225 ± 0.14	40.83 ± 30.7
10	0.26 ± 0.18	53.3 ± 40.0	0.37 ± 0.11	61.87 ± 19.2
15	0.25 ± 0.16	55.42 ± 38.2	0.33 ± 0.09	67.5 ± 9.35

F.C.S. Batch No. = 20F

A foetal calf serum concentration of 10 or 15% was found to give optimum growth at clonal concentrations.

Variation of basal medium.

Another parameter tested was the variation of the basal medium for growth (see Table 3.3). Cell concentrations used were again 20 and 40 cells per 16mm well. Media tested included DME + 5% foetal calf serum, MEM + 5% foetal calf serum and DME:Hams F12 (1:1) + 5% foetal calf serum.

TABLE 3.3 : Growth of RPMI-2650 in a variety of basal medium

Initial cell density per 16mm well	medium + 5% fcs	mean colony area	CFE
20 cells	DME	0.18 ± 0.08	36.4 ± 29.0
	MEM	0.10 ± 0.06	57.0 ± 2.8
	DME:F12 (1:1)	0.25 ± 0.07	132.0 ± 27.2
40 cells	DME	0.11 ± 0.04	22.71 ± 12.13
	MEM	0.22 ± 0.09	25.23 ± 10.6
	DME:F12 (1:1)	0.25 ± 0.029	93.1 ± 23.2

Note : F12 = Hams F12 medium

FCS Batch No. = 20F

The basal medium giving optimum growth of RPMI-2650 at clonal concentrations was a mixture of DME and Hams F12 (1:1). This was also reported for higher concentrations of cells by Murphy (1986). The mixture of the two media DME and Hams F12 seems to supply the optimum combination of nutrients for this cell line. All assays presented in this thesis, however, were carried out using MEM + 5% fcs as this experiment (Table 3.3) was performed towards the end of the project.

Variation of gaseous atmosphere

An attempt was made to grow RPMI-2650 cells in a gaseous atmosphere of 92% N_2, 3% O_2 and 5% CO_2 in the hope of improving clonal growth. The first experiment was set up at one cell density, 100 cells per 60mm plate in two gas atmospheres 5% CO_2 and 92% N_2; 3% O_2, 5% CO_2. The second experiment included two cell densities 15 and 30 cells per 16mm well in the two gas atmospheres.

TABLE 3.4 : Growth of RPMI-2650 cells in varying gas atmospheres

Initial cell density per 60mm plate	100 cells	
Gas	Mean colony area	CFE
5% CO_2; 95% Air	0.052 ± 0.01	32.2 ± 12.03
92% N_2; 3% O_2; 5% CO_2	0.04 ± 0.013	9.7 ± 6.5

n = 6

Initial cell density per 16mm well	15 cells		30 cells	
Gas	Mean colony area	CFE	Mean colony area	CFE
5% CO_2; 95% Air	0.67 ± 0.16	145.5 ± 33.3	0.81 ± 0.08	93.4 ± 13.9
92% N_2; 3% O_2; 5% CO_2	0.4 ± 0.26	62.2 ± 9.1	0.75 ± 0.14	76.7 ± 14.15

n = 12

A gas atmosphere of 92% N_2, 3% O_2 and 5% CO_2 appeared to be inhibitory when compared with growth in 5% CO_2.

Altered trypsinization methods

In an attempt to minimize the amount of trypsin introduced into stock cultures and clonal growth experiments, an experiment was set up using an altered trypsinization procedure, that is a low temperature trypsinization technique. Test cell concentrations used were 20, 30 and 40 cells per 16mm well and cells were subcultured at 4°C and 37°C for 15 minutes.

TABLE 3.5 : Growth of RPMI-2650 at varying temperature of trypsinization

Temperature of trypsinization	4°C (15 minutes)		37°C (15 minutes)	
Test cell conc. cells per 16mm well	Mean colony area	CFE	Mean colony area	CFE
20 cells	0.11 ± 0.03	73.0 ± 13.2	0.25 ± 0.46	21.43 ± 8.52
30 cells	0.12 ± 0.085	37.38 ± 16.85	0.11 ± 0.066	20.93 ± 9.85
40 cells	0.11 ± 0.019	48.9 ± 16.6	0.1 ± 0.05	15.6 ± 7.0

n = 12

Using the low temperature trypsinization technique a higher colony forming efficiency (CFE) was obtained for cells subcultured at 4°C.

Effect of DEAE dextran pre-coating on growth of RPMI-2650

An experiment was set up to test the effect of DEAE dextran pre-coating on growth of RPMI-2650 at clonal concentrations. This was reported to stimulate growth of RPMI-2650 in Murphy (1986). Cells were set up at clonal densities, 20 and 40 cells per 16mm well in MEM + 5% fcs. Wells were pre-coated with 100 gml^{-1} DEAE dextran for 3 hours at 37°C and washed with phosphate buffered saline. Control wells were uncoated.

TABLE 3.6 : Growth of RPMI-2650 with and without DEAE dextran pre-coating

Test cell conc. cells per 16mm well + MEM + 5% fcs	20		40	
+/- DEAE dextran (100µg ml^{-1}; 3 hours; 37°C)	Mean colony area	CFE	Mean colony area	CFE
-	1.01 ± 0.55	39.1 ± 1.2	1.11 ± 0.38	23.6 ± 8.32
+	0.84 ± 0.39	57.9 ± 13.2	0.85 ± 0.50	30.5 ± 14.5

n = 12

There was an increased colony forming efficiency with DEAE dextran pre-coated wells. These results suggest that DEAE dextran pre-coating or inclusion in the medium may be a useful addition for cloning work.

3.1.4. Effect of conditioned medium on clonal growth of RPMI-2650

A number of experiments were designed to evaluate the effect of conditioned medium on the clonal growth of RPMI-2650 cultures (see Table 3.7). Conditioned medium was collected as described (Section 2.9) or alternatively as described in Note, Table 3.7.

In experiment 1, a screening assay was set up in 60mm plates and conditioned medium:MEM + 5% fcs (1:2) tested. Conditioned medium (CM), both 24 and 48 hour, from RPMI-2650 wild type cells was used, and growth was compared with growth in MEM + 5% foetal calf serum and MEM + 1% foetal calf serum (fcs).

Experiment 2 was designed for a smaller surface area (16mm) and a lower cell density (10 and 25 cells). Growth was again compared with growth in MEM + 5% fcs. RPMI-2650 conditioned medium was collected as described (see Note, Experiment 2, Table 3.7) and was again used in the ratio 1:2 with MEM + 5% fcs.

Experiment 3 was again set up in 16mm wells and cell concentrations used were 10 and 20 cells. Growth was again compared with growth in MEM + 5% fcs Conditioned media was used with MEM + 5% fcs in the ratios 1:1 and 1:2 as specified (see Table 3.7 and Note, Experiment 3).

Experiment 4 contained cell densities of 20 and 40 cells per 16mm well. Growth was compared with growth in MEM + 5% fcs and conditioned medium was used with MEM + 5% fcs in the ratios 1:2 and 2:1 as described (see Table 3.7, Experiment 4).

TABLE 3.7 : Effects of conditioned medium on RPMI-2650 growth in monolayer

Experiment 1

Initial cell density per 60mm plate	medium or conditioned medium	Mean colony area	CFE
50 cells	MEM + 5% fcs	0.15 ± 0.03	22.0 ± 5.7
	RPMI CM (24 hour)	0.18 ± 0.042	24.0 ± 2.8
	RPMI CM (48 hour)	0.13 ± 0.0	16.0 ± 0.0
100 cells	MEM + 1% fcs	0.10 ± 0.011	10.7 ± 1.53
	MEM + 5% fcs	0.20 ± 0.007	15.0 ± 7.1
	RPMI CM (24 hour)	0.13 ± 0.0	22.0 ± 0.0
	RPMI CM (48 hour)	0.14 ± 0.0	17.0 ± 0.0
500 cells	MEM + 5% fcs	0.185± 0.049	16.5 ± 4.9
	RPMI CM (24 hour)	0.22 ± 0.0	20.0 ± 0.0

Note : Conditioned medium (CM) was used in the ratio 1:2 respectively with MEM + 5% f.c.s.

F.C.S. Batch No. = 20F

CM was collected from cultures containing serum-free medium

TABLE 3.7 (cont'd) : Effects of conditioned medium and medium on RPMI-2650 growth in monolayer

Experiment 2

Initial cell density per 16mm well	medium or conditioned medium	Mean colony area	CFE
10 cells	MEM + 5% fcs	0.14 ± 0.21	6.67 ± 10.3
	RPMI CM 24 hour (1:2) 10^2	0.58 ± 0.38	14.0 ± 5.5
	10^3	0.83 ± 0.91	18.0 ± 10.0
	10^4	0.56 ± 0.52	15.0 ± 10.5
25 cells	MEM + 5% fcs	0.27 ± 0.18	7.3 ± 5.9
	RPMI CM 24 hour (1:2) 10^2	0.23 ± 0.34	5.3 ± 7.9
	10^3	0.52 ± 0.17	16.8 ± 8.2
	10^4	0.66 ± 0.31	13.0 ± 3.83
	MEM + 5% fcs	0.66 ± 0.12	13.0 ± 5.0
	RPMI CM 48 hour (1:2) 10^2	1.12 ± 0.55	9.0 ± 3.83
	10^3	0.58 ± 0.31	12.0 ± 2.5
	10^4	0.78 ± 0.45	11.2 ± 1.8

Note : Conditioned medium was used with MEM + 5% fcs in the ratio described. It was collected from tissue cluster dishes (24 wells) from 10^2, 10^3 and 10^4 cells, that is, initial seeding concentration cell suspensions were made up in MEM - FCS.

FCS Batch No = 20F

TABLE 3.7 (cont'd) : Effects of conditioned medium on RPMI-2650 growth in monolayer

Experiment 3

Initial cell density per 16mm well	medium or conditioned medium	Mean colony area	CFE
10 cells	MEM + 5% fcs	0.58 ± 0.21	71.0 ± 18.0
	RPMI CM (48 hour) 1:2	0.55 ± 0.20	96.7 ± 27.3
	RPMI CM (48 hour) 1:1	0.19 ± 0.12	65.0 ± 21.0
20 cells	MEM + 5% fcs	0.47 ± 0.23	51.7 ± 23.0
	RPMI CM (48 hour) 1:1	0.40 ± 0.08	47.0 ± 22.0

Note : Cells were set up in MEM + 5% fcs to attach for 24 hours before medium was removed and CM:MEM + 5% fcs added.

Conditioned medium (CM) was used with MEM + 5% fcs in the ratio described.

F.C.S. Batch No = 20F

TABLE 3.7 (cont'd) : Effects of conditioned medium on RPMI-2650 growth in monolayer

Experiment 4

Initial cell density per 16mm well	medium or conditioned medium	Mean colony area	CFE
20 cells	MEM + 5% fcs	0.053 ± 0.074	4.6 ± 5.0
	RPMI CM (48 hour) 1:2	0.12 ± 0.075	10.83 ± 8.2
	RPMI CM (48 hour) 2:1	0.043 ± 0.08	2.1 ± 2.6
40 cells	MEM + 5% fcs	0.09 ± 0.095	6.25 ± 6.35
	RPMI CM (48 hour) 1:2	0.11 ± 0.09	7.3 ± 4.7
	RPMI CM (48 hour) 2:1	0.02 ± 0.05	0.42 ± 0.97

Note : Conditioned medium (CM) was used with MEM + 5% fcs in the ratio described.

F.C.S. Batch No = 20F

Summary of Findings

There appeared to be a small increase in colony forming efficiency (CFE) with a combination of 24 hour conditioned medium and MEM + 5% fcs in Experiment 1, which was consistent for three cell concentrations, 50, 100 and 500 cells. This was also apparent in Experiment 2 for conditioned media, particularly for CM from 10^3 cells. However, this was only apparent for CM collected at 24 hours and was not apparent for 48 hour CM.

In experiment 3, the ability of RPMI-2650 CM to stimulate cell growth at a very low cell population density is assessed. Cell concentrations used were 10 and 20 cells.
48 hour conditioned medium only was used in combination with MEM + 5% fcs in the ratio described. Stimulation was not apparent and the standard errors were large. In Experiment 4, 48 hour conditioned medium was again used with MEM + 5% fcs as described. Cell concentrations were again low, 20 and 40 cells per 16mm well. When the CM was used in a higher ratio with MEM + 5% fcs (2:1 respectively) it appeared to be inhibitory. These results suggest that a small amount of conditioning may be useful for growth at clonal densities. Conditioned media derived after 24 hours appeared to be preferable to 48 hour CM.

3.1.5 Effects of 3T3 feeder layers on clonal growth of RPMI-2650

The purpose of these experiments was to determine if the presence of 3T3 feeder cells increased the colony forming efficiency of RPMI-2650 cells in monolayer. Feeder cells were treated with mitomycin C to arrest their growth and set up 24 hours in advance as described (Section 2.8).

The first experiment was intended as a screening assay in 60mm plates to determine the validity of the test (see Table 3.8). Test cells were seeded at three concentrations 5×10^2, 1×10^3 and 1.5×10^3 cells per plate. Feeder layer concentrations used were 0, 1×10^4, 5×10^4 and 1×10^5 cells per plate. In the next experiment (Table 3.8, Experiment 2), cells were set up at clonal densities in 16mm wells. Two test cell concentrations were seeded, 50 and 100 cells per well and feeder layer concentrations used were 0, 1×10^4, 5×10^4 and 1×10^5 cells per well. Experiment 3 (Table 3.8 continued) included an internal control, that is wells containing feeder layers but no indicator test cells. This was in order to be assured that colonies counted were in fact derived from test cells and not from feeder layers. Test cells were seeded at 50 cells per 16mm well and feeder layer concentrations used were 0, 5×10^3, 1×10^4 and 5×10^4 cells per well. The next experiment (Experiment 4) was similar to Experiment 3 and also included an internal control. In Experiment 5, conditions used approximated cloning conditions, test cell concentrations were 20 and 40 cells per 16mm well and feeder layer concentrations used were 0, 5×10^3, 1×10^4 and 5×10^4 cells per well. It was thought that by lowering the test cell concentration with unaltered feeder layer concentrations, a feeder effect might become more apparent. This experiment also contained internal controls.

TABLE 3.8 : Growth of RPMI-2650 cells in the presence of 3T3 feeder cells

Experiment 1

Test layer conc. cells per 60mm plate	500		1000		1,500	
Feeder layer conc. per 60mm plate	CFE	Mean Colony Area	CFE	Mean Colony Area	CFE	Mean Colony Area
0	18	0.29	10	0.17	8	0.12
1×10^4	25.6	0.36	17.5	0.086	12.4	0.096
5×10^4	25.6	0.11	15.2	0.085	16.0	0.098
1×10^5	24.4	0.11	20.6	0.111	No results	

Experiment 2

Test layer conc. cells per 16mm well	50		100	
Feeder layer conc. per 16mm well	Mean Colony Area	CFE	Mean Colony Area	CFE
0	0.17 ± 0.066	22.6 ± 7.6	0.14 ± 0.03	43.5 ± 9.2
1×10^4	0.14 ± 0.037	49.0 ± 18.4	0.12 ± 0.02	40.0 ± 1.4
5×10^4	0.26*	52.0*	0.19 ± 0.02	40.0 ± 8.02
1×10^5	0.17*	74.0*	0.164 ± 0.028	45.3 ± 4.9

Note : * indicates that only one well was counted. In all other cases n = 6 for calculation of standard error of mean (S.E.M.)

F.C.S. Batch No = 20F

In experiment 1, no statistics were available

TABLE 3.8 (cont'd) : Growth of RPMI-2650 cells in the presence of 3T3 feeder cells.

Experiment 3

Test layer conc. cells per 16mm well	0 cells		50 cells	
Feeder layer conc. per 16mm well	Mean Colony Area	CFE	Mean Colony Area	CFE
0	0.0	0.0	0.204 ± 0.056	32.34 ± 9.06
5×10^3	0.009 ± 0.008	3.0 ± 5.48	0.28 ± 0.055	88.6 ± 17.6
1×10^4	0.059 ± 0.03	1.46 ± 2.02	0.27 ± 0.046	93.08 ± 18.12
5×10^4	0.12 ± 0.14	1.34 ± 1.64	0.15 ± 0.02	58.8 ± 11.0

Experiment 4

Test layer conc. cells per 16mm well	50 cells	
Feeder layer conc. per 16mm well	Mean colony area	CFE
0	0.39 ± 0.095	26.0 ± 4.5
5×10^3	0.46 ± 0.17	31.5 ± 8.48
1×10^4	0.48 ± 0.15	40.6 ± 7.96
5×10^4	0.47 ± 0.12	41.6 ± 14.4

n = 12

Note : At each feeder concentration in Experiment 4, controls without cells gave no colony counts.

In experiment 3, there was some background readings from feeder layers.

F.C.S. Batch No = 20F

TABLE 3.8 (cont'd) : Growth of RPMI-2650 cells in the presence of 3T3 feeder cells

Experiment 5

Test layer conc. cells per 16mm well	20		40	
Feeder layer conc. cells per 16mm well	Mean colony area	CFE	Mean colony area	CFE
0			0.43 ± 0.26	10.0 ± 6.12
5×10^3	0.315 ± 0.12	36.25 ± 17.02	0.35 ± 0.10	48.1 ± 19.19
1×10^4	0.56 ± 0.11	85.6 ± 31.0	0.47 ± 0.11	66.0 ± 19.25
5×10^4	0.25 ± 0.089	90.6 ± 44.76	0.33 ± 0.24	35.0 ± 15.4

n = 6

Note : At each feeder concentration controls without cells gave no colony counts.

F.C.S. Batch No = 20F

Summary of Findings

From the first experiment, it appeared that the colony forming efficiency was increased in the presence of mitomycin C treated 3T3 feeder layers. This increase was consistent for the three test cell concentrations. In Experiment 2 (see Table 3.8), the colony forming efficiency was again increased in the presence of feeder layers at a test cell concentration of 50 cells per well. However, at 100 cells per well this increase was not apparent. A test cell concentration of 100 cells per well may have been too high to allow experimental data to be collected and for this reason a test cell concentration of 50 cells per well or lower was used in the following experiments. In Experiments 3 and 4, a test cell concentration of 50 cells per well only was seeded. There was an increase in colony forming efficiency again in the presence of 3T3 feeder cells in both experiments. In experiment 3, a feeder layer concentration of 1×10^4 cells per well gave maximum stimulation. In this experiment there were some background readings with feeder layers but these were not significant. In Experiment 4, maximum stimulation was obtained at a feeder concentration of 5×10^4 cells per well but again a feeder concentration of 1×10^4 cells per well gave a good increase in colony forming efficiency. There were no background readings in this experiment.

The final experiment in this series again showed increased colony forming efficiencies in the presence of 3T3 feeder layers. Maximum stimulation was obtained with 5×10^4 feeder cells per well in the case of a test cell concentration of 20 cells per well and with 1×10^4 feeder cells per well in the case of a test cell concentration of 40 cells per well.

In Experiments 3, 4 and 5 strict controls were adhered to and this removed any possibilities of the colonies being other than RPMI-2650 colonies.

All of the information derived from these experiments pointed to a feeder effect on RPMI-2650 cells by mitomycin C treated 3T3 cells in monolayer.

It is therefore suggested that 3T3 feeder cells be considered in any strategy for facilitating cloning of RPMI-2650 cells.

3.1.6. Effects of RPMI-2650 feeder layers on clonal growth of RPMI-2650

The purpose of these experiments was to determine if the presence of RPMI-2650 feeder cells increased the CFE of RPMI-2650 cells in monolayer. Feeder cells were treated with mitomycin C to arrest their growth and they were set up 24 hours in advance as described in Section 2.8.

The first experiment (see Table 3.9, Experiment 1) was designed to test the feeder effect of RPMI-2650 (feeder layer concentrations were 5×10^4 and 1×10^5 per 16mm well) on RPMI-2650 cells (test cell concentrations were 10 and 50 cells per well). Test cells were also plated without feeder layers. It was found that these feeder layer concentrations were inappropriate for this test as there was a decrease in CFE. This was attributed to the high concentration of feeders causing either a depletion of growth factors in the medium or an accumulation of inhibitory products causing cessation of cell proliferation.

The second experiment was altered because of this information and a larger surface area for growth was chosen. Feeder layer concentrations were as in Experiment 1, with a concentration of 1×10^4 cells per 60mm plate in addition and test cell concentrations were 50 and 100 cells per 60mm plate. Test cells were also plated without feeder layers.

In the third experiment (see Table 3.9, Continued), test cell concentrations were varied and one feeder layer concentration only was examined. Test cells plated were 10, 20, 30, 40 and 50 cells per 16mm well with and without feeder layers and the feeder layer concentration was 5×10^4 cells per 16mm well.

Experiments 4, 5 and 6 (Table 3.9 continued) were designed with lower feeder layer concentrations, 0, 1 x 10^3, 5 x 10^3 and 1 x 10^4 cells per 16mm well. All of these experiments contained a test cell concentration of 20 cells per well and a control with no test cells for each feeder layer concentration but experiments 4 and 6 also contained a test cell concentration of 30 cells and 40 cells per well respectively.

TABLE 3.9 : Growth of RPMI-2650 cells in the presence of RPMI feeder cells

Experiment 1

Test layer conc. cells per 16mm well	10		50	
Feeder layer conc. per 16mm well	Mean colony area	CFE	Mean colony area	CFE
0	0.36 ± 0.10	45.0 ± 16.4	0.24 ± 0.09	30.0 ± 15.0
5×10^4	0.086 ± 0.086	18.3 ± 19.0	0.12 ± 0.14	10.67 ± 10.25
0	0.415 ± 0.13	52.0 ± 12.0	0.12 ± 0.14	10.67 ± 10.25
1×10^5	0.032 ± 0.07	2.0 ± 4.5	0.03 ± 0.07	4.0 ± 7.3

n = 12

Experiment 2

Test layer conc. cells per 60mm plate	50		100	
Feeder layer conc. per 60mm plate	Mean colony area	CFE	Mean colony area	CFE
0	0.022 ± 0.0044	17.0 ± 4.24	0.022 ± 0.002	14.5 ± 0.71
1×10^4	0.0314*	42.0*	0.036 ± 0.014	19.5 ± 6.4
5×10^4	0.052*	56.0*	0.045 ± 0.00021	49.5 ± 13.4
1×10^5	0.043*	100.0*		

Note : * indicates that only one plate was counted. In all other cases,

n = 3 for calculation of S.E.M.

F.C.S. Batch No = 20F

TABLE 3.9 (cont'd) : Growth of RPMI-2650 cells in the presence of RPMI feeder cells

Experiment 3

Feeder layer conc per 16mm well	0		5×10^4	
Test layer conc. cells per 16mm well	Mean colony area	CFE	Mean colony area	CFE
10 cells	0.28 ± 0.23	65.0 ± 35.0	0.11*	90.0*
20 cells	0.14 ± 0.03	28.3 ± 2.9	0.115 ± 0.05	67.5 ± 38.9
30 cells	0.18*	17.0*	0.15 ± 0.10	39.5 ± 4.95
40 cells	0.15 ± 0.113	23.75 ± 19.44	0.0845 ± 0.006	36.25 ± 8.84
50 cells	0.26*	8.0*	0.123 ± 0.081	47.0 ± 4.2

Note : * indicates that only one well was counted. In all other cases, n = 12 for calculation of S.E.M.

F.C.S. Batch No = 20F

TABLE 3.9 (cont'd) : Growth of RPMI-2650 cells in the presence of RPMI feeder cells

Experiment 4

Test layer conc. cells per 16mm well	20		30	
Feeder layer conc. per 16mm well	Mean colony area	CFE	Mean colony area	CFE
0	0.0	0.0	0.0	0.0
1×10^3	0.22 ± 0.07	57.5 ± 20.2	0.33 ± 0.06	46.1 ± 23.0
5×10^3	0.28 ± 0.033	293.3 ± 61.2	0.28 ± 0.07	257.8 ± 92.7
1×10^4	0.305 ± 0.09	66.25 ± 20.0	0.24 ± 0.12	63.3 ± 15.6

Experiment 5

Test layer conc. cells per 16mm well	20	
Feeder layer conc. per 16mm well	Mean colony area	CFE
0	0.24 ± 0.08	50.0 ± 28.2
1×10^3	0.22 ± 0.08	36.0 ± 18.16
5×10^3	0.21 ± 0.08	46.7 ± 13.9
1×10^4	0.074	8.75 ± 2.5

n = 6

Note : At each feeder concentration controls without cells gave no colony counts.

F.C.S. Batch No = 20F

TABLE 3.9 (cont'd) : Growth of RPMI-2650 cells in the presence of RPMI feeder cells

Experiment 6

Test layer conc. cells per 16mm well	20		40	
Feeder layer conc. per 16mm well	Mean colony area	CFE	Mean colony area	CFE
0	0.06 ± 0.27	1.25 ± 4.4	0.032 ± 0.10	0.94 ± 2.6
1×10^3	0.093 ± 0.045	19.17 ± 6.6	0.14 ± 0.042	13.75 ± 4.43
5×10^3	0.33 ± 0.13	76.25 ± 31.6	0.37 ± 0.066	75.83 ± 24.06
1×10^4	0.47 ± 0.11	110.6 ± 14.7	0.41 ± 0.07	104.4 ± 10.6

n = 12

Note : At each feeder concentration controls without cells gave no colony counts.

F.C.S. Batch No = 20F

Summary of Findings

As already discussed in this Section, there was no increase in CFE, in Experiment 1, in the presence of feeder layers. This was attributed to the high feeder layer concentration and low surface area for growth causing either depletion of growth factors or an accumulation of inhibitory products. In Experiment 2, there were definite signs of an increased CFE in the presence of mitomycin C treated RPMI-2650 feeder cells, particularly at feeder layer concentrations of 5×10^4 and 1×10^4 cells per 60mm plate. However at a test cell concentration of 50 cells per plate, it was difficult to interpret as in many cases only one plate was counted due to contamination See Table 3.9, Experiment 2). It could be said however, that the direction was in favour of an increased CFE with feeder layers. Experiment 3 also gave good indications of an increased CFE with feeder layers, although again in some instances only one plate was counted.

In Experiment 4, there was a significant increase in CFE in the presence of 5×10^3 feeder cells per 16mm well. This was consistent with both test cell concentrations. This was consolidated in Experiment 6 where maximum stimulation was obtained at a feeder layer concentration of 1×10^4 cells per 16mm well. This was again consistent for two test cell concentrations and internal controls gave no colony counts which identified colonies as arising from test cells and not feeder cells.

Apart from Experiments 1 and 5, evidence was accumulating in favour of an autostimulatory feeder effect of RPMI-1650 cells on RPMI-2650 cells in monolayer.

It was difficult to interpret mean colony area results for both Section 3.1.5 and 3.1.6 as this appeared to be a variable parameter and the validity of these values measured by the Image Analyser at clonal sizes must be questionable. However, there was a slight indication of an increased mean colony area overall in the presence of RPMI-2650 feeder layers.

The results presented in this section provide evidence that RPMI-2650 feeder layers may also be a useful stratagem in overcoming the difficulties of cloning RPMI-2650 cells in monolayer.

3.2.1 Primary Samples

A glioma sample was obtained in BME + 5% foetal calf serum + penicillin (300 units ml^{-1}), streptomycin (300 g ml^{-1}), tetracycline (150 g ml^{-1}) and fungizone (50 g ml^{-1}).

The sample was incubated at 37°C in this antibiotic mixture for 30 minutes to minimize microbial contamination and washed in sterile phosphate buffered saline in a laminar flow three times, before being set up as explants (see Materials and Methods, Section 2.16). Explants were refed at 3-4 day intervals. There was cellular growth from the explants and after 4 weeks it was possible to subculture one flask, a total cell count of 1 x 10^5 cells being obtained (See Fig. 20).

This primary sample was subcultured for a period of over one year in culture, and deteriorated around passage number 16. It was not known why the sample eventually deteriorated. Two vials of this sample remain frozen in storage at passage number 15.

3.2.2 Effect of SW1088 feeder layers on growth of SW1088 cells in agar.

SW1088 is an established astrocytoma cell line. The purpose of the experiments was to determine if the presence of SW1088 feeder cells increased the colony forming efficiency (CFE) of SW1088 cells physically separated from the feeders in a double layer agar assay system. Feeder cells were set up 24 hours in advance as described in Section 2.8, and agar layers and indicator SW1088 cells were added as described in Section 2. In Experiments 1, 2 and 3 (see Table 3.10), cells were plated without feeder layers to estimate the 'cut-off point' of growth of SW1088 in agar. In Experiment 1, cells were set up at concentrations of 3.3×10^3, 4.3×10^3, 1.7×10^4 and 3.3×10^4 cells per 35mm dish. Cell densities in Experiment 2 were 0.93×10^4, 2.8×10^4, 0.93×10^5 and 2.8×10^5 cells per 35mm dish. Cell densities in Experiment 3 were 0.33×10^3, 1×10^3, 1.66×10^3, 1×10^4, 1.66×10^4 and 0.33×10^5 cells per 35mm dish. Cells were passaged 48 hours before assay and all colonies greater than 50μ were counted.

In the presence of feeder layers an experiment was set up to compare the CFE of SW1088 cells, in the presence of untreated live SW1088 feeder cells and mitomycin C treated SW1088 feeder cells. Cells were set up at concentrations of 5×10^3, 1×10^4 and 5×10^4 cells per 30mm plate and feeder cells were set up between 5×10^3 and 1×10^5 cells per 30mm plate (see Table 3.11).

An experiment was also set up in monolayer to compare CFE in monolayer. Cells were set up at densities of 2.5×10^3, 5×10^3, 1×10^4, 2×10^4 and 4×10^4 cells per 35mm dish and cells trypsinized and counted after 12 days.

In addition, an experiment was set up to compare the growth of SW1088 cells in monolayer in varying serum concentrations, 0.5, 1.5, 3.5, 5.0 and 10.0% fcs (see Table 3.13). Initial cell density was 7 x 10^4 cells per 35mm plate and cells were trypsinized and counted after 12 days.

TABLE 3.10 : Growth of SW1088 cells in agar without feeder layers

Experiment 1

Initial cell density per 35mm dish	CFE
3.3×10^3	0
4.3×10^3	0
1.67×10^4	0
3.3×10^4	1.45

Experiment 2

Initial cell density per 35mm dish	CFE
0.93×10^4	0
2.8×10^4	2.5
0.93×10^5	1.3
2.8×10^5	0.4

Experiment 3

Initial cell density per 35mm dish	CFE
0.33×10^3	0
1×10^3	0
1.66×10^3	0
1×10^4	0.24
1.66×10^4	0.58
0.33×10^5	0.30

n = 3

Note : Cells were passaged 48 hours before assay to standardise assay conditions.

All colonies greater than 50μ were counted

CFE % = percentage colony forming efficiency

TABLE 3.11 : Colony forming efficiencies of SW1088 cells in the presence of SW1088 feeder cells

Initial cell density per 30mm plate	feeder concentrations/30mm plate				
		A		B	
	0	5×10^3	5×10^4	5×10^4	1×10^5
5×10^3	0	0	0	6.75	5.75
1×10^4	0	0	0	1.25	1.87
5×10^4	0.475	0.15	0	0	0.15

Note : n = 3

All colonies greater than 50μ were counted

A = untreated live feeders (SW1088 cells)

B = mitomycin C treated SW1088 feeders

Unfortunately, no statistics are available for these results

TABLE 3.12 : Growth of SW1088 cells in monolayer

Initial cell density per 35mm dish	mean Final cell density (12 days)
2.5×10^3	0
5×10^3	1.5×10^4
1×10^4	2.25×10^4
2×10^4	4.5×10^4
4×10^4	6×10^4

Note : Cells were growing in BME + 10% fcs

n = 3

SW1088 cells; P36 were used

No statistics are available

TABLE 3.13 : Varying serum concentration on SW1088 growth

Initial cell density per 35mm plate : 7×10^4 cells

% foetal calf serum	mean cell density (12 days)
0.0	3×10^4
0.5	10.5×10^4
1.5	22.5×10^4
3.5	24.75×10^4
5.0	33.75×10^4
10.0	41.25×10^4

Note : n = 2

No statistics are available

Summary of Findings

Table 3.10 shows that SW1088 cells form colonies in agar without feeder layers only at concentrations of 10^4 per 35mm dish or higher. As cell density increases colony forming efficiency reaches a maximum and then decreases. The three experiments gave reasonably consistent results.

Table 3.11 shows the results in the presence of feeder layers. Feeders used were SW1088 cells, either untreated live cells or mitomycin C treated SW1088 cells. There was very little or no growth with live feeders probably due to either depletion of growth factors or accumulation of inhibitory products due to high cell densities.

Mitomycin C treated feeders appeared to give CFEs above controls at feeder concentrations of 5×10^4 cells/30mm plate and 1×10^5 cells/30mm plate and test cell densities of 5×10^3 and 1×10^4 cells/30mm plate. The optimum combination of feeders and test cells from Table 3.11 appeared to be a feeder layer concentration of 5×10^4 cells per 30mm plate and a test cell density of 5×10^4 cells per 30mm plate. At higher concentrations of both these parameters, CFE was decreased. In contrast, in monolayer (Table 3.12) SW1088 cells will form colonies down to concentrations of 5×10^3 per 35mm plate.

Table 3.13 shows the effect of foetal calf serum concentration on cell growth. Growth was improved with increasing foetal calf serum percentage.

All of this work was completed as preliminary work for improving cloning conditions of SW1088 cells.

3.2.3 Transforming growth factor production of SW1088 cells and brain cells

In addition to the work previously described for SW1088 cells and the primary brain sample, an experiment was set up to compare the transforming growth factor production from these cells (See Table 3.14). Control medium was BME + 5% FCS and BME + FCS was used for culturing both SW1088 cells and brain samples. Conditioned medium was collected 48 hours after addition of serum free medium. Also included in this experiment was conditioned medium from a known transforming growth factor producer, RPMI-2650 and two of its isolated clones (See Table 3.14). Control medium in this case was MEM + 5% FCS and MEM-FCS.

Normal rat kidney (NRK) passage 23 cells were used as indicator cells (See Section 2.18).

Clone A was derived from RPMI-2650 cells (P50) in monolayer. Clone B was derived from RPMI-2650 cells (P50) in agarose.

TABLE 3.14 : Comparison of transforming growth factor production from samples of brain, SW1088 cells and RPMI-2650 cells and its clones.

Sample	Mean number of colonies
BME - FCS	8.6 ± 3.05
BME + FCS (5%)	35.0 ± 1.41
Brain sample CM	41.0 ± 4.24
SW1088 cell CM	42.0 ± 5.3
MEM - FCS	6.3 ± 3.2
MEM + FCS (5%)	22.5 ± 3.53
RPMI-2650 CM	36.5 ± 4.95
Clone A CM	23.3 ± 2.31
Clone B CM	15.0 ± 4.24

Note : Clone A was derived from RPMI-2650 cells (P50) in monolayer
Clone B was derived from RPMI-2650 cells (P50) in agarose

All conditioned medium was collected from cultures containing serum-free medium.

n = 3

Summary of Findings

RPMI-2650 conditioned medium gave an increased mean number of colonies, compared with control medium as expected. Clones A and B also gave higher colony counts than the control medium but less than RPMI-2650 wild type.

It also appeared from this experiment that both the brain sample conditioned media and SW1088 conditioned media contained a transforming growth factor as colony counts were greater than controls. It would be well worthwhile to investigate this transforming growth factor further.

3.3 Mutagenesis Results

In addition to preparing random clones, it was decided to obtain, by mutagenesis, cell variants selected for particular phenotypes, namely 6-thioguanine resistant (6-TG^r) cells.

EMS Kill Curve

Ethylmethanesulphonic acid (EMS) was chosen as a mutagen. To choose an EMS concentration which would generate mutants but would not drastically reduce cell viability was necessary. The highest possible EMS concentration consistent with high viability would generate more mutant (see Materials and Methods, Section 2). Initial cell survival was estimated by trypsinizing and counting, viability being estimated by use of trypan blue (16 hour exposure to EMS).

There was a slight drop in viability in RPMI-2650 between 500 and 750 gml^{-1} EMS.

For cell recovery, $25cm^2$ flasks were inoculated with 5×10^5 cells for each EMS concentration. $25cm^2$ flasks were then trypsinized and counted after 7 days.

To test cloning efficiency, 60mm plates were inoculated with 1×10^3 cells per plate in duplicate for each EMS concentration. After ten days, plates were washed with PBS, stained with Leishmann's stain and colonies counted on an Image Analyser. The colony forming efficiency dropped dramatically between 100 and 250 gml^{-1}. It appeared that the optimal EMS concentration for mutagenesis was between 100 and 250 gml^{-1}.

Thioguanine-Kill curve RPMI-2650.

To select for thioguanine resistant mutants, a kill curve was used to choose a thioguanine concentration.

RPMI-2650 cells were seeded at a concentration of 1 x 10^5 cells per 60mm plate. Thioguanine was made up as 100mM stock in 1N NaOH and filter sterilized with a 0.22µm filter.

Cellular resistance to thioguanine is almost always associated with a complete loss of hypoxanthine - guanine phosphoribosyl transferase (HGPRT) activity (Meyer et al., 1980). Therefore, 6-thioguanine was chosen as the selective agent for HGPRT mutants.

Selection of 6-thioguanine resistant mutants

To select for 6-thioguanine resistant mutants, RPMI-2650 cells were again treated with mutagen, EMS, in large 75cm^2 flasks at chosen concentrations, 100µg/ml and 200µg/ml and set up in flasks and 100mm plates with thioguanine containing medium, after the expression time. Expression times chosen were 6, 8, 10 and 12 days after EMS treatment. There appeared to be very little difference between colony formation for expression times, although later expression times appeared somewhat better.

Four plates (100mm) were set up with 2.5 x 10^5 cells per plate, and incubated at 37°C in 5% CO_2/95% air for 7-14 days or until colonies formed. A lower cell concentration is recommended by most authors, but as RPMI-have a low cloning efficiency, a concentration of 2.5 x 10^5 cells per plate was chosen.

After colonies had formed, they were marked and cloned (see Materials and Methods) into multi-well plates and serially

propagated into vessels of increasing size.

To show these were actually HGPRT mutants, hypoxanthine aminopterin-thymidine (HAT) medium was used. Mutants lacking HGPRT, in the presence of aminopterin, a folate antagonist, will not survive as the 'de novo' pathway is blocked. Normal wild type cells will survive if supplied with thymidine and hypoxanthine as they have the required salvage enzymes (Giles and Ruddle, in Kruse and Patterson, 1973).

It was found in three separate experiments in 35mm plates and 25cm^2 flasks that these cells would not survive in the presence of HAT medium, whereas in ordinary medium without HAT, cells proliferated. This demonstrated that these cells were actually HGPRT mutants.

TABLE 3.15 : Ethylmethanesulphonic acid kill curve

EMS Conc. ($\mu g\ ml^{-1}$)	Cell Count
Control (no EMS)	2.4×10^7
50	1.7×10^7
100	2.5×10^7
250	3×10^5
500	0
750	0
1000	0

It appeared that 100$\mu g\ ml^{-1}$ did not drastically reduce cell viability, whereas 250$\mu g\ ml^{-1}$ did.

3.4.1 Colony Forming Efficiencies of Clones of RPMI-2650 in Monolayer

For a culture to be considered a pure culture it would seem reasonable to suppose that the cells composing it should all have about the same rate of growth (Willmer, 1935). Therefore, to examine possible clonal variations in growth rates a number of experiments were set up to test the colony forming efficiences (CFE) of clones of RPMI-2650 in monolayer.

For the evaluation of CFE in monolayer, cells were set up in 30mm plates (Table 3.16). Three experiments were designed using two cell concentrations 5×10^3 and 1×10^4 cells per plate (see Table 3.16, Experiments 1, 2 and 3).
The final experiment also tested two additional cell concentrations 1×10^3 and 5×10^4 cells per plate. As it was impossible to include all the samples in any one particular experiment, it was necessary to overlap samples in separate experiments. If all twenty cell suspensions were tested simultaneously, this would have significantly increased the possibility of inaccuracy or cross-contamination.

Plates were incubated for a minimum of 7 days and stained. The number and size of colonies was estimated with an Image Analyser (see Section 2). Growth was expressed as a percentage of growth of RPMI-2650 wild type cells.

TABLE 3.16 : Colony forming efficiencies of clones of RPMI-2650 in monolayer

Initial cell density per 30mm plate :	5×10^3		1×10^4	
cells/clone	Mean colony area	CFE	Mean colony area	CFE
Experiment 1				
RPMI-2650	100.0 ± 4.83	100.0 ± 38.1	100.0 ± 21.05	100.0 ± 63.5
Clone 10(P16)	53.1*	69.23*	68.42 ± 15.8	138.5 ± 1.4
Clone 14(P16)	55.2 ± 20.7	21.1 ± 2.3	68.42 ± 7.4	21.1 ± 4.04
Clone 18	131.03 ± 13.8	109.6 ± 42.3	136.8 ± 73.7	157.7 ± 69.2
Clone 12	103.4 ± 41.4	25.0 ± 5.96	68.4 ± 41.4	38.46 ± 0.38
Clone 10(P15)	63.4 ± 11.7	153.8 ± 32.7	63.2 ± 7.9	211.5 ± 12.5
Experiment 2				
RPMI	100.0*	100.0*	100.0 ± 8.6	100.0 ± 1.25
Clone 18(P18)	135.0*	312.8*		
Clone 12(P13)	122.5 ± 8.7	10.6 ± 2.13	61.5 ± 26.1	4.9 ± 0.66

TABLE 3.16 (cont'd) : Colony forming efficiencies of clones of RPMI-2650 in monolayer

Initial cell density per 30mm plate :	1×10^3		5×10^3		1×10^4		5×10^4	
cells/clone	Mean colony area	CFE	Mean colony area	CFE	Mean colony area	CFE	Mean colony area	CFE
RPMI P61	100	100	100	100	100	100	100	100
Clone 9(P13)	52.8	18.2			52.9	38.5	96.1	146.7
Clone 4(P13)	71.9	16.4	47.5	5.33	54.9	18.8	144.4	65.33
RPMI	100	100	100	100	100	100		
Clone 2			88.7	90.9				
Clone 11							88.8	192.0
Clone 14			53.9	8.0	200.0	139.8		

TABLE 3.16 (cont'd) : Colony forming efficiencies of clones of RPMI-2650 in monolayer

Initial cell density per 30mm plate :		5×10^3		1×10^4	
	cells/clone	Mean colony area	CFE	Mean colony area	CFE
Experiment 3					
	RPMI	100.0 ± 17.0	100.0 ± 16.4	100.0 ± 5.5	100.0 ± 19.4
	Clone 14(P16)	141.1 ± 8.04	181.5 ± 37.9	172.4 ± 72.4	109.3 ± 48.2
	Clone 12(P12)	35.7 ± 4.3	3.08 ± 2.05	56.9 ± 7.24	3.11 ± 1.48
	Clone 2(P17)	57.14 ± 6.25	7.49 ± 5.13	36.21 ± 12.1	1.48 ± 0.78
	Clone 18(P14)	110.7 ± 10.34	95.9 ± 16.4		
	Clone 13	60.71 ± 21.4	9.23 ± 7.7	153.4 ± 62.1	10.62 ± 2.78
A	Clone 11(P15)	46.43*	43.08*	72.4 ± 10.34	66.6 ± 17.9
B	Clone 11(P15)	56.9 ± 10.0	6.15 ± 0	75.86 ± 15.5	16.1 ± 2.05

Note : All cells were trypsinized 48 hours before assay unless otherwise stated

A = trypsinized 5 days before assay

B = trypsinized 12 days before assay

* indicates that only one plate was counted. In all other cases n = 3 for calculation of S.E.M.

F.C.S. Batch No. = 116

Summary of Findings

During subculturing of stocks, it was apparent from microscopic observation that some clones had a slower growth rate than others. This applied particularly for clones 4 and 8. Clone 4 was eventually lost in culture.

In the experiments it was apparent that some clones gave consistently higher colony forming efficiencies. It could be said that clone 18 and clone 10 gave consistently high CFEs, whereas clone 12 consistently gave a lower CFE than the wild type RPMI-2650. It was difficult to interpret some of the results. This was mainly attributed to the inability to test all samples simultaneously and the necessity for cells to be in identical states before testing. Cultures were sub-cultured 48 hours before testing unless otherwise stated in an attempt to standardize conditions.

However, results may also be affected by passage number, culture conditions before assay (medium etc), trypsinization conditions etc. However, in spite of all these difficulties, it was apparent that there were clonal variations with regard to growth rate in monolayer.

3.4.2 Colony Forming Efficiencies of clones of RPMI-2650 in agar

The cloning efficiency of cells in semi-solid media is usually regarded as a measure of the degree of loss of anchorage dependence (Vasiliev and Gelfand, 1981) and anchorage independence seems to be correlated with oncogenicity (Barrett et al., 1979).

For these reasons a number of experiments were designed (Table 3.17) to compare the abilities of subpopulations to grow in agar. The clonogenic assay was used (Section 2.14) and cells were suspended in the upper layer of a double-layer agar assay system and colonies counted microscopically after 7-10 days with the use of a graticule. Colonies greater than 50μ were counted. Samples were set up in triplicate.

Cells were plated at concentrations of 5×10^3 to 1×10^6 cells in agar (see Table 3.17). As with the monolayer assay there were difficulties in manipulating a number of cell suspensions.

Growth was again expressed as a percentage of growth of RPMI-2650 wild type.

TABLE 3.17 : Colony forming efficiencies of clones of RPMI-2650 in agar

	Initial cell density per 30mm plate :	5×10^4	1×10^5	5×10^5	1×10^6
	Cells/clones	CFE	CFE	CFE	CFE
Expt1	RPMI P64	100.0 ± 25.8	100.0 ± 17.5	100.0*	100.0*
	Clone 10 (P18)	61.6 ± 9.9	80.7 ± 8.8	95.2 ± 42.8	
	Clone 18 (P17)		147.4 ± 35.1	171.4 ± 38.1	100.0 ± 28.6
	Clone 2 (P20)	0.0 ± 0.0	12.3*	52.4 ± 9.5	
	Clone 13 (P18)	0.0 ± 0.0	24.6 ± 12.3	90.5 ± 17.6	76.2 ± 6.2
	Clone 8 (P6)	77.5 ± 17.7	82.5 ± 11.4	128.6 ± 11.4	
	Clone 14 (P19)	252.5 ± 53.7	149.1 ± 26.3	190.5 ± 30.5	
	Clone 11 (P16)	190.8 ± 26.3	121.0 ± 30.0	123.8 ± 13.3	
	Clone 12 (P12)	61.63 ± 7.9	68.4 ± 17.5	80.9 ± 41.9	
	Clone 18	7.55 ± 5.96	103.5 ± 10.2	104.8 ± 4.3	
	Clone 4	4.77	47.4 ± 2.98	71.4 ± 5.24	
	Clone 10	4.0*	12.63*	80.95 ± 8.09	
Expt2	RPMI	100.0 ± 19.4	100.0 ± 0.45	100.0 ± 1.67	
	Clone 12	61.1 ± 5.5	66.3 ± 22.5	65.15*	
	Clone 2 (P18)	0	9.2 ± 5.6	8.94 ± 4.5	
	Clone 14 (P17)	11.1 ± 7.4	30.3 ± 7.5	42.4 ± 3.03	

Note : * indicates that only one plate was counted. In all other cases, n = 3 for calculation of S.E.M.

All colonies > 50μ were counted

TABLE 3.17 (cont'd) : Colony forming efficiencies of clones of RPMI-2650 in agar

Initial cell density per 30mm plate :	5×10^3	1×10^4	5×10^4	1×10^5	5×10^5	1×10^6
A Cells/clones	CFE	CFE	CFE	CFE	CFE	CFE
RPMI P62	100	100	100			
Clone 18 (P14)	0	93.3	87.7			
Clone 4 (P11)	0	0	33.9			
Clone 14 (P12)	0	0	32.3			
Clone 9 (P12)	0	0	100			
RPMI (P60)	100	46.7	135.4			
B						
RPMI			100	100	100	100
Clone 13			0	0	20	43.6
Clone 10			0	0	53.3	32.7
Clone 14a*			0	0	70.8	100
Clone 14b*			0	0	60	

Note : A = F.C.S. Batch No. 116 (except *)
B = F.C.S. Batch No. 30Q
*a = 116
*b = 30Q

Summary of Findings

Cells were plated at concentrations between 5×10^3 and 1×10^6 per plate. At the higher concentrations of 5×10^5 and 1×10^6 cells per plate, counting of colonies became rather difficult due to crowding of colonies. However results were obtained (see Table 3.17) and growth was expressed as a percentage of growth of the RPMI-2650 wild type. Four separate experiments were set up on separate days and this may explain the variation between different experiments which was not found within an experiment. In Experiment 1, clones 11 and 14 gave higher colony forming efficiencies than the wild type RPMI-2650 in agar. Clone 18 also showed good growth patterns in all experiments. Clones 2, 4 and 13 generally gave low colony forming efficiencies as did clone 12.

Despite the difficulty in interpretation, it was established that all clones had the capacity for anchorage-independent growth but it appeared that with the exception of clones 11, 14, and 18, wild type RPMI-2650 cells generally grew better in agar than its subpopulations. However, it was demonstrated that clonal variations were present for this property.

3.4.3 Clonal variation in transforming growth factor production

Two experiments (see Section 2.18) were set up initially at an indicator concentration of 6×10^3 cells per 30mm plate but no colony growth was observed. It was thought that the indicator cells concentration was too low, therefore this was increased to 2×10^4 cells per 30mm plate (see Table 3.18). Both 48 and 96 hour conditioned media were tested. Conditioned medium (CM) was collected as described in Section 2.9.

A number of experiments were set up to compare the TGF activity in RPMI-2650 subpopulations. This was carried out in order to establish whether the RPMI-2650 population consisted of small subpopulations of high growth factor producers and a balance of low to zero producers or whether a homogeneous population with regard to TGF production was present. If a high producing subpopulation was present, this would have an important implication for the isolation and purification of TGFs.

TABLE 3.18 : Transforming growth factor activity in RPMI-2650 clones

Sample Experiment 1	48 hr CM CFE	96 hr CM CFE
Clone 1		3.12 ± 0.07
Clone 2	2.1 ± 0.3	3.7 ± 1.0
Clone 3		2.5 ± 0.51
Clone 9		3.03 ± 0.54
Clone 10	1.36 ± 0.28	2.2 ± 0.3
Clone 11	3.9 ± 0.69	3.83 ± 0.44
Clone 12	3.3 ± 0.45	3.95 ± 0.18
Clone 13	1.89 ± 0.08	2.25 ± 0.29
Clone 14	3.44 ± 0.41	
Clone 15	4.0 ± 0.4	3.74 ± 0.57
Clone 18		3.5 ± 0.34
Clone 20	1.45 ± 0.22	1.31 ± 0.35

Experiment 2	Colony No*	48 hr CM CFE	96 hr CM CFE
RPMI	100.0 ± 15.8	1.27 ± 0.2	
Clone 2	112.1 ± 28.2	1.43 ± 0.36	2.45 ± 0.38
Clone 9			1.86 ± 0.38
Clone 10	41.8 ± 3.8	0.53 ± 0.05	1.84 ± 0.66
Clone 11	85.9 ± 24.3	1.1 ± 0.31	
Clone 12	47.6 ± 17.5	0.61 ± 0.22	2.81 ± 0.71
Clone 14	154.8 ± 41.4	1.97 ± 0.53	
Clone 15	72.8 ± 28.2	0.93 ± 0.36	2.1 ± 1.2
Clone 20	82.2 ± 6.5	1.05 ± 0.083	2.05 ± 0.36

Note : NRK = P24
n = 3
F.C.S. Batch No = 116
NRK indicator cell concentration = 2×10^4 cells/30mm plate
* Growth expressed as a percentage of growth of RPMI-2650 wild type

Summary of Findings

In Experiment 1, clones showing highest TGF activity were clones 11, 12, 14 and 15 and this was consistent for both 48 hour and 96 hour CM.

In Experiment 2, CM samples from the different subpopulations were compared with CM samples from RPMI-2650 wild type cells. 48 hour CM samples were also expressed as a percentage of growth of RPMI-2650 wild type (see Column 1, Table 3.18, Experiment 2). Clone 14 again showed a high TGF activity as compared with the wild type. Clones 11 and 15 again showed reasonable TGF activity, this activity being more apparent when 96 hour CM was tested. Clone 12 again gave a high TGF activity for 96 hour CM but this was not the case for the 48 hour CM. It was generally found that CFEs were higher for CM collected at 96 hours. At 96 hours, cultures would be nearer to confluency and this may be a factor in increased production of transforming growth factors.

The information collected does establish that RPMI-2650 cells and its clones produce a TGF and that this production is not a uniform property. It was clearly shown that clonal variations existed and there was not a homogeneous population with regard to TGF production. However, it was also established that the population did not contain subpopulations of high growth factor producers so that TGF production seems to be a general property of the cells.

3.4.4 Clonal variation in TGF-β production

The soft agar response of normal rat kidney (NRK) clone 49F cells to TGF-β requires the presence of Epidermal Growth Factor (EGF) (Roberts et al., 1981)

NRK 49F cells were used as indicator cells at a concentration of 2 x 10^4 cells per 30mm plate. EGF was used at a concentration of 2ng/ml.

Four experiments were set up (Table 3.19) to compare the TGF-β activity of clones of RPMI-2650. Both 48 hour and 96 hour conditioned media were tested. As it was impossible to test all the samples together it was necessary to overlap samples from separate experiments.

Control samples were included in the absence of EGF.

TABLE 3.19 : Transforming growth factor β assay

Experiment 1	48 hour CM		96 hour CM	
Sample	Colony No.*	CFE	Colony No.*	CFE
RPMI	100.0 ± 26.33	1.6 ± 0.42	100.0 ± 11.23	1.7 ± 0.2
Clone 2	106.9 ± 1.62	1.7 ± 0.03	111.1 ± 8.28	1.9 ± 0.14
Clone 10	150.2 ± 10.55	2.4 ± 0.17		
Clone 11	133.86 ± 9.06	2.1 ± 0.14	88.36 ± 1.41	1.5 ± 0.02
Clone 12	104.64 ± 14.84	1.65 ± 0.23	114.9 ± 8.42	2.0 ± 0.14
Clone 13	75.6 ± 27.36	1.2 ± 0.43	88.27 ± 14.72	1.51 ± 0.25
Clone 14			134.68 ± 17.5	2.3 ± 0.3
Clone 18	116.64 ± 15.29	1.8 ± 0.24		
Clone 20	67.94 ± 10.77	1.1 ± 0.17	109.03 ± 24.44	1.86 ± 0.42
RPMI (-EGF)	0		0	
Experiment 2				
RPMI	100.0 ± 26.5	0.62 ± 0.082	100.0 ± 15.9	0.615± 0.095
Clone 10	158.1 ± 49.68	0.98 ± 0.31		
Clone 12			92.64 ± 23.6	0.57 ± 0.15
Clone 13	97.02 ± 22.21	0.6 ± 0.14	108.5 ± 40.4	0.67 ± 0.25
Clone 14	205.3 ± 40.9	1.3 ± 0.25		
Clone 18	138.1 ± 15.98	0.86 ± 0.1	129.45**	0.8
Clone 20	74.53 ± 19.9	0.46 ± 0.12		
RPMI (-EGF)	0		0	

TABLE 3.19 (cont'd) : Transforming growth factor β assay

Experiment 3

	48 hour CM		96 hour CM	
	Colony No.*	CFE	Colony No*	CFE
RPMI	100.0 ± 17.42	3.06 ± 0.53	100.0 ± 2.14	3.96 ± 0.085
Clone 2	60.06 ± 27.37	1.84 ± 0.84	105.3 ± 15.12	4.2 ± 0.6
Clone 10	178.64 ± 35.38	5.5 ± 1.1		
Clone 11	324.6 ± 68.66	9.9 ± 2.1		
Clone 12	145.45 ± 37.45	4.4 ± 1.15	203.03 ± 9.74	8.04 ± 0.39
Clone 13	221.36 ± 8.7	6.8 ± 0.27	323.82 ± 25.9	12.8 ± 1.03
Clone 14	138.6 ± 10.38	4.24 ± 0.32	258.4 ± 13.99	10.23 ± 0.55
Clone 18			53.5**	2.12
RPMI (-EGF)	0		0	

Note : * Growth expressed as a percentage of growth of RPMI-2650 wild type

Concentration of EGF = 2ng/ml : NRK 49F Indicator cell

** = One plate only.

Concentration = 2×10^4 cells/30mm plate

other n = 3

TABLE 3.19 (cont'd) : Transforming growth factor β activity in RPMI-2650 clones

Experiment 4	48 hour CM		96 hour CM	
Sample	Colony No.*	CFE	Colony No.*	CFE
RPMI (P40)	100.0 ± 15.24	0.54 ± 0.082		
Clone 1				0.53 ± 0.04
Clone 2	101.8 ± 22.1	0.56 ± 0.12		0.86 ± 0.27
Clone 9				0.83 ± 0.04
Clone 10	151.3 ± 25.4	0.83 ± 0.14		1.28 ± 0.05
Clone 11	84.1 ± 10.91	0.46 ± 0.06		0.4 ± 0.04
Clone 12	161.9 ± 28.64	0.88 ± 0.16		1.02 ± 0.07
Clone 13	108.2 ± 11.0	0.59 ± 0.06		0.70 ± 0.15
Clone 15	164.4 ± 18.1	0.90 ± 0.1		0.89 ± 0.03
Clone 18				0.66 ± 0.13
Clone 20	116.82 ± 26.0	0.64 ± 0.14		0.96 ± 0.07

Note : * Growth expressed as a percentage of growth of RPMI-2650 wild type. There was no sample of 96 hour CM for RPMI-2650 which explains the absence of figures in column 3.

NRK 49F : P40

n = 3

F.C.S. Batch No = 116

Summary of Findings

From the results obtained (see Table 3.19, Experiments 1 to 4), it could be stated that TGF-β activity was present for all subpopulations and also interestingly all clones compared favourably with the wild type RPMI-2650. This was not found to be the case for other parameters, for example the TGF assay (Section 3.4.3) or CFE in monolayer and agar (Section 3.4.1).

No growth was obtained in the absence of EGF and this emphasizes that TGF-β requires the presence of EGF for its activity.

There did not appear to be any overwhelming differences between results obtained for 48 hour CM and 96 hour CM although the balance seemed to be slightly in favour of the 96 hour CM.

These results also establish that clonal variation is present with regard to TGF-β activity.

It did not appear from these experiments that there was any relationship between the previous TGF results and these TGF-β results.

3.4.5 Clonal variation in autostimulatory activity

Autostimulating growth factors may be produced by neoplastic cells. These are transforming polypeptides produced by a transformed cell for which the cell has its own functional cellular receptors. This information has been used to explain the lesser requirement of neoplastic cells for an exogenous supply of growth factors.

To compare production of these growth factors by clones of RPMI-2650, experiments were set up in soft agar. The assay for detecting autostimulatory activity was similar to that described for TGF activity (see Section 2.18). A double layer agar assay system was again used but indicator cells used were of 2×10^4 cells per 30mm plate.

The growth of RPMI-2650 colonies in agar was an indication of autostimulatory activity. Two experiments were set up to compare the autostimulatory activity of RMPI-2650 clones. Conditioned media was again obtained at 48 hours and 96 hours. Colonies were again counted microscopically with the aid of a graticule.

TABLE 3.20 : Autostimulatory activity of RPMI-2650 clones

Experiment 1	48 hour CM		96 hour CM	
Sample	Colony No*	CFE	Colony No*	CFE
RPMI	100.0 ± 12.34	2.49 ± 0.31	100.0	2.72
Clone 1				
Clone 2	72.6 ± 20.6	1.81 ± 0.51	76.1 ± 20.3	2.07 ± 0.55
Clone 9			36.4 ± 13.5	0.99 ± 0.37
Clone 10	83.0 ± 16.6	2.07 ± 0.41	106.4 ± 8.8	2.89 ± 0.24
Clone 11	103.3 ± 16.6	2.57 ± 0.41	78.0 ± 8.23	2.12 ± 0.22
Clone 12	102.6 ± 21.6	2.55 ± 0.54	75.8 ± 18.3	2.06 ± 0.50
Clone 13	119.7 ± 34.0	2.98 ± 0.85	99.6 ± 23.27	2.71 ± 0.63
Clone 14	127.7 ± 30.3	3.18 ± 0.75		
Clone 15	88.2 ± 9.96	2.2 ± 0.25	54.82± 4.4	1.49 ± 0.12
Clone 18				
Clone 20	96.7 ± 15.6	2.41 ± 0.39	33.97 ± 7.1	0.92 ± 0.19

RPMI indicator cell concentration = 2×10^4 cells/30mm plate

TABLE 3.20 (cont'd) : Autostimulatory activity of RPMI-2650 clones

Experiment 2	48 hour CM		96 hour CM	
Sample	Colony No*	CFE	Colony No*	CFE
RPMI	100.0 ± 27.6	3.68 ± 1.01	100.0 ± 42.8	3.61 ± 1.54
Clone 1			67.84 ± 3.2	2.45 ± 0.12
Clone 2	37.5 ± 5.4	1.38 ± 0.2	32.7 ± 2.4	1.18 ± 0.09
Clone 9			20.5	0.74
Clone 10	76.3 ± 9.1	2.81 ± 0.33	65.9 ± 8.2	2.38 ± 0.3
Clone 11	47.3 ± 8.8	1.74 ± 0.32		
Clone 12	161.4 ± 10.9	5.94 ± 0.4	136.2 ± 37.1	4.92 ± 1.34
Clone 13	70.0 ± 6.8	2.58 ± 0.25	61.0 ± 1.04	2.2 ± 0.04
Clone 14	95.6 ± 10.2	3.52 ± 0.38		
Clone 15	113.7 ± 10.8	4.18 ± 0.4	67.0 ± 9.2	2.42 ± 0.33
Clone 18			10.8	0.39
Clone 20	60.9 ± 7.4	2.24 ± 0.27	73.2 ± 29.5	2.64 ± 1.07

Note : *Growth expressed as a percentage of growth of RPMI-2650 wild type

n = 3

F.C.S. Batch No. = 116

RPMI indicator cell concentration = 2×10^4 cells/30mm plate

Summary of Findings

Autostimulatory activity was present (see Table 3.21) for all subpopulations and was quantitated by growth of RPMI-2650 colonies in agar. Activity did not generally compare as favourably with the wild type RPMI-2650 as that of TGF-β but was, however, still apparent for each subpopulation. It was noted that clonal variation was present. Clone 12 appeared to show consistently high autostimulatory activity, whereas clones 2 and 9 showed consistently lower activity. No differences in activity were obvious for CM collected at 48 hours or 96 hours.

3.4.6 Morphology

To investigate clonal variation another parameter, morphology was looked at (see Figs. 2 to 19). Clones were grown in 25cm^2 flasks to the same stage of growth and density in the same medium and on the same substrate before photographs were taken. It can be seen that these isolated subpopulations may be distinguished morphologically. This emphasizes that clonal variation is present with regard to morphology. Particular subpopulations could be identified by their morphology. However, criteria for identification other than morphology is necessary.

From the results presented in this thesis, no connections between morphology and any other properties were apparent but further investigations on this would be useful research.

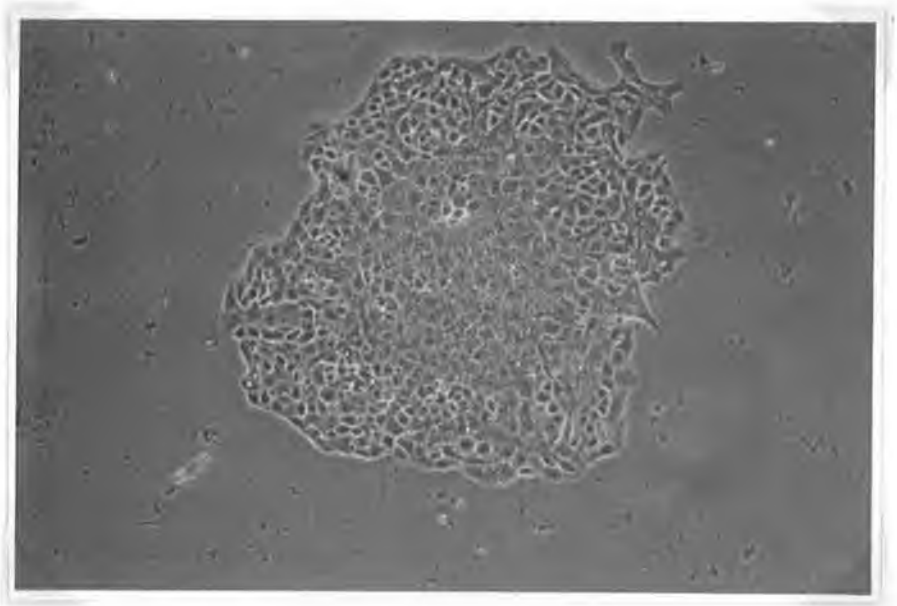


Fig. 1 : RPMI colony 10X phase 1

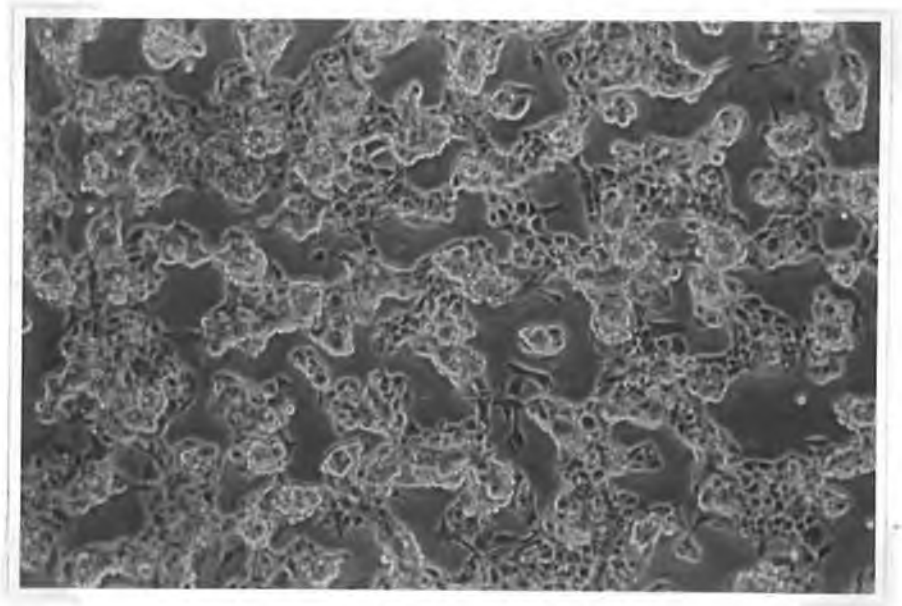


Fig. 2 : Clone 1. P7 10X phase 1

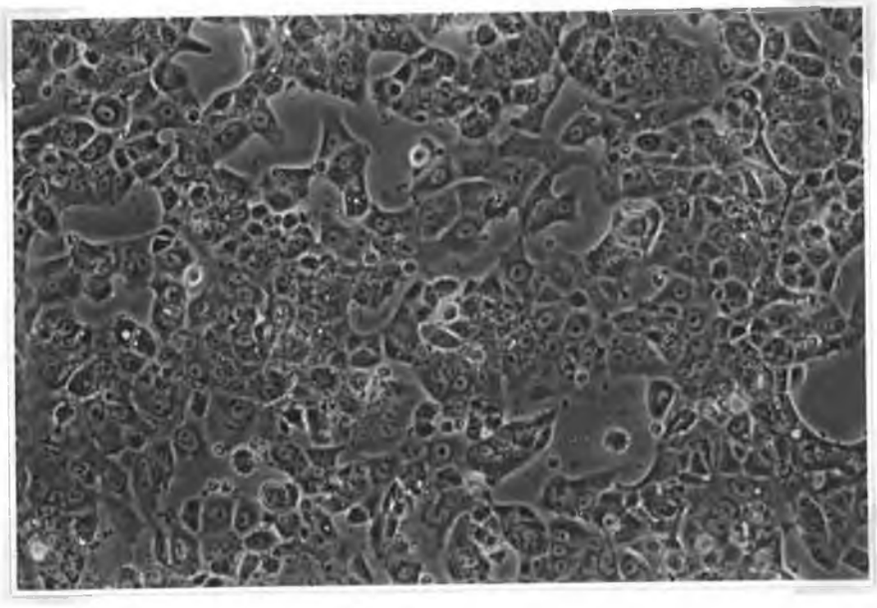


Fig. 3 : Clone 2. P9. 20X phase 2

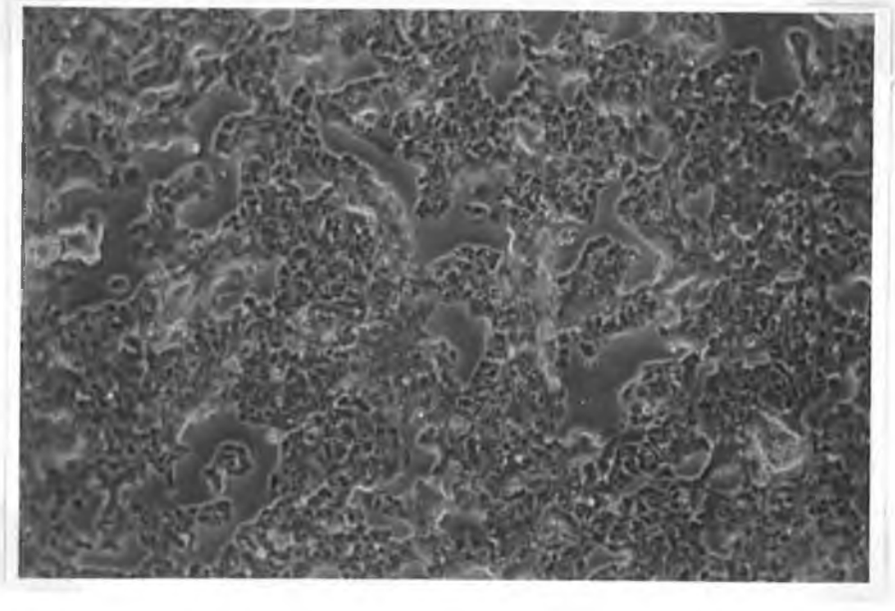


Fig. 4 : Clone 3. P8. 10X phase 1

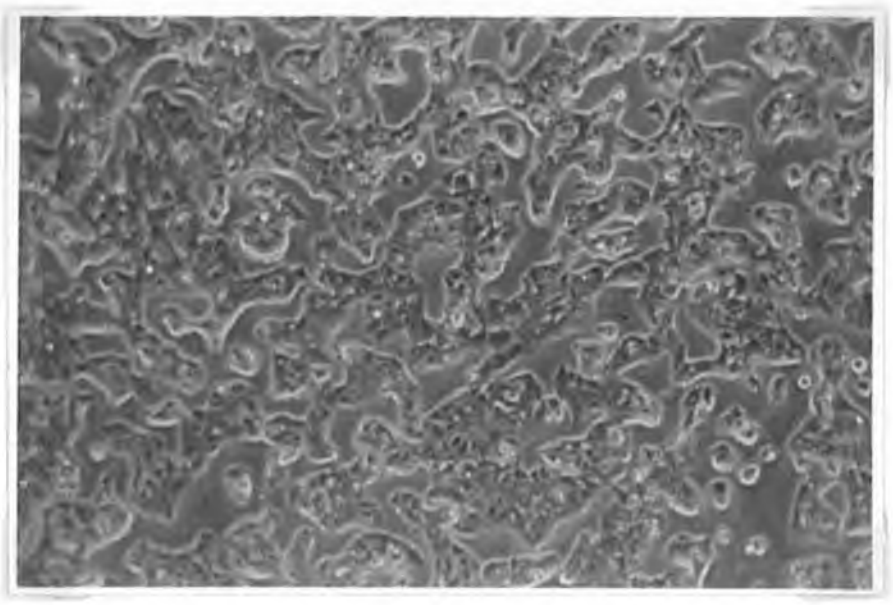


Fig. 5 : Clone 4. P5. 10X phase 1

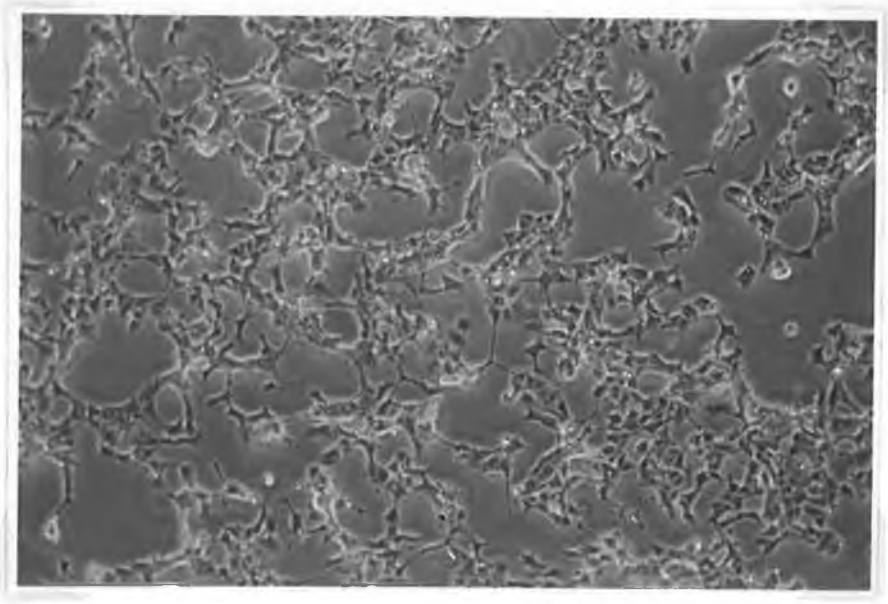


Fig. 6 : Clone 5. P4. 10X phase 1

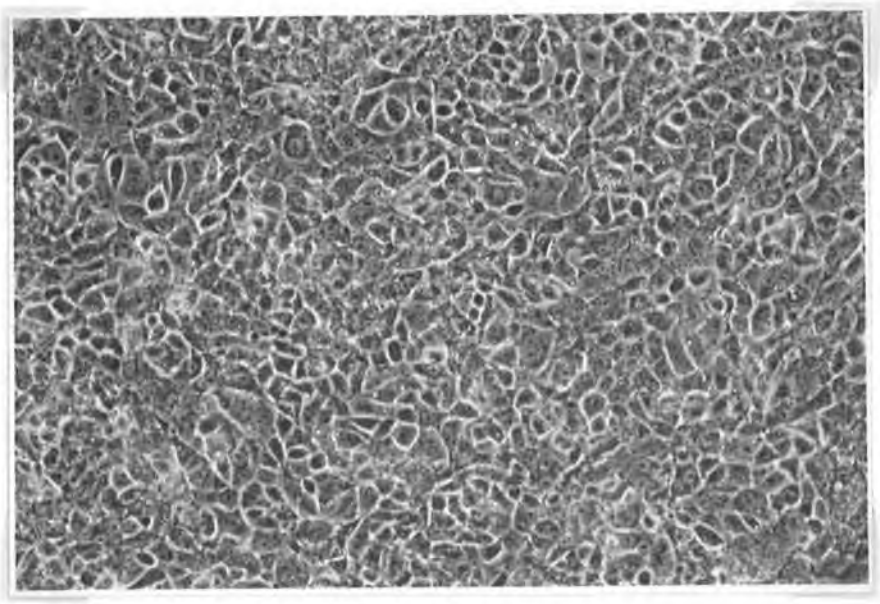


Fig. 7 : Clone 6. P7. 20X phase 2

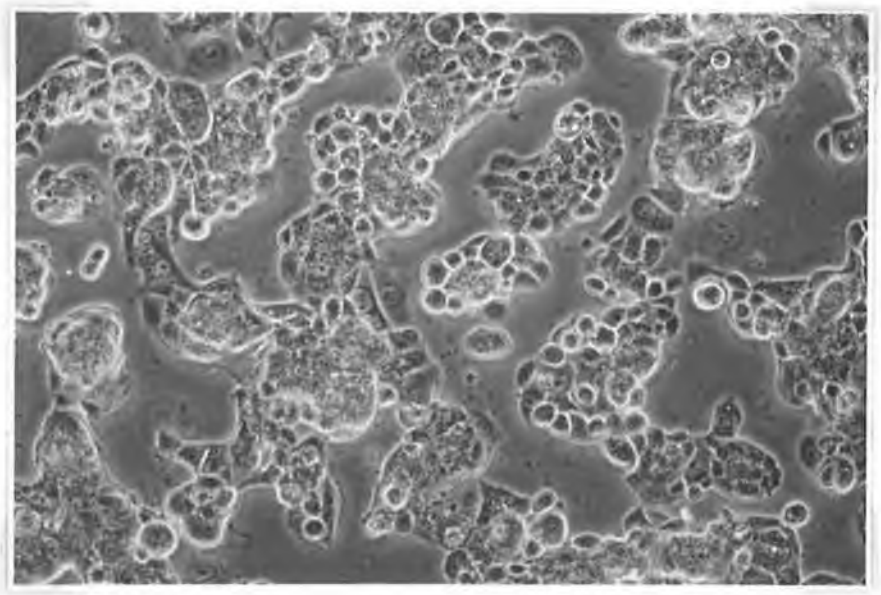


Fig. 8 : Clone 7. P6. 20X phase 2

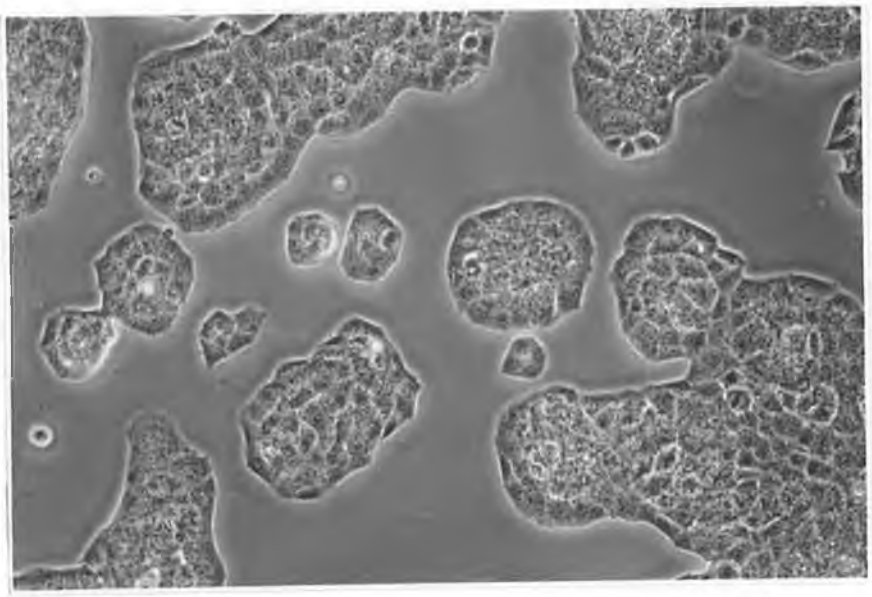


Fig. 9 : Clone 8. P6. 20X phase 2

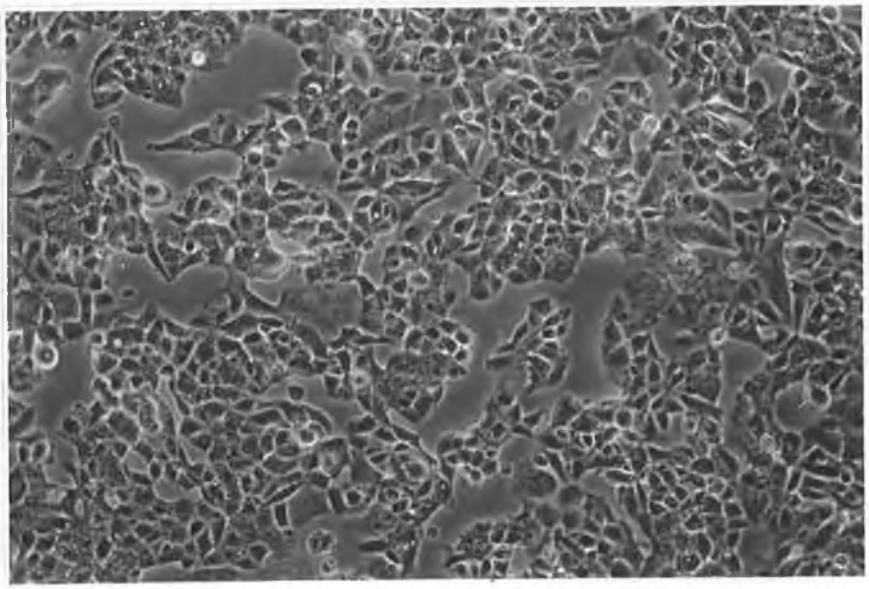


Fig. 10 : Clone 9. P9. 20X phase 2

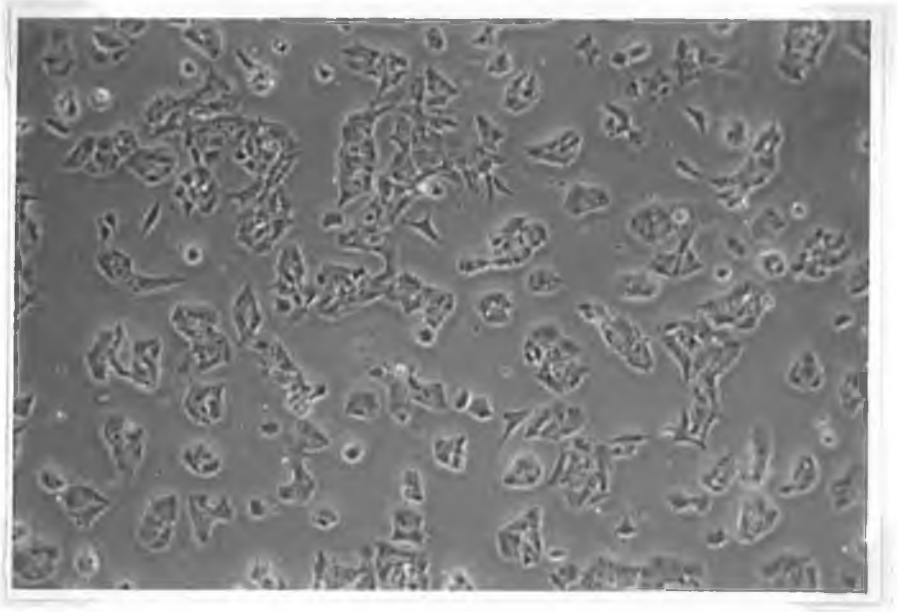


Fig. 11 : Clone 10. P4. 10X phase 1

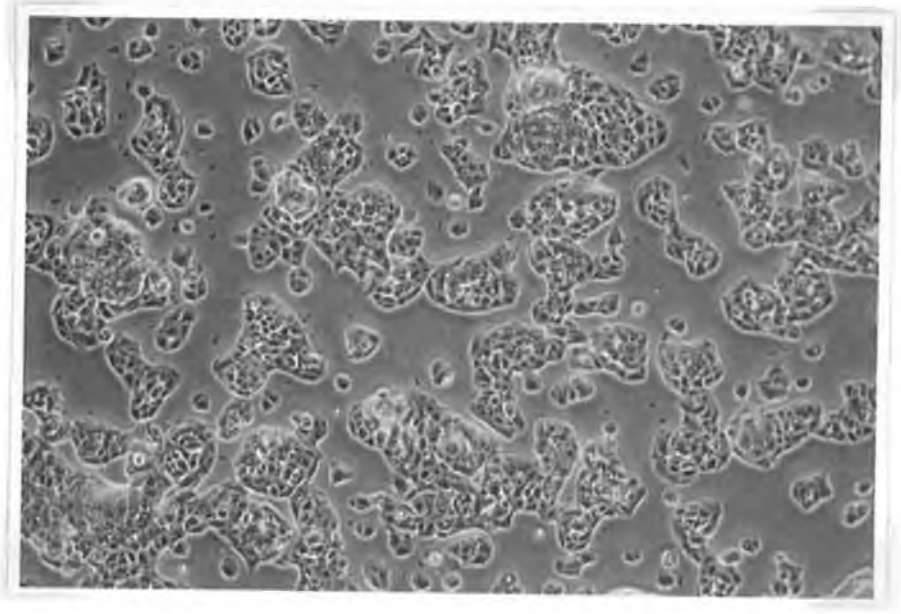


Fig. 12 : Clone 11. P4. 10X phase 1

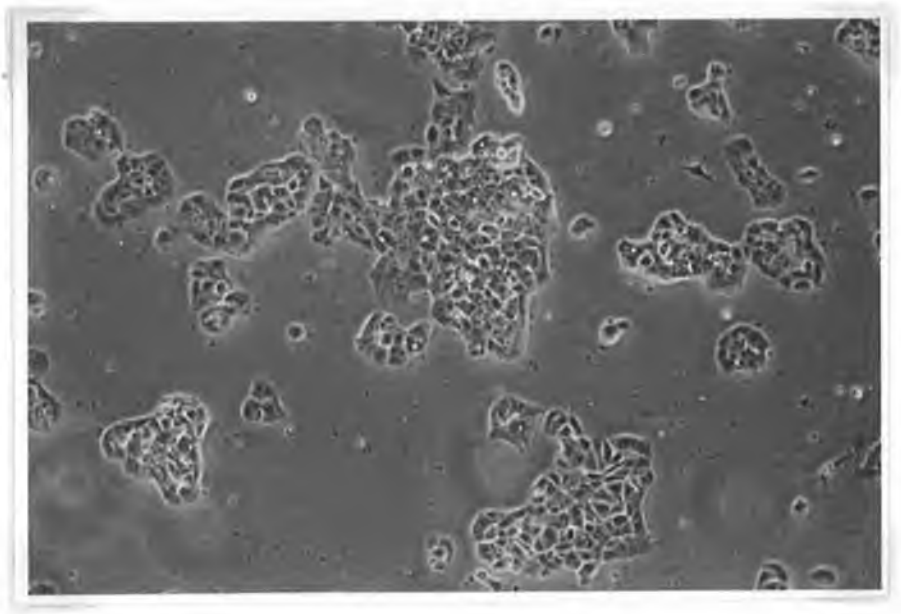


Fig. 13 : Clone 12. P4. 10X phase 1

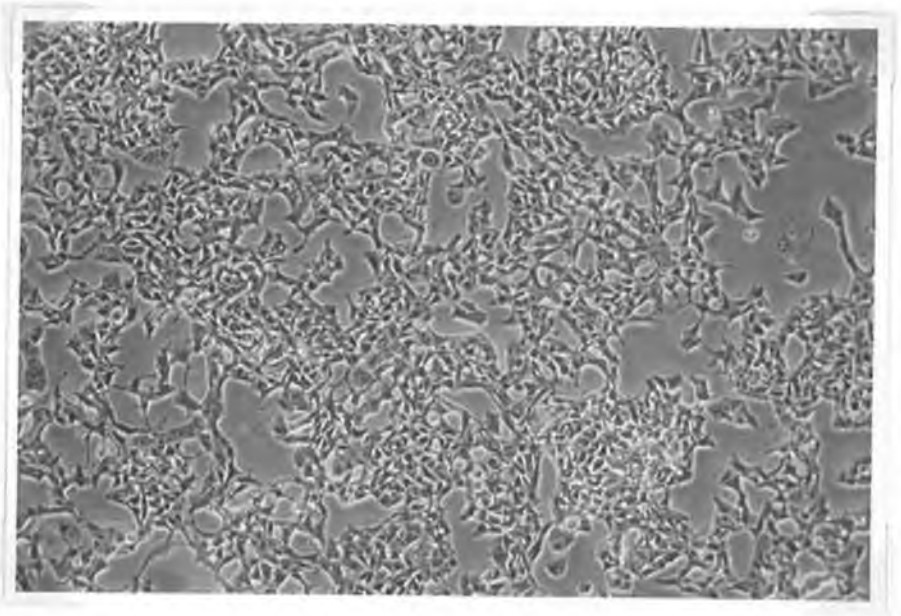


Fig. 14 : Clone 13. P4. 10X phase 1

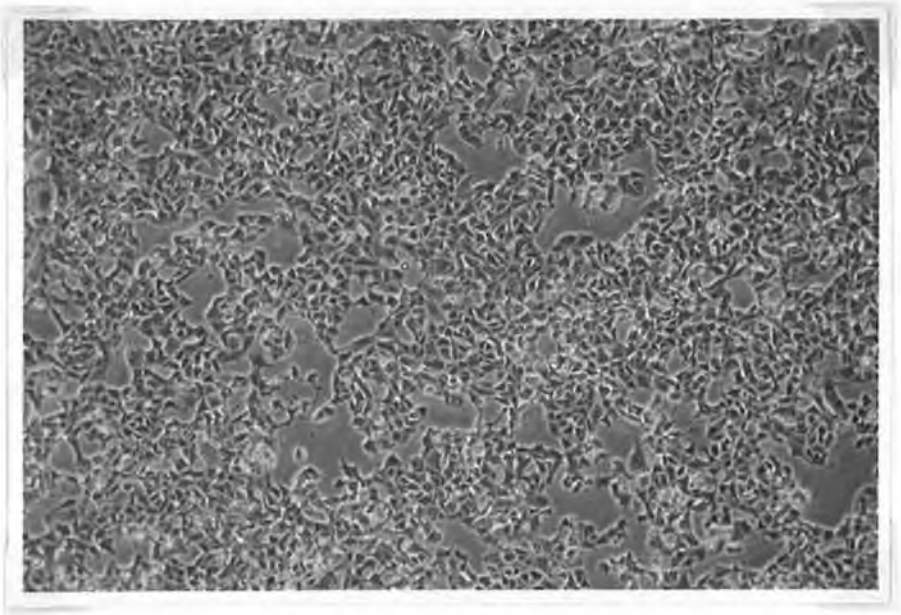


Fig. 15 : Clone 14. 10X phase 1

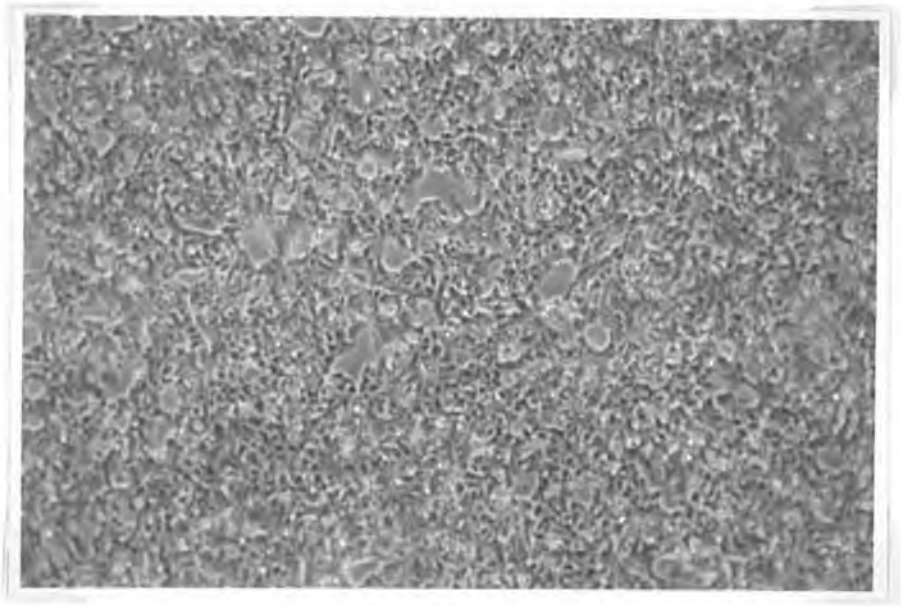


Fig. 16 : Clone 15. P5. 10X phase 1

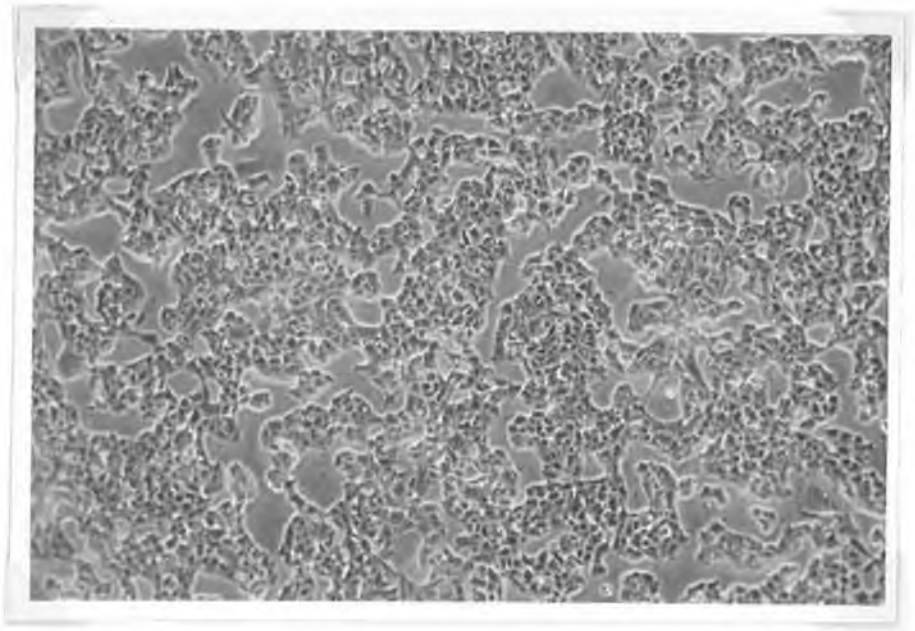


Fig. 17 : Clone 16. 10X phase 1

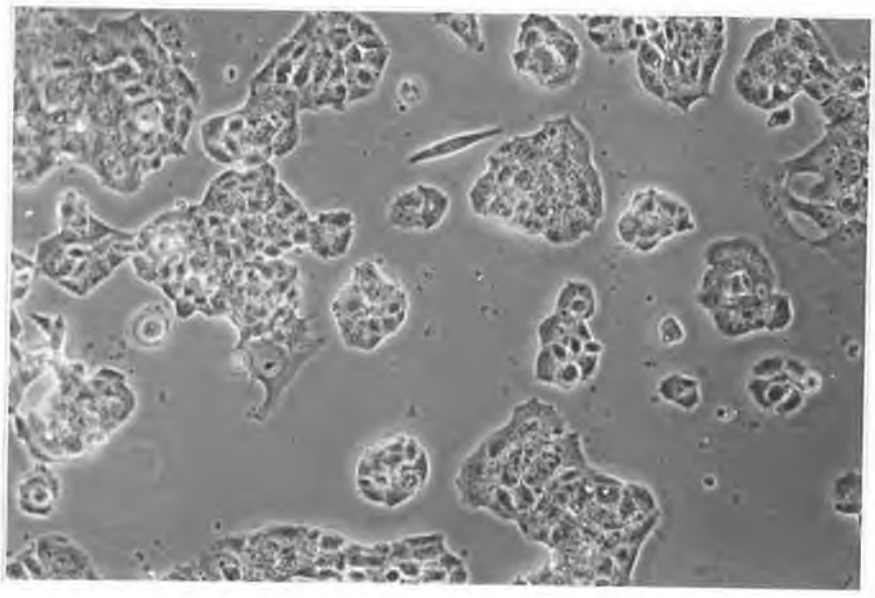


Fig. 18 : Clone 19. P4. 20X phase 2

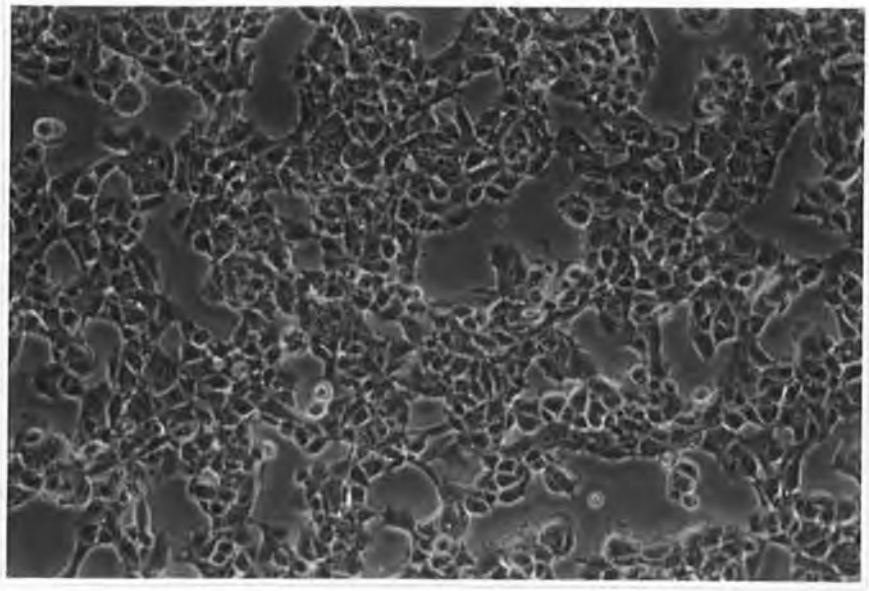
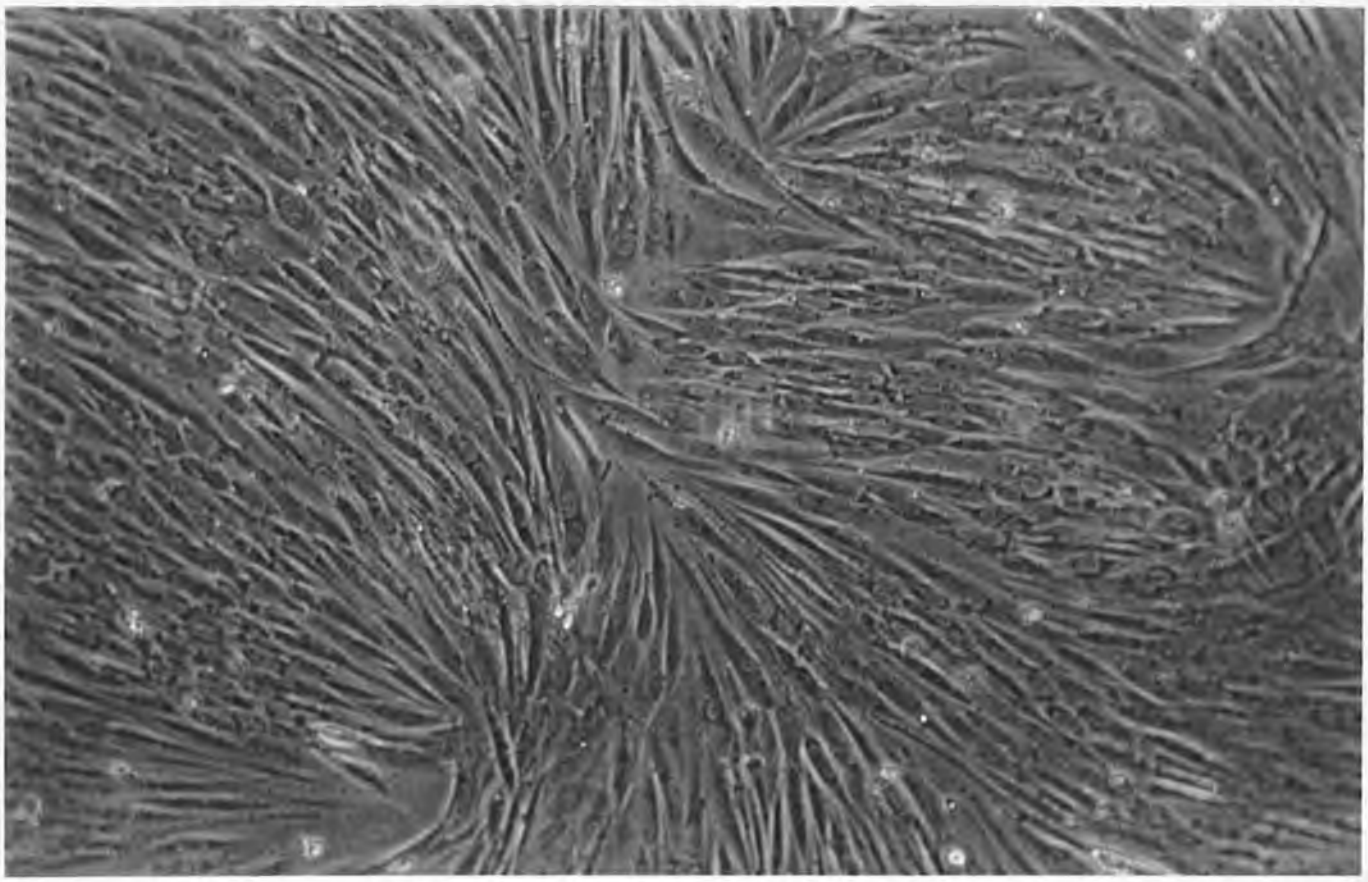


Fig. 19 : Clone 20. P4. 20X phase 2



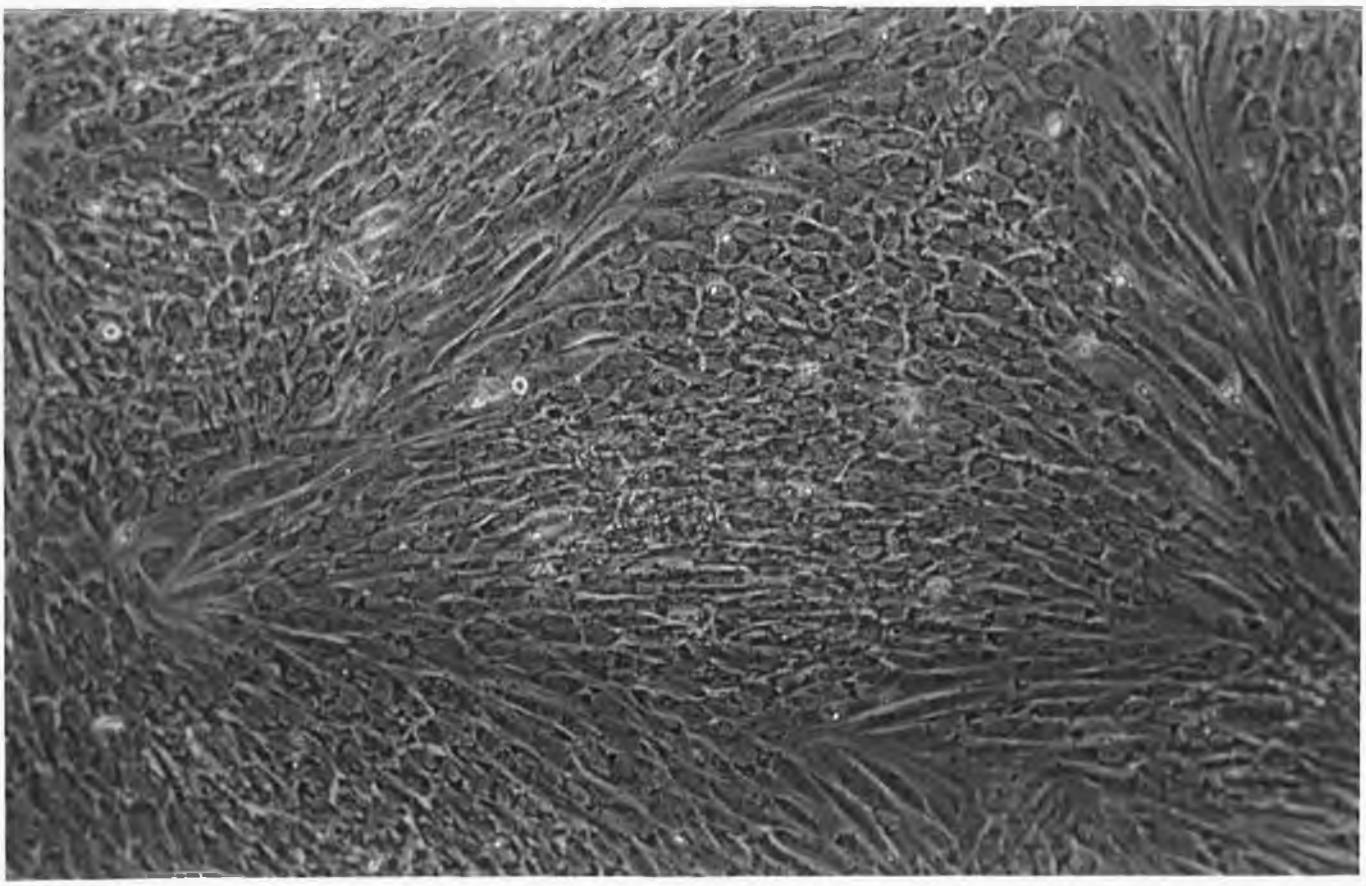


Fig. 20 : Primary brain sample. 10X phase 1

SECTION 4

DISCUSSION

4. DISCUSSION

The primary aim of this thesis was to develop a system for isolating clones and to investigate clonal variations in a number of parameters. As presented in detail in the introduction, studies on clonal populations of cells have yielded important information in cancer research (Fidler et al., 1978; Kripke et al., 1978; Dexter 1978; Sweeney et al., 1982; Heppner et al., 1978; Nister et al, 1986; Freshney, 1980).

RPMI-2650 is a quasi-diploid epithelial cell line which is available at a low passage level. It was therefore thought that this cell line would be more representative of human tumours than commonly used lines such as HeLa cells and therefore an attempt was made to clone this cell line, which has a low colony forming efficiency.

The introduction of 96 multi-well tissue culture dishes greatly simplified the recovery of individual colonies as the smaller surface area facilitated the serial propagation of clones. The time taken to generate a sufficient quantity of cells from a clone by serial propagation was usually rather long due to the relatively long generation time.

A number of parameters with potential for facilitating the cloning procedure were examined, including effects of serum concentration and batch variation of media, addition of conditioned media, effect of temperature of trypsinization, effects of DEAE dextran, effects of different gas atmospheres and effects of feeder layers (3T3 and RPMI-2650).

A foetal calf serum concentration of 15% was found to give optimum growth (see Results, Section 3, Table 3.2) at clonal concentrations; this may be of some value in cloning. Growth of RPMI-2650 in monolayer and agar was affected by the serum batch used; therefore, it was necessary to screen batches for optimum growth (see Materials and Methods, Section 2.15; Results, Table 3.1). It may also be necessary to develop a screening assay for growth in agar as growth in monolayer and agar may be differently affected by particular batches of foetal calf serum (fcs). In the work presented in this thesis, batch numbers were screened in monolayer, but when cloning in agar or for other experimental work with agar, it may be advantageous to screen batches in an agar screening assay. In this thesis, selection of batches of fcs depended on availability as well as CFE results.

The basal medium giving optimum growth of RPMI-2650 at clonal concentrations was a mixture of DME and Hams F12 (1:1). This was also reported for higher concentrations of cells by Edel Murphy (1986).

The mixture of the two media (DME and Hams F12) seem to supply the optimum combination of nutrients for this cell line.

To analyze the effect of conditioned medium (CM) on clonal growth of RPMI-2650, sparse cultures of RPMI-2650 were treated with a number of combinations of MEM and MEM:CM (see Results, Section 3.1.4, Table 3.7). The need for 'conditioning' the medium by the living cells used in tissue culture was emphasized by Earle et al., (1954). They suggested that it may be of great importance to supply the cell with a culture medium already adjusted by the

growth of cultures of living cells. Experiments were set up to assess the ability of RPMI-2650 CM to stimulate cell growth at a very low cell population density. In these experiments (see Table 3.7) there was a small increase in colony forming efficiency (CFE) with a combination of CM:MEM + 5% fcs (1:2). When CM was used in a higher ratio with MEM + 5% fcs there was no increase in CFE. It may be that a small amount of conditioning is useful for growth, especially at clonal densities.

A medium conditioned by one type of cell may also sometimes exert a beneficial effect on other cells. It was shown that epidermal cells grow in a medium conditioned by fibroblasts much better than in a non-conditioned medium (Rheinwald and Green, 1975). Also, epithelial cultures may produce growth-stimulating substances for fibroblasts (Howard, Scott and Bennett, 1976) and medium conditioned by BALB/3T3 cells stimulates the proliferation of cultured bovine endothelial cells (Birdwell and Gospodarowicz, 1979). This conditioning by one cell type or another may be worth investigating for RPMI-2650, that is, conditioned medium from other cell lines may be tested for the ability to stimulate growth of RPMI-2650 cells.

It was hoped that an altered trypsinization procedure would improve the clonal growth of RPMI-2650. A low temperature typsinization technique was used routinely for preparation of clonal inocula by Agy et al., (1981) as this method minimizes the amount of trypsin introduced into cultures and therefore, may be less damaging to the cell. An experiment was set up to test this (see Table 3.5) and there appeared to be success with this as a higher colony forming efficiency was seen from cells trypsinized at 4°C.

A complex of stimulating environmental factors is needed to induce the transition of a population into the growing state. This complex includes the substratum, and it was hoped to improve cell attachment and growth by pre-treating the surface for attachment. Previous work by McKeehan and Ham (1976), showed increased cloning efficiency of human and chick fibroblasts by coating of the plastic surface with polylysine or other basic polymers. Also it was shown by Murphy (1986) that DEAE dextran pre-coating improved growth of RPMI-2650. It was decided to repeat this experiment but at clonal densities (Section 3.1.3, Table 3.6) and a small increase in colony forming efficiency was seen with DEAE dextran pre-coating. The inclusion of DEAE dextran as a medium component may also be of value in improving cloning efficiencies of RPMI-2650.

Poste et al., (1982), reported that the use of a gaseous atmosphere of 5% O_2; 5% CO_2 and 90% N_2 significantly enhanced the growth of B16 melanoma cells. In contrast, it was found that colony forming efficiencies for RPMI-2650 (see Section 3.1.3, Table 3.4) were somewhat reduced under these conditions but this would need to be verified by further experiments.

'Feeder' effects of pre-established cells on the clonal growth of other cells are well known. In 1955, Puck and Marcus attempted to use feeder cells, which were themselves incapable of multiplication, to supply conditioned factors. They suggested that these feeder cells could supply a single multiplying cell with the necessary growth factors to allow multiplication into a colony. Feeder layers have been found to improve attachment and growth of a number of epithelial cell types (Rheinwald and Green, 1975). Also

it has been shown (Taylor-Papadimitriou, Shearer and Stoker, 1977;

Taylor-Papadimitriou et al., 1978) that the rate of growth of human mammary epithelial cells and of calf lens epithelium may be increased by feeder layers of fibroblasts. In this thesis, a number of experiments were set up (see Sections 3.1.5 and 3.1.6) in the hope that feeder layers of 3T3 and RPMI-2650 cells would provide an improved substrate for attachment as well as growth promotion factors for RPMI-2650 cells at clonal densities. To arrest growth of feeder cells and make them incapable of multiplication, mitomycin C was used. Mitomycin C selectively inhibits DNA synthesis in both bacteria and mammalian cells (Kersten, 1975). In five experiments set up with 3T3 feeder layers (fibroblast cells) a higher colony forming efficiency was obtained in each case with feeder layers (see Section 3.15; Table 3.8).

The first experiment was a screening assay in 60mm plates to assess possible stimulation of RPMI-2650 growth by 3T3 feeder layers (see Section 3.1.5; Table 3.8). The following four experiments were set up at clonal densities in multi-well plates (16mm well diameter) with and without feeder layers. From the results presented, it appears that 3T3 fibroblast feeder layers may be a useful addition for improving cloning efficiencies of RPMI-2650. The mechanism of this feeder effect is currently not understood.

An attempt was also made to assess the feeder layer stimulation of RPMI-2650 by the same cell type, in the hope that RPMI-2650 would produce autocrine growth factors, that is, hormone-like substances secreted by a cell, for which the cell itself has functional external receptors (Sporn and Todaro, 1980). In the first experiment (see Section 3.1.6, Table 3.9) there was no increased colony forming efficiency with feeder layers, possibly due to a high concentration of feeders causing depletion of growth factors

in the medium or an accumulation of inhibitory products causing cessation of cell proliferation. The second experiment was a screening assay in 60mm plates. There appeared to be some increase in colony forming efficiency due to feeder layers, but in some cases, there was only one plate counted, so results were inconclusive here.

The following experiments were set up at clonal concentrations in multi-well plates (16mm well diameter) and with the exception of experiment 5, an increase in colony forming efficiency was seen in the presence of RPMI-2650 feeder layers. It appears from the work on feeder layers that both 3T3 and RPMI-2650 feeder layers are useful for improving cloning efficiencies of RPMI-2650 cells. The exact mechanisms of these feeder effects are not currently known.

In addition, some work on feeder effects was done on SW1088 cells in a double layer agar assay system (see Section 3.2.2., Tables 3.10 and 3.11). SW1088 cells were used as feeder cells, both live untreated cells and mitomycin C treated feeder layers were tested. In the presence of live SW1088 feeder cells, there was little or no growth probably due to either depletion of growth factors or accumulation of inhibitory products due to high cell densities. In the presence of mitomycin-C treated feeder layers, there appeared to be increased colony forming efficiencies of SW1088 cells, especially at a feeder cell concentration of 5×10^4 cells per 30mm plate and a cell density of 5×10^3 cells per 30mm plate. At higher concentrations of test cells and feeder cells, there appeared to be inhibition. These results demonstrated improved cloning efficiencies of SW1088 cells with SW1088 feeder cells in agar. Further research would be worthwhile on these cells, both in agar and monolayer.

For isolation of clones, a single cell suspension was desired at subculture to ensure an accurate cell count and uniform growth on re-seeding. This was essential where cells were to be isolated as clones. A mixture of trypsin and EDTA was used to subculture cells. Cloning was carried out in monolayer, as there was a higher efficiency of growth in monolayer than in agar. For monolayer cultures, the cell yield is proportional to the available surface area of the flask or well. Small volumes and multiple replicates are best performed in multi-well dishes.

When cells were plated as a single cell suspension at low densities in 60mm plates, colonies of such cells tended not to fuse but to remain apart, that is they grew as discrete colonies, allowing a clone to be isolated.

The introduction of multi-well plates revolutionized the approach to tissue culture and greatly assisted in the recovery of clones. The 96-well microtitration plate used in the cloning procedure had a $32mm^2$ growth area and a capacity for 0.1 or 0.2ml medium and up to 10^5 cells. The applications of this technology also include cytotoxicity assays of potential toxins, antibody, virus and drug titration and assay of anticancer drugs.

Clones may also be seeded directly into a $25cm^2$ plastic flask standing on end, but there was little success with this method for RPMI-2650 cells. This method may be more appropriate for other cell lines, for example cell lines with larger cells or faster growth rates.

Advantages of cloning in agar include the exclusion of any normal cells, as most normal cells will not form colonies in suspension

with the same high efficiency as virally transformed cells. The isolation of colonies of transformed cells is possible by this method, although it is a more difficult procedure in practice.

The system found to be the best for isolating clones involved plating cells at a concentration of 50-100 per 60mm plate. When the colonies had reached macroscopic size, they were removed by trypsinization by the cloning ring method (see Materials and Methods, Section 2.20) and transferred to 96 well multi-well tissue culture dishes, 32mm^2 growth well area and subsequently transferred to vessels of increasing surface area.

Other attempts at cloning included disaggregating cultures into single cell suspensions and dispensing cell suspension into the wells of a 96-well microtest plate, adding on the average one cell per well. This was not successful, mainly due to contamination problems due to the need for regular feeding and also the low colony forming efficiency of RPMI-2650 cells.

At a certain stage of colony growth, the colony could be subcultured into the same well and clones serially propagated. This may also be a valid method for cloning. An attempt in this project was made to clone RPMI-2650 cells in agarose (see Section 2). This was found to be a difficult procedure in practice. One clone was isolated in this manner (clone B) and some TGF results were obtained for this clone (see Table 3.14). It would be interesting to investigate variations in clones isolated from different sources, for example agar or monolayer.

In addition to isolating clones of RPMI-2650 cells, it was thought that selection of a variant cell type of RPMI-2650 would be

worthwhile research. It was thought that comparisons could be made of certain properties for example TGF production, autostimulatory activity and growth in monolayer and agar with RPMI-2650 clones. A thioguanine resistant mutant of RPMI-250 was isolated (see Section 2.17). However, the colony forming efficiency of this mutant was extremely low compared with wild type RPMI-2650 cells and it was found to be impractical at the time to grow these cells for collection of conditioned media. However, further research in this area may be warranted.

Clones of RPMI-2650 were isolated and frozen in liquid nitrogen when there was a sufficiently high cell density. A number of parameters were investigated for clonal variation including morphology (see Figs. 2 to 19). Morphology is the simplest and most direct technique used to identify cells. However, this has some shortcomings which need to be recognised. These include response to different culture conditions, that is the cellular morphology may alter with a different environment. Alterations in the substrate and the constitution of the medium can affect cellular morphology (Freshney, 1980; Gospodarowicz, 1978 and Coon and Cahn, 1966). An example of this can be seen with fibroblasts from hamster kidney or human lung or skin. They assume multipolar or bipolar shapes and are well spread on the culture surface but at confluence they are bipolar and less well spread (Freshney, 1980). Also, mouse 3T3 cells and human glial cells grow like multipolar fibroblasts at low cell density, but become epithelial-like at confluence (Freshney, 1980). Comparative observations should always be made at the same stage of growth and cell density, in the same medium and growing on the same substrate (Freshney, 1980).

It can be seen (Figs. 2 to 19) these isolated clones may be distinguished, but their similarity underlines the need for criteria for identification other than morphology. However, this apparent heterogeneity would appear to substantiate the statement by Fidler et al., (1978) that a neoplasm should no longer be considered a uniform entity. The investigation of possible morphological correlations with any properties, for example TGF production, autocrine secretion or thymidine incorporation, would be useful research. It was suggested by Nister et al., (1986) that there was a morphological correlation with glioma PDGF production. Most of the high producing clones were found among "immature" looking, tightly packed cells, whereas low producing clones had a more astrocytic or glia-like morphology.

The results presented in this thesis do not suggest any correlation between morphology and any other property. However, until more studies are carried out in this area, no definite correlations can be made.

It was found by Nister et al., (1986) that a high degree of glioma PDGF production was found predominantly in clones derived from cells of high passage levels. It would be of value to investigate any correlations with low or high passage clones with other properties for example TGF production. It would also be of value to investigate the metastatic capacity of high and low passage clones. There are a number of methods available for this, including the ability to grow in agar (Freedman et al., 1974; Shin et al., 1975 and Barrett et al., 1977). It is still not known whether unique metastatic cells pre-exist in the tumour population or whether they arise during metastasis by a process of adaptation

to local environmental conditions. It may be that repeated passage *in vitro* provides an opportunity for variant cell types to arise.

Another area of research with huge potential is the study of heterogeneity of the tumour cell population with regards to drug sensitivity. Morphologically similar tumours do not always uniformly respond to single therapeutic agents. It has been demonstrated by Shapiro *et al.*, (1981) and Yung *et al.*, (1982) that clones from a single glioma biopsy displayed differing chemosensitivities *in vitro*. Also Kornblith *et al.*, (1981) have demonstrated considerable variation in the sensitivity of glioma cultures to BCNU. Established clones would be a useful tool for research into testing sensitivity of subpopulations to therapeutic agents. This may provide valuable information on predicting drug responses. However, it still remains to be demonstrated whether there is any correlation between drug sensitivity *in vitro* and response to chemotherapy *in vivo* (Freshney, 1980). Also it may be that even though the responses of isolated subpopulations are known, the response of the entire tumour may be unpredictable.

For a culture to be considered a pure culture it would seem reasonable to suppose that the cells composing it should all have about the same rate of growth (Willmer, 1935). On this basis, a number of experiments were set up to test the colony forming efficiencies (CFE) of clones of RPMI-2650 in monolayer. A number of experiments were set up in 16mm wells but there was a lot of inconsistency, possibly due to the small surface area and low cell densities, so it was decided to use 30mm plates for calculation of CFE in monolayer (see 3.16).

However, there was still some inconsistency in results, although some clones gave indications of having consistently high CFEs; these included clones 10 and 18. From observation during sub-culturing of stocks it was obvious that some clones were more slow growing then others; these included clones 4 and 8. Clone 4 was eventually lost in culture.

Among the transformed growth properties, anchorage independence seems to be the best correlation with oncogenicity, most lines that do not form tumours in animals have a low ability to form colonies in semi-solid media (Shin et al., 1975; Freedman et al., 1974; Barrett et al., 1979). The cloning efficiency of cells in semi-solid media is usually regarded as a measure of the degree of loss of anchorage dependence (Vasiliev & Gelfand; 1981). Subpopulations (clones) were tested for their ability to grow in agar (see Section 2.14). From the results presented (see Table 3.17, Section 3.5.1), it appeared that most of the subpopulations had the capacity for anchorage- independent growth. Some patterns emerged, although there was a lot of inconsistency. From observation of colony size and appearance, four clones consistently appeared large and healthy in semi-solid media. These were clones 10, 11, 14 and 18. Clone 12 gave reasonable colony forming efficiencies in agar but colonies were obviously smaller in size although no quantitative measurements were made.

The transforming effect of medium conditioned by RPMI-2650 clones was assessed by the transforming growth factor assays (see Section 2). Two assays were used, the transforming growth factor (TGF) assay and the transforming growth factor (TGF-β) assay, for which indicator cells were normal rat kidney (NRK) and normal rat kidney clone 49F cells respectively (NRK49F). The soft agar response of

NRK 49F cells to TGF-β required the presence of epidermal growth factor (EGF) (Roberts et al., 1981) and a concentration of 2ng/ml EGF was used. An indicator cell concentration of 2 x 10^4 cells per 30mm plate was used for both TGF and TGF-β assays. In the TGF experiments (see Section 3.5.2, Table 3.18) some clones appeared to give generally higher colony forming efficiencies than others, these included clones 11, 12, 14 and 15. However, there was a lot of inconsistency in results, which may depend on timing of conditioning, concentration of cells available for conditioning etc. In TGF-β experiments, no growth was observed in the absence of EGF. In the presence of EGF, clones 10 and 12 appeared to give consistently high colony counts. However, in both these assays no definite conclusions could be drawn. A number of factors would need to be taken into account for the inconsistency of results. The precise conditions for collection of conditioned medium may need to be re-evaluated. For collection of conditioned medium, cells were grown in 25cm^2 flasks to approximately 90% confluence and cells washed with phosphate buffered saline (PBS) and serum free medium added. Conditioned medium (CM) was then collected after 48 hours and 96 hours. This conditioned medium was then spun at 3000 rpm for 20 minutes and supernatant collected. For more accurate results, it might be desirable in future to make precise cell counts for collecting CM. A number of parameters of interest could be varied for collection of CM. These include : varied source cell density with constant volume of medium, varied volume of medium with constant cell density, varied volume of medium and varied cell density and also the time of exposure of medium to source cells. It was found that concentration (ultrafiltration) of conditioned media was probably unnecessary as colony growth was obtained from unultrafiltered conditioned media. What these results do clearly show, however, is that production of TGF-α, TGF-β

and autostimulatory activity is a general property of RPMI-2650 cells. The work presented here allows us to reject the hypothesis that the RPMI-2650 population consists of a small sub-population of high-level producers, and a larger population of low or zero level producers.

Autostimulating growth factors may be produced by neoplastic cells and the nature of these factors has been established only for a few cell lines in vitro. Sporn and Todaro (1980) proposed the term 'autocrine secretion' for a type of self stimulation, where a transforming polypeptide is produced by a transformed cell and the cell has its own functional cellular receptors for this polypeptide. This hypothesis may explain the lesser requirement of neoplastic cells for an exogenous supply of growth factors. Experiments were set up in soft agar to compare production of these growth factors by clones of RPMI-2650. It has already been established that RPMI-2650 cells produce an autocrine growth factor (Dooley and Clynes, 1986) but it is not known whether subpopulations of RPMI-2650 may produce more or less autostimulators than others. The assay used was similar to that described for TGF activity in a double layer agar assay system but indicator cells used were RPMI-2650 cells at a concentration of 2 x 10^4 10^4 cells per 30mm plate (Section 3.5.4, Table 3.21). It appeared that clones 2, 9 and 18 were giving generally low colony forming efficiencies whereas clone 12 was giving high colony forming efficiencies. However, no definite conclusions could be made here. Again conditions of collection of conditioned media may have affected results.

Isolated subpopulations (clones) can be a useful research tool as it is generally accepted that most neoplasms have a unicellular

origin and clonal growth pattern. In most cases it is impossible to tell which particular cell variant, present in the parent normal tissue has been the progenitor of the neoplastic clone. It would be useful research to investigate the metastatic potentials of subpopulations (clones) as this may give some indications into the origin of a tumour *in vivo*. Although the clonogenic cells in many tumours may be relatively anaplastic, they may give rise to progeny which can undergo terminal differentiation. These cells are no longer malignant as they have lost the capacity to proliferate but they can make a substantial contribution to the bulk of the tumour. Further investigations into subpopulations may give some clues into the development of a tumour population. The results presented in this thesis, both morphological and biochemical, add to the evidence that malignant cells in culture are not homogeneous. This work *in vitro* adds to the evidence that an established tumour should not be regarded as a mass of relatively homogeneous malignant cells.

SECTION 5

REFERENCES

5. REFERENCES

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ABBREVIATIONS

RPMI-2650	Rosewell Park Memorial Institute - cell line
SW1088	Astrocytoma - cell line
PBS	Phosphate buffered saline
FCS	Foetal calf serum
TGF	Transforming growth factor
CM	Conditioned medium
No.	Number
PDGF	Platelet derived growth factor
EGF	Epidermal growth factor
TV	Trypsin versene
MEM	Minimal essential medium
DME	Dulbecco's modified Eagle's medium
Hepes	4-(2-hydroxyethyl-1-piperazine ethane sulphonic acid)
EDTA	Ethylene diamine tetra acetic acid
DEAE	Diethylaminoethyl
CFE	Colony forming efficiency
	$= \frac{\text{Number of colonies}}{\text{number of cells plated}} \times 100$
BCNU	1,3-bis-(2-chloroethyl)-1-nitrosourea
EMS	Ethyl methane sulphonate
TG	Thioguanine
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
HAT	Hypoxanthine aminopterin thymidine
HeLa	Helen Lane cells - the first epithelial-like cell line (epitheloid carcinoma)
Balb/3T3	3T3-like cells derived from Balb/c mouse embryos
BME	Basal essential medium (Eagle)
Conc.	Concentration

DEFINITIONS

Passage	The transfer or subculture of cells from one culture vessel to another.
Passage number	The number of times a culture has been subcultured.
Primary culture	A culture started from cells, tissue or organs taken directly from an organism and before the first subculture.
Subconfluent	All of the available substrate is not covered.
Confluent	All of the available substrate is covered.
Colony Forming Efficiency	$\frac{\text{The number of colonies}}{\text{the number of cells plated}} \times 100$
Clone	A population of cells derived from one cell
Explant	A fragment of tissue transplanted from its original site and maintained in an artificial medium.
Substrate	The matrix or solid underlay upon which a monolayer culture grows.
Neoplastic	A new, unnecessary proliferation of cells giving rise to a tumour.