A STUDY OF THE GROWTH AND DIFFERENTIATION OF THE HUMAN ADENOCARCINOMA OF THE COLON CELL LINE HT-29

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Doctor of Philosophy

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The HT-29 human colon adenocarcinoma cell line, like many epithelial cells, displays an undifferentiated phenotype when cultured on plastic substrata. Biochemical markers of differentiation, such as brush border associated enzymes and carcinoembryonic antigen were expressed at very low levels. The differentiation-inducing effects of the culture of HT-29 cells on collagen type I gels were evaluated, and were assessed by morphological appearance, brush border associated enzyme activities and the secretion of CEA. The effect that this more physiological environment had on their chemosensitivity to a panel of chemotherapeutic agents was determined, so as to indicate whether this system could be used to improve the selectivity of screening for novel anticancer agents.

Initial studies were performed on HT-29 cells derived from cells seeded directly from plastic substrata onto the collagen gels (designated Non-PPC gels). Their time of exposure to the collagen was limited to the time course of a single experiment and the results suggested that a longer, more permanent exposure might produce a more pronounced differentiation.

HT-29 cells were then passaged continuously on collagen gels for a minimum of 10 passages prior to experimentation (designated PPC gels). The same parameters were measured, and compared to those for the cells grown on plastic and on the non-passaged collagen gels (Non-PPC) from the original studies. Permanently passaged cells displayed a similar degree of morphological differentiation as the non-passaged cells, with both culture conditions resulting in a more pronounced differentiation than that achieved by culture on plastic. It was noted that the morphological differentiation observed was very heterogeneous, a situation also seen in xenografted tumours *in vivo*. The activity of alkaline phosphatase and the production of CEA was higher in the cells passaged on collagen (PPC) than the cells cultured on non-passaged collagen gel (Non-PPC) and plastic. The biochemical determination of aminopeptidase activity showed that collagen gel culture enhanced the activity in both non-passaged and passaged HT-29 cells above that of the cells cultured on plastic. However, immunocytochemical localization of aminopeptidase and sucrase-isomaltase of samples of cells grown on the various substrata for 7, 14, 21 and 28 days showed a reduction in both enzymes in the cells grown on collagen gels when compared to cells grown on plastic. The reason for the discrepancy between the two assays for aminopeptidase is at this stage unexplained.

Although, there was evidence to suggest that the culture of HT-29 cells on collagen gels was capable of inducing morphological and biochemical markers of enterocytic differentiation, there were no differences in the chemosensitivity of the different cell groups to a panel of anticancer agents.

Preliminary studies suggested that the ability of the cells to polarize by their culture on porous filter chambers without any exogenous ECM was sufficient to enhance HT-29 differentiation and the onset of differentiation was probably correlated with the production of ECM by the cells themselves.

Keywords : HT-29 cells, collagen type I, differentiation, chemosensitivity, continuous subculturing.

Carlyle

TO GRANDAD CHARLIE

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

AlP	alkaline phosphatase
AMP	2-amino-2-methyl-1-propanol
APAAP	alkaline phosphatase anti-alkaline phosphatase
APN	aminopeptidase N
BM	basement membrane
CAM	cellular adhesion molecule
CEA	carcinoembryonic antigen
dH_2O	double distilled water
DMEM	dulbecco's modified Eagle's medium
DMF	N,N,-dimethylformamide
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetra-acetic acid
EHS	engelbreth-holm swarm
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
FCS	foetal calf serum
5-FU	5-fluorouracil
g	acceleration due to gravity
g	grammes
h	hour
H&E	haematoxylin and eosin
$HT29_1$	human colon adenocarcinoma cells - 1st batch
$HT29_2$	human colon adenocarcinoma cells - 2nd batch
ih	intrahepatic
ip	intraperitoneal
IU	international units
iv	intravenous
K562	human chronic myelogenous leukaemia cells

L	litre
LM	light microscopy
M	molar
mab	monoclonal antibody
min	minutes
ml	millilitre
mM	millimolar
mRNA	messenger RNA
MUF	methylumbelliferone
MV	microvilli
n	sample size
NaB	sodium butyrate
Non-PPC	non-permanently passaged on collagen
P	passage
P_2	pellet number 2
PBS	phosphate-buffered saline
PE	plating efficiency
PPC	permanently passaged on collagen
ppm	parts per million
rpm	revolutions per minute
RPMI 1640	Roswell Park Memorial Institute medium 1640
RR	ruthenium red
s	seconds
S_1	supernatant number 1
sc	subcutaneous
SD	standard deviation
SEM	scanning electron microscopy
SI	sucrase-isomaltase
TBS	tris-buffered saline

TCNU	tauromustane,(1-(2-chloroethyl)-3-/2 (dimethylaminosulfonyl)ethyl/-1-nitrosurea)
TEM	transmission electron microscopy
TGF-ß	transforming growth factor-ß
TL	lower threshold
Tris	tris (hydroxymethyl)aminomethane
TU	upper threshold
TX-100	triton X-100
µl	microlitre
w/o	without
x	times

SECTION 1 : INTRODUCTION

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1.I General introduction

The investigations which will be discussed during the course of this thesis were performed within a cancer chemotherapy research group which was interested in the discovery and development of novel anticancer agents. Several sites within malignant cells were considered to be possible targets by the research group: these included the plasma membrane, key enzyme systems and DNA. Once novel agents were synthesised it was essential that their antineoplastic capabilities were determined before they could be submitted for clinical evaluation. Within our research group this was achieved by using *in vitro* screening models consisting of malignant cell lines. This is the method used by many laboratories including the National Cancer Institute in the United States (Alley *et al.*, 1988). The lack of success at producing anticancer agents which are useful against the solid tumours such as those of the colon lead us to explore the possibility that the environment in which the cells were cultured may determine the cells response to exogenous agents. A hypothesis was developed which suggested that our general lack of success with the solid tumour screening models may be a direct result of the restrictive nature of the *in vitro* environment, leading to the culture and use of unrepresentative cell lines. We aimed to evaluate the effects of collagen gel culture on growth, differentiation and the chemosensitivity of the human adenocarcinoma of the colon cell line, HT-29 (Fogh and Trempe, 1975).

1.II Colonic epithelium as a model system for studying the control of differentiation

1.II.i Why study a colon adenocarcinoma-derived cell line ?

There has been a gradual improvement in sanitation and medical care which has lead to increased longevity, but with it a corresponding increase in the age-associated diseases. One of these diseases is cancer, which is responsible for 22 % of all deaths in the United States (Silverberg and Lubera, 1989). The colorectum is the second most common site, after the bronchi and lungs, in the developed countries (Burkitt, 1971). In the United States of America alone, in 1989, 151,000 new cases of colorectal cancer were anticipated, with more than 61,000 expected to die of the disease (Mayer *et al.*, 1989). A

further 100,000 were expected to develop colon cancer in 1990 (Silverberg and Lubera, 1989).

Tumorogenesis is a complex, multistage process (Nowell, 1976). In experimental models the process has been broken down into at least three distinct steps : initiation, promotion, and progression (Farber and Cameron, 1980). It would appear that several physiological controls must be overridden in order for tumour progression to occur (Weinberg, 1989). This idea has been supported by the findings that several cellular oncogenes work in collaboration in order to induce malignant transformation (Land *et al.*, 1983; Ruley, 1983; Land *et al.*, 1986). The theory of multistep tumorigenesis "would propose that each step in the tumorigenic process reflects a mutation leading to the activation of one or other cellular oncogene; the resulting activated oncogenes then work together to induce the full neoplastic phenotypes of the cell" (Weinberg, 1989). Activated *c-ras* oncogenes have been found in a number of preneoplastic human tumour models (Liu *et al.*, 1987), including those of the colon (Vogelstein *et al.*, 1988), and these results would suggest that *c-ras* activation is a relatively early event in tumour progression. Colon adenocarcinomas have been shown to express higher levels of *c-ras* ^{Ha} and *c-ras* ^{Ki}, as well as *c-fos* and *c-myc* than normal tissue (Slamon *et al.*, 1984). *N-myc, L-myc* and *p53* have also been shown to act synergistically with *c-ras* in *in vitro* transformation assays (Eliyahu *et al.*, 1984), and are therefore candidate genes for late activation and collaboration. The primary genetic defect in hereditary colon carcinomas is located on chromosome 5q21-q22 (Bodmer *et al.*, 1987). Aberrations have also been found on the p-arm of chromosome 17 (Reichmann *et al.*, 1981; Kroes *et al.*, 1987; Fearon *et al.*, 1987) which is were the gene for the nuclear tumour antigen p53 (Lane and Crawford, 1979) is located. Analysis of the expression of p53 in adenomas and adenocarcinomas of the colon has shown that enhanced expression would seem to be associated with more advanced tumour progression (Van den Berg *et al.*, 1989). This would support the earlier ideas that p53 may be involved in late activation and possible collaboration with *c-ras*. However, the wild-type gene product of the p53 gene is known to function as a tumour suppressor (Wolf and Rotter, 1985; Finlay *et al.*, 1989), and initially it was not understood why either a single deletion of one allele of chromosome 17

leaving one functionally active allele, or overexpression of the p53 gene product could lead to malignancy. It was recently shown that most tumours with allelic deletions at chromosome 17.13.1, also contain p53 point mutations resulting in amino-acid substitutions in the retained allele (Nigro *et al.*, 1989). The authors also found that transformation could occur without deletion if mutation of one allele had occurred so that both mutant and wild-type alleles were expressed. It was suggested that the mutant p53 product may bind to the wild-type product creating an inactive oligomeric complex. Allelic deletions of chromosome 17 occurs in many tumour types, including 60% of colon tumours. Allelic loss of chromosome 18q is the second most common in colorectal tumours and is lost in over 70% of colorectal cancers, and approximately 50% of late adenomas (Vogelstein *et al.*, 1988). A candidate tumour suppressor gene has been located in this region, designated the DCC gene and found to encode a protein with considerable homology to the cell adhesion molecules (Fearon *et al.*, 1990). It is thought that the reduced or absent expression of this protein observed in almost all colorectal malignancies results in altered cell-cell and/or cell-extracellular matrix interactions which may lead to pathogenesis. By considering the data generated with regard the tumorigenesis of colorectal cancer, Fearon and Vogelstein (1990) have suggested a model which may explain the multistage process which results in colorectal cancer (figure 1).

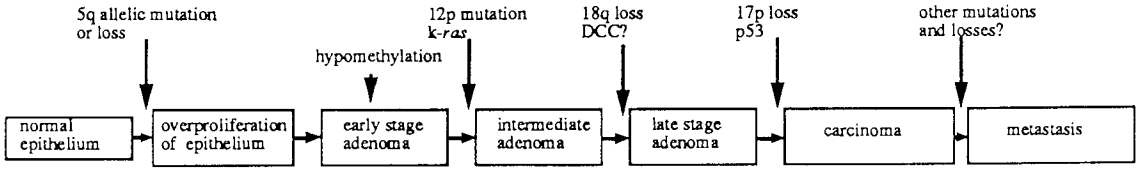


Figure 1 : Genetic model for colorectal tumorigenesis (adapted from Fearon and Vogelstein, 1990).

Colorectal cancer kills a large number of people every year, but it only accounts for 11-13 % of all cancer-related deaths (Silverberg and Lubera, 1989), which helps to illustrate the seriousness, and enormity of this disease when taken as a whole. Unfortunately, it has been shown that an estimated 66% of patients with colorectal cancer will be found to have serosal invasion (Dukes' stage B_2) or regional spread (Dukes' stage C) by the time of diagnosis. These patients are considered to have a higher risk of disease recurrence, and therefore often serve as the basis of clinical trials in order to determine the effectiveness of adjuvant therapy (Mayer *et al.*, 1989). Surgery remains the oldest and most tested therapeutic treatment available for colon cancer, but despite the major advances in this field, it still remains relatively limited in its ability to effect a cure (Eilber, 1985). Surgery is only really effective at the primary site of the tumour, and its usefulness is almost entirely dependent on the stage of tumour progression reached at the time of diagnosis. It remains fairly ineffective when dealing with advanced tumours and metastatic neoplasms. Radiation therapy also has its limitations, which are similar to those of surgery. It has been known for some time that it is often necessary to use chemotherapy in conjunction with surgery and radiotherapy, combinations that have been proved to be important in the treatment of local spread and distant metastases (Ramming and Haskell, 1985).

There has already been almost 50 years of research into cancer chemotherapy, and yet systemic chemotherapy is only minimally useful, particularly in the treatment of solid tumours like those of the colorectum. 5-Fluorouracil (5-FU), a fluorinated pyrimidine, was first introduced into clinical trials over 25 years ago, and it still remains the most effective single agent in the treatment of advanced colorectal cancer, but only producing partial response rates of 15-20 %. Several other anti-neoplastic agents have been tested for their efficacy in the treatment of colorectal cancer, but only mitomycin and the nitrosoureas have been found to have marginal activity comparable to 5-FU (Mayer *et al.*, 1989). Combinations of drugs given in conjunction with 5-FU, although originally thought to be more effective (Moertel *et al.*, 1975), have since been shown not to be (Buroker *et al.*, 1985; Richards *et al.*, 1986). Recent studies have concentrated on improving the efficacy of the single agent use of 5-FU, with groups focusing on the drugs effects on cellular metabolism, thought to be due to its binding to thymidylate

synthetase, leading to the depletion of thymidine, and consequently to a reduction in DNA synthesis. Several clinical trials have been conducted, in which the patients have received 5-FU and folinic acid, which enhances the binding of 5-FU to thymidylate synthetase. An improvement in therapeutic activity was seen in most of the trials (Petrelli *et al.*, 1987; Erlichman *et al.*, 1988; Gastrointestinal Tumor Study Group, 1989), but not in all of them (Valone *et al.*, 1989). There remains considerable controversy as to whether postoperative chemotherapy is beneficial in the treatment of colorectal cancer, with different groups producing conflicting evidence. There are ongoing studies at present, which are trying to solve this dilemma once and for all, and to determine the best postoperative management of patients with regional colorectal cancer (Mayer *et al.*, 1989). It is therefore apparent that colorectal cancer is very refractory to treatment and a considerable improvement in its treatment is essential. This is most likely to be achieved by understanding the cell and molecular nature of the perturbations which result in malignancy. The search for new anticancer agents needs to be based on a thorough understanding of specific tumour cell and molecular biology. The methods used to screen novel anticancer agents and their limitations are discussed further in section 1.IV.i.

Most of the anticancer agents presently used in the clinic have antiproliferative actions on malignant cells and it is this property which also results in the characteristic toxicity experienced by certain normal tissues which have a high proliferative capacity; for example the bone marrow, the gastrointestinal tract, and the hair follicles. Cancer was thought for a long time to be due to uncontrolled cell proliferation resulting in an expanded tumour cell population which had escaped the normal homeostatic controls of the tissue of origin. It is now believed that the error which causes cancer results from a block in the cells ability to differentiate normally. There has therefore been great interest in the control mechanisms for cellular differentiation in order that this knowledge may be used to produce chemicals which are capable of inducing the differentiation of malignant undifferentiated cells to their mature counterparts (Pierce and Verney, 1961; Pierce and Speers, 1988). The ultimate fate of these differentiated cells, with their reduced proliferative potential, would be death, as is seen in their normal counterparts. These

ideas for novel anticancer agents will be discussed in section 1.IV.ii. It is necessary to understand how differentiation is controlled in normal tissues in order to understand how this process has been perturbed during the development of a malignancy. A better understanding of the normal regulation of differentiation will also help in the synthesis of useful differentiating agents which may have clinical efficacy, possibly in conjunction with the standard antiproliferative agents.

1.II.ii Stem cells and tissue kinetics of the intestinal epithelium

It is essential when studying the differentiation of a malignant cell line to compare this to the normal tissue of origin. The stage of differentiation reached by the cells at the point of malignant transformation is also an important characteristic of the tumour and a factor which determines features such as morphology, differentiation potential and degree of malignancy. This is demonstrated nicely by cell lines derived from blood cell precursors. For example the HL60 cell line which was derived from a human promyelocytic leukaemia retains the ability to differentiate into both granulocytes and monocytes under certain conditions (Collins *et al.*, 1977; Collins *et al.*, 1978; Gallagher *et al.*, 1979). A thorough understanding of the cell population composition within the tissue of origin is essential as it gives the researcher a reference point to which the differentiation of malignant cells can be compared. The hierarchy of the intesinal epithelium has been studied extensively and the separate populations of cells have been well characterized (Cheng and Leblond, 1974; Wright, 1978; Potten and Morris, 1988). The tissue as a whole can be roughly divided up into the stem cell compartment and the maturing cell compartment.

Stem cells combine two very special properties that other cells may have separately. These two properties are the ability to generate progeny that are either the same as themselves or different i.e. asymmetric division, and the ability of self-renewal . In vertebrates, the evidence would suggest that it is environmental factors that control asymmetric division (Wolpert, 1988). There are distinct populations of cells within each tissue, each serving a specific function. The function of the stem cell population is to repopulate the tissue after cell loss, either natural or otherwise, and generally they are

thought to compose only a small fraction of the total cell population. Each mouse intestinal crypt is thought to contain about 20 functional, slowly cycling stem cells, with 60 rapidly cycling clonogenic cells, which can be termed potential stem cells (Wright, 1978). This is out of a total crypt cell population of 250 cells (Potten and Morris, 1988). Cell populations can be compartmentalized according to Gilbert and Lajtha (1965), and by combining several compartments it is possible to produce a sequence that adequately describes a specific tissue. Figure 2 shows the most likely sequence of compartments for the intestinal epithelia.

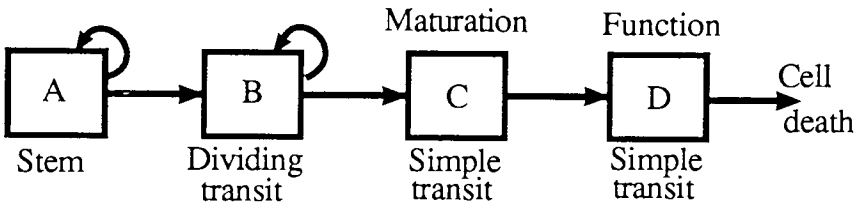


Figure 2 : The proposed sequence of compartments of cell populations for the intestinal epithelium (after Wright and Alison, 1984a).

It comprises of a stem cell compartment containing proliferating cells which not only renew themselves, but also feed cells into the next compartment. These stem cells are localized in the intestinal crypts. The dividing transit population, found in the mid-crypt region, provides a means of cell amplification, feeding cells into the simple transit compartments (maturation and function). The cells within these compartments are nonproliferative, terminally differentiated and are found in the intestinal villi (Wright and Alison, 1984b). After the terminally differentiated villus cells have migrated to the top of the villus tip, they are exfoliated into the gut lumen, to be replaced by further migrating and maturing cells. Figure 3 shows the spatial and proliferative organization of the mouse small intestinal epithelium.

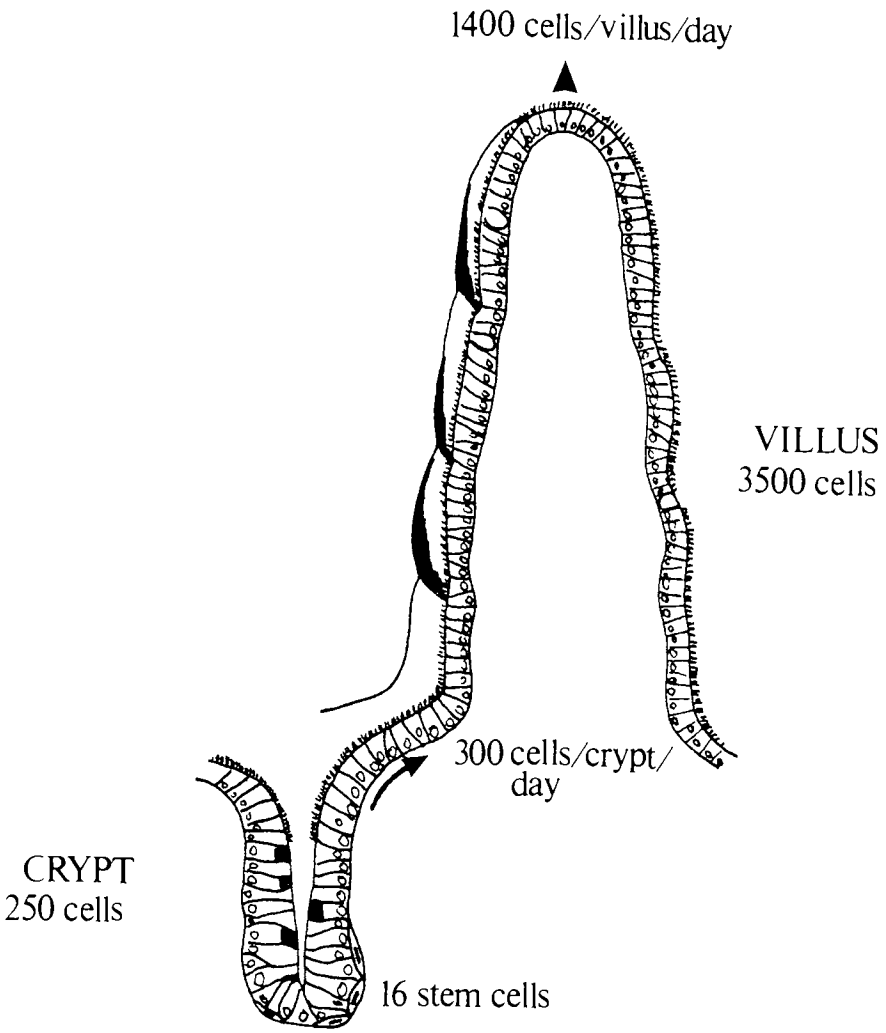


Figure 3 : A diagrammatic representation of the organization of the mouse small intestine (adapted from Potten and Morris, 1988).

It should be noted here that the small intestine is most commonly used as an example of intestinal proliferation and differentiation and it is true to say that it has been studied more extensively than other areas of the gastrointestinal mucosa. It may seem inappropriate to discuss the population dynamics of the small intestine in the introduction to work that was carried out on a colonic tumour cell line, but it will become clear later in this introduction why this has been done and it is not just because a larger body of research exists about the small intestine.

1.II.iii Regulation of differentiation of epithelial cell populations

Why should there be so much interest in the regulation of differentiation of epithelial cell populations? Firstly, they are very interesting from a purely cell biological point of view because of their highly ordered and uniquely polarized ultrastructure, which in turn determines their function. Secondly, and more importantly for researchers interested in cancer, epithelial cell populations give rise to approximately 90% of human malignancies, where there are often perturbations in the control of normal epithelial differentiation (Wright and Alison, 1984c). In studying the differentiation of epithelial cell populations, because of the inherent complexity, it has been necessary to work with primary cells and cell lines *in vitro*, so that the putative inducers can be studied separately. To this end, a wide variety of possible inducers of differentiation have been studied in such systems, and a brief review of the more recent advancements that have been made so far in the field of epithelial differentiation will be presented in section 1.III. I will concentrate in this section on what is known generally about differentiation as a process and more specifically on that which is known about the control of intestinal differentiation.

During the process of differentiation the cell undergoes many changes which can be outlined as follows :

1. Signal reception and transformation,
2. selective alterations in the genetic material,
3. differential gene expression,
4. organization of gene expression programs and
5. intercellular coordination of cell differentiation within developmental programs of tissues, organs and organisms (Nover, 1982).

The extracellular milieu provides a constant source of information which is interpreted through signal reception and transformation systems of the cell membrane and cytoplasm to the sites of gene expression. In the opposite direction, the gene products constitute the cytoplasm, and components of the extracellular matrix (ECM) and thus may in turn lead to changes in gene expression patterns within that cell (Nover, 1982). Bissell *et al* ., (1982) described a model that postulated an intimate relationship between the ECM on the one hand, and the cytoskeleton and the nuclear matrix on the other hand. Bissell called

this relationship, "dynamic reciprocity", and is based on existing literature which would suggest that the ECM exerts " physical and chemical influences on the geometry and the biochemistry of the cell via transmembrane receptors so as to alter the pattern of gene expression by changing the association of the cytoskeleton with the mRNA and the interaction of the chromatin with the nuclear matrix. This, in turn, would affect the ECM, which would affect the cell, which....." (Bissell *et al.*, 1982). The process of differentiation begins as soon as an egg is fertilized, and continues throughout the life of that organism. From his work on the differentiation of the three germ layers in the embryo, Grobstein (1955) initially termed the process by which different, but closely associated cell populations had an effect on each other in order to produce new and different cell types during development, "embryonic induction". In his earlier work (Grobstein,1953), he had shown how tissue interactions could effect morphogenesis. During the last 35 years, the subject of cell-cell and cell-ECM interactions have been the centre of considerable research efforts, and as many reviews have shown, the mesenchyme often plays a predominant role in controlling the differentiation of epithelia (Bissell *et al.*, 1982) ; for a general discussion see section 1.III.

After birth and during adult life, the small intestinal epithelium is maintained as a typical steady-state tissue, with a proliferating crypt stem cell population which differentiates to produce the four main cell types of the mature villus. These are described in more detail in section 1.II.v. In addition to the epithelial cell population there also exists a population of pericryptal fibroblasts which are capable of proliferating and migrating (Kaye *et al.*, 1968; Marsh and Trier, 1974 a and b; Maskens *et al.*, 1979). Histological observations have shown that there is a close relationship between the epithelial and mesenchymal cell populations during the development of the gut (Mathan *et al.*, 1972; Burgess, 1976). More recent work has shed light on the mesenchymal influences upon the differentiation of both embryonic intestinal endodermal cells and epithelial progenitor cells of the gut by using recombinations of mesenchyme (or stroma) and endoderm (or epithelium) cultured *in ovo* or *in vitro* (Kedinger *et al.*, 1983; Lacroix *et al.*, 1985; Haffen *et al.*, 1986; Kedinger *et al.*, 1986; Kedinger *et al.*, 1987). Haffen *et al.*, (1987) in their review concluded that "from the related studies on epithelial-mesenchymal interactions during

development of the digestive tract, emerges the concept that mesenchyme from the small intestine displays like the stroma of neonates, inductive influences on epithelial differentiation". They also noted that even though isolated gut endodermal cells were capable of some self-differentiation as already observed by Le Douarin *et al* (1968), their complete differentiation into the enterocytic, goblet, endocrine and paneth cell lineages, as well as villus morphogenesis and enzyme expression, was dependent on contact with intestinal mesenchyme. Another important aspect was the effects that the endodermal and crypt cells had on the mesenchymal morphogenesis, which resulted in the organization of typical circular and longitudinal smooth muscle layers. The chick gut endoderm can cause the differentiation of rat skin and lung fibroblasts into intestinal smooth muscle, which suggests that the endoderm causes a specific morphogenetic signal that is not species specific, and can be considered instructive (Haffen *et al.*, 1987). Although it would appear that a considerable advancement has been made in our understanding of the regulation of the differentiation of the gut, there still remains a number of fundamental questions to be answered. The inductive signals remain to be characterized, as do the modes of signal transduction from the extracellular milieu to the site of gene expression. The roles that the ECM (Kedinger *et al.*, 1987) and hormones (Lacroix *et al.*, 1985) may play in regulating the differentiation of the intestine are also being examined more closely.

1.II.iv Differentiation of the HT-29 human colon adenocarcinoma cell line *in vitro*

The HT-29 cell line was originally derived in 1964 from a moderately well differentiated primary adenocarcinoma of the human colon (Fogh and Trempe, 1975). When grown *in vitro,* in medium containing 25 mM glucose, these cells exhibit characteristics of an anaplastic, undifferentiated cell line with a doubling time of 24 hr, forming a tightly packed multicellular layer on tissue culture plastic. At the TEM level these cells show few features of enterocytic differentiation, and grow as unpolarized cells with little evidence of tight junctions, desmosomes, or microvilli. Brush-border associated enzyme activities are corresponding low or absent (Pinto *et al*, 1982).

Changes in metabolism

There has been considerable research into the control of differentiation of this cell line during the past 26 years, particularly since the discovery that enterocytic differentiation could be induced by simply altering the nutrient supply. Pinto *et al* (1982), showed that by replacing the standard tissue culture medium containing 25 mM glucose with identical medium containing 5 mM galactose and no glucose, these normally undifferentiated cells, could be induced to develop a morphology similar to that of normal intestinal epithelial cells, exhibiting apical microvilli-lined brush-borders and associated tight junctions between adjacent cells. These morphologically differentiated cells also showed characteristics of functional intestinal differentiation, with an increase in the activity of the brush-border associated enzymes, aminopeptidase, alkaline phosphatase, maltase and sucrase-isomaltase. There were also distinct changes in the metabolic profile of these cells. The differentiated cells were shown to have a reduced hexose consumption and lactic acid production, both suggesting reduced glycolytic activity. Another interesting feature of the differentiated cells was their decreased accumulation of glycogen. This was particularly significant, especially in relation to a previous study by Rousset *et al.*,(1979), which showed that there were very low levels of glycogen in the epithelium of normal adult colon and rectum, but much higher levels in the colon of the human foetus up to 30 weeks of gestation. The levels of foetal colonic glycogen decreased with foetal age, and from 30 - 36 weeks there were almost no glycogen positive cells. Their study included human primary colon carcinomas and xenografts of two human cell lines, HT-29 and HRT-18 (Bernard, 1959). They found that the carcinomas regularly expressed levels of glycogen higher than normal adult colon, and similar to the amount seen in the foetal colonic tissue. This was also the case for the two cell line xenografts studied. The expression of sucrase-isomaltase activity in foetal colon, adult small intestine, HT-29 xenografts, and not in adult colon lead to the suggestion that the HT-29 cell line has foetal origins and retains the capacity to differentiate into mature cells more representative of the adult small intestine than the colon (Zweibaum *et al.*, 1983).

In their 1980 paper, Rousset *et al.*, showed that the accumulation of glycogen in human intestinal carcinoma cell lines *in vitro* was correlated with the phase of growth, with levels increasing at confluency in each cell line, although individual cell lines exhibited distinctly different amounts of glycogen. The levels of glycogen seen in the corresponding xenografts were comparable to that seen *in vitro* during the exponential growth phase for each cell line studied. The levels of glycogen stored by the xenografts was not effected by fasting the mice, which was in direct contrast to that which was seen in the liver, were levels fell drastically during fasting. The highest levels of glycogen seen were associated with the slowest rate of cell division in both *in vitro* and *in vivo* studies. The authors suggested that the increase in glycogen in malignant intestinal cells could be due to a reversion to the foetal enzyme patterns for glycogen metabolism, a situation that is seen in malignant liver cells which express foetal liver isoenzymes (Sato *et al.*,1973). They also suggested that there may be an impairment in the extracellular control of glycogen metabolism, and that this area needed further studies to elucidate the role that vasoactive intestinal peptide (VIP), which had been shown to activate cyclic AMP-dependent protein kinases in HT-29 cells (Marvaldi *et al..*, 1979), may play in glycogen metabolism. Chastre *et al.*, (1985), demonstrated that the enterocytic differentiation observed in the HT-29-18 subclone (Huet *et al.*, 1987) when grown in galactose-containing medium, was associated with a reduction in their capacity to generate cAMP after forskolin treatment and cell surface receptor activation by VIP. Taken together with the authors previous work on rat intestinal development, their results lead them to propose that VIP receptor activity was an indicator of intestinal cell maturation. Further studies by Luis *et al.* (1987), described the cyclohexamide-induced enhancement of VIP binding to the undifferentiated form of the subclone, HT-29-D4 (Fantini *et al.*, 1986). They explained this result, in which an inhibitor of protein synthesis resulted in the increase of a cell-surface receptor glycoprotein, by proposing that exposure to the drug caused a deregulation in the balance between internalization and recycling of unoccupied VIP receptors to and from an intracellular pool. Also in 1987, VIP receptors were located on the nuclei of HT-29 cells (Omary and Kagnoff,1987). These receptors may have been translocated from the plasma membrane, in a similar manner as

hypothesized for the insulin receptor (Podlecki *et al.*, 1987) and the EGF receptor (Raper *et al.*, 1987), or that the site of synthesis of the VIP receptors may be on the nuclear envelope, as reported for the vesicular stomatitis G protein (Puddington *et al.*, 1985).

Elaborate studies into the metabolic changes that take place during HT-29-D4 differentiation, using multinuclear magnetic resonance spectroscopy, were undertaken by Galons and his co-workers (Galons *et al.*, 1989; and Galons *et al.*, 1990). In their first study, they demonstrated that there were no significant differences between the energy status of undifferentiated and differentiated HT-29-D4 cells, but there were elevated levels of phosphorylcholine in the starved, differentiated cells, suggesting there had been an induction of fatty acid utilization as energy substrates. This result supported previous observations by Agris and Campbell (1982), which showed an increase in phosphorylcholine in leukaemic cells which had been induced to undergo erythroid differentiation by exposure to DMSO. The authors were able to state from their results that the differentiation observed in HT-29-D4 cells was correlated with a large increase in oxidative metabolism, although further studies were required in order to determine the fluxes occurring through the tricarboxylic acid cycle in these cells. Their second study, (Galons *et al.*, 1990), measured the rate of glycolysis, lactate production and glycogen synthesis in response to VIP, in HT-29 cells anchored on a polystyrene beaded microcarrier system, using ^{13}C nuclear magnetic resonance spectroscopy. Their results showed that VIP did not seem to play a very important role in the regulation of glycogen metabolism as it only acted transiently in the activation of glycogen phosphorylase.

The work on glycogen storage was further supported by a study (Zweibaum *et al.*, 1985), in which it was shown that HT-29 cells could be induced to differentiate in a similar manner as to that induced by growth in galactose, by removing all sources of hexose. Initially, the switch from glucose containing medium to medium devoid of hexose caused considerable cell loss, which gradually decreased, to select for a subpopulation which when passaged would readily attach and begin to grow at a markedly reduced growth rate of 10 days compared to 24-28 hr of the undifferentiated HT-29. Upon reaching confluency, these hexose-deprived cells formed a polarized monolayer which expressed a typical enterocytic morphological and enzymatic

differentiation. These differentiated cells stored very low levels of glycogen, which increased back to control levels if these cells were re-exposed to glucose containing medium, during a process which the authors called dedifferentiation.

Also in 1985, Wice *et al* , carried out an elaborate set of studies which correlated specific biochemical changes with the regulation of glucose deprivation induced enterocytic differentiation of HT-29 cells. They assayed the undifferentiated and differentiated cells for intracellular nucleotide levels, and found that the accumulation of UDP-N-acetylhexosamines was concomitant with the inability of these cells to differentiate. They found elevated levels of UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine in post-confluent HT-29 cells grown only in glucose or fructose (used as a control because the cells can utilize it at the same rate as glucose). Further support for the results was given by the finding that the levels of these two metabolites remained constant during the spontaneous enterocytic differentiation of the Caco-2 cell line (Fogh *et al*, 1977), which can occur in the presence of glucose. Why should the accumulation of UDP-N-acetylhexosamines be involved in the regulation of differentiation of HT-29 cells ? The authors suggested that because 1) the sugar composition of cell-surface glycoproteins change as a function of cell growth and transformation, 2) UDP-N-acetylhexosamines are important intermediates in the glycosylation of cell-surface glycoproteins, 3) the brush-border enzymes are glycoproteins 4) the differentiation of HT-29 and Caco-2 occurs after confluency and 5) the differentiation of HT-29 can be regulated by altering the carbon source, it would seem likely that the glycosylation of proteins is closely associated with the ability of these cells to differentiate (Lennarz, 1980; Hubbard and Ivatt, 1981; and Kenny and Maroux, 1982 for reviews on the biochemistry of glycoproteins and proteoglycans). However, it should be bourne in mind that different subclones of HT-29 are apt to give different results, a phenomenon that is highlighted by the fact that Galons *et al.*, (1989), did not show an increased accumulation of UDP-N-acetylhexosamines in undifferentiated HT-29-D4 cells compared with differentiated HT-29-D4.

A study by Ogier-Denis *et al.*, (1988), was designed to investigate whether an overall alteration of protein glycosylation in HT-29 cells, was associated with their inability to differentiate. Their experiments revealed that during a short exposure (10 min), the

undifferentiated HT-29 cells incorporated less D-[2-^{3}H] mannose, and during a longer exposure (24 hr) more of the radioactivity was seen to accumulate in the high mannose glycopeptides. Also, analysis of the high mannose oligosaccharides showed that there was an accumulation of a particular species of these in the undifferentiated cells, which was not seen in the differentiated cells. The authors stated that the glycosylation pattern observed in the undifferentiated cells was consistent with an impairment of the trimming of high mannose into complex glycans. It can be concluded from their work that N-glycan processing was correlated with the state of enterocytic differentiation of HT-29 cells. Earlier work by Simon-Assmann *et al.*, (1987), had also shown that changes in the synthesis of glycosaminoglycans (GAGs), were correlated to the onset of enterocytic differentiation of HT-29 cells. GAGs are long polyanionic carbohydrate chains composed of repeating disaccharide units (uronic acid and hexosamine) which form the ground substance of the extracellular matrix, and are always linked covalently to proteins, forming proteoglycans (with the exception of hyluronic acid,the only non-sulphated GAG). HT-29 cells were differentiated by substituting the glucose for inosine (2.5 mM), a nucleotide which had previously been shown to support mammalian cell growth (Wice *et al.*, 1981,1985). The differentiated HT-29 cells incorporated 20 times and 4.5 times more [^{3}H] glucosamine and [^{35}S] sulphate respectively, and they contained 5 times more uronic acid, than the undifferentiated cells. Not only was there this increase in overall GAG synthesis in the differentiated cells, there was also the expression of a new class of chondroitin, (CS4), with a simultaneous reduction in and a charge density modification to heparan sulphate. These changes in CS4 levels paralleled observations reported for avian cornea, in which differentiation was associated with an overall increase in chondroitin levels (Toole and Trelstad, 1971). The apparent ability of these cells to alter their extracellular environment, as suggested by these studies, correlates well with the idea of "dynamic reciprocity" as advocated by Bissell *et al.*, (1982), and already discussed in section 1.II.iii.

An aspect of HT-29 differentiation that has been largely neglected is the study of the possible inductive effects of individual components of the extracellular matrix. In particular, the effects of culture on collagen type I gels, which has in many cells lines

been shown to induce differentiation (see section 1.III). The reason for there being very little interest in this area of differentiation induction, for the HT-29 cell line, could be that those experiments published to date have produced rather disappointing results. Phillips *et al.*, (1988), showed that growth on either laminin, fibronectin, collagen type I or IV did not enhance the differentiation brought about the alteration in the hexose source, from glucose to galactose. In another study, Richman and Bodmer (1988), demonstrated that increased levels of mucus were only seen in HT-29 cells grown on mesenchymal cell lawns. There were very little changes seen in cells grown embedded within collagen type I gel, when compared to cells grown on uncoated tissue culture plastic. Pignatelli and Bodmer (1989), also showed that the culture of HT-29 cells that they used did not bind very well to collagen type I, and this may explain the lack of results in their 1988 study. As already stated, different batches of HT-29 cells from different sources, and different clones and subclones of the same parental line, are likely to display intrinsic features which will lead to different results, and due caution must be taken when comparing results from different research groups. Therefore, although it would seem an unfruitful area of work to pursue, the role of collagen type I and other ECM components, in the differentiation of HT-29 cells should not be dismissed before it has been fully investigated. For these reasons I chose to investigate the role that the ECM component collagen type I may play on the intestinal differentiation of the human adenocarcinoma of the colon cell line, HT-29.

Polarization

An important morphological characteristic of epithelial cells is their arrangement into a polarized cell sheet and very little is known about how this organization into a polarized epithelium is controlled, but the use of a cell-line such as HT-29, whose polarized differentiation can be induced *in vitro,* has lead to a higher degree of understanding, which interestingly correlates well with studies on the development of the foetal intestinal polarization. Work by both Remy *et al.*, (1984), and Le Bivic *et al.*, (1988), have highlighted the development of intracellular lumina with associated apical markers (for example antigen 517) in undifferentiated HT-29 cells, which would appear to be the

source of the apical membranes observed in the polarized, differentiated cells. This situation is very similar to that which is observed during the histogenesis of crypts and villi in the rat small intestine (Mathan *et al.*, 1976). During such foetal development, the layer of undifferentiated epithelial cells lining the lumen, forms secondary lumina by the fusion of cytoplasmic vesicles, called vacuolar apical compartments (VACs), by Rodriguez-Boulan and Nelson, (1989), adjacent to the junctional complexes between adjacent cells. The lumina become lined with micro-villi, gradually enlarge in size, and finally fuse together and with the main lumen, forming rudimentary villi, which are lined with a layer of columnar cells. The similarity between this situation in the developing intestine and that which is observed in differentiating HT-29 cells, has resulted in this cell line being used in the study of lateral diffusion in polarized membranes (Magnusson *et al.*, 1987; Gustafsson *et al.*, 1988; Rabenandrasana *et al.*, 1990; Magnusson *et al.*, 1990). It is evident that the HT-29 cell line retains the ability to differentiate morphologically into polarized enterocytes and it is this characteristic which was considered to be important when designing the collagen type I experiments for my investigations, in which the cells were cultured on either attached gels or gels which were allowed to float freely in the media. The latter gels were used to test whether or not being able to derive nutrients from a putative basolateral surface compared to being forced to feed from the apical surface as is the case when cultured on plastic and attached collagen gels, could induce intestinal differentiation above that induced by the collagen alone. The effects of being able to selectively feed from one surface without the possible inductive ECM effects were also determined by using inert porous filter inserts.

Differentiating agents

HT-29 cells have also been used to study the effects of putative differentiation-inducing agents, to be discussed in section 1.IV.ii. Sodium butyrate (2 mM) was shown to induce increased levels of alkaline phosphatase, a brush-border associated enzyme, used as a marker of intestinal differentiation in HT-29 cells. This increase was enhanced by the simultaneous exposure to a hyperosmolar environment, brought about by the addition of sodium chloride (40 mM) to the culture medium (Herz *et al.*, 1981). Although their

results were indicating that differentiation may be occurring in response to NaB, they did not study any morphological changes that may have been taking place, or the expression of any other markers of intestinal differentiation (see section 1.II.v). Also, an important finding was that all of the responses observed were reversible upon removal of NaB. However, Augeron and Laboisse (1984), produced a range of permanently differentiated clones of HT-29 cells by exposing the parental cells to 5 mM NaB for 9 days, followed by trypsinization and subculturing in NaB for a further 14 days. The cells were then returned to control culture medium, in which focal patches of differentiated cells emerged 10 - 12 days later. These "flat foci" of morphologically differentiated cells remained stable in culture for longer than 2 years. Several clones were characterized, and clones were distinct from one another for a number of properties. For example, the 16E clone produced a thin layer of mucus at the free surface of the cells, where as the 19A clone produced domes, representing transepithelial transport. A panel of human colorectal cancer cell lines were used to determine the effects of NaB (2 mM) on the activity brush-border associated hydrolases (Chung *et al.*, 1985). The results varied from cell line to cell line, with some being more responsive than others. The results for HT-29 showed overall enzyme levels being relatively low in comparison to the other cell lines tested. As expected, NaB induced a marked increase in the activity of alkaline phosphatase, along with slightly increased sucrase and dipeptidyl aminopeptidase IV activities, no change in lactase and trehalase, and a reduction in aminopeptidase activity. An interesting point to note was that very few cell lines showed an increase in their activity of aminopeptidase in response to NaB. More recently, Tanaka *et al.*, (1990), showed that by exposing HT-29 cells to 1,25-dihydroxyvitamin D3 (or one of its analogues),which alone caused growth inhibition only and no differentiation, at the same time as NaB (2mM), they could enhance the differentiation seen with NaB alone and prolong the reversal time once the NaB was removed. It is therefore apparent that HT-29 cells are, like other cells *in vitro* (Griffin *et al.*, 1974 ; Chou, 1979 ; Kim *et al.*, 1980 ; Tsao *et al.*, 1982 ; Morita *et al.*, 1982) responsive to the differentiation-inducing effects of NaB, although the effects are not permanent (except in the case of the Augeron and Laboisse clones), and the cells are free to revert to their undifferentiated form upon removal of the drug.

This overview of the wide variety of studies that have been performed on the HT-29 cell line has hopefully shown the usefulness of this cell line in different areas of research. However, most are related to intestinal differentiation in some way, and the struggle to determine what mechanisms control this process in the normal tissue, and the possible aberrations associated with malignant transformation. These cells have shown themselves to be very useful in the study of alterations in metabolic status, enzyme biosynthesis and processing, organization of cell polarity and membrane targeting of membrane-associated proteins, and growth factor and secretagoue production. It can be said that the HT-29 cell is a very versatile cell, which lends itself well to a vast range of research possibilities, and these attributes have resulted in its extensive use during the past 26 years.

1.II.v Markers of intestinal cell differentiation

It has been shown in many studies (see section 1.II.iv) that the human adenocarcinoma of the colon cell line HT-29 (Fogh and Trempe, 1975), produces differentiated cells whose characteristics are similar to those of mature small intestinal epithelial cells. It is therefore appropriate to use markers of small intestinal differentiation when studying these cells. For this reason, the small intestinal system and its markers will be discussed here.

Elaborate studies on the differentiation of the various intestinal epithelial cells have been performed, which describe in detail the kinetic changes and the subtle morphological alterations that take place as the cells migrate up from the area of proliferation in the crypts, to the sites of maturation in the villi (for an excellent description of these events see Cheng and Leblond, 1974). Only a brief outline of the morphological markers of intestinal differentiation, that can be seen at the electron microscope level will be given here.

The proliferative stem cells of the intestinal crypts are undifferentiated, and they exhibit very few features that could distinguish one cell that will become an absorptive enterocyte, from another that may be destined to become a goblet cell. These cells, although undifferentiated, do display polarized organelles, a characteristic of epithelial

cells. These cells are dividing continuously, and in doing so, the cells above them are forced to migrate upwards and out of the crypt. Migrating cells mature as they move up the length of the villous and as they do they gradually loose their proliferative potential. When maturation is complete, and the cell has acquired its final functional phenotype, it is said to be terminally differentiated and can no longer divide. The cell number along the length of the crypt-villous axis is maintained ay a reasonably constant level by the continual cycle of cell division, maturation and finally cell loss at the villous tip.

The morphological appearance of the fully mature intestinal cell will very much depend on the lineage that it is has originated from. The four main main cell types seen in the intestine, are the columnar or absorptive cell displaying a brush border, the mucin-secreting goblet cell, the entero-endocrine cells with their small, usually dense basal granules, and the paneth cells with larger electron-dense granules (see Figure 4).

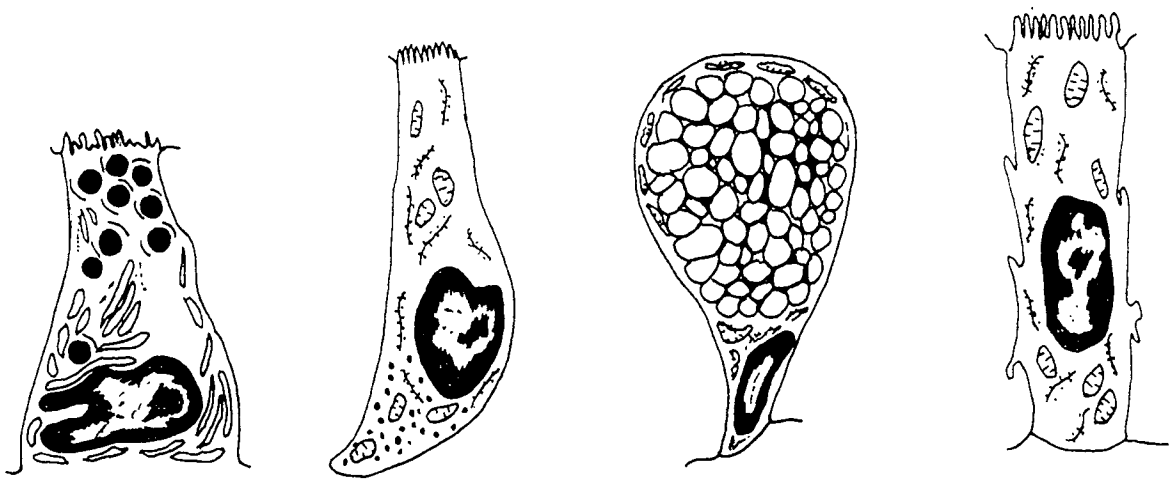


Figure 4 A diagrammatic representation of the four cell lines of the small intestine. (adapted from Wright and Alison, 1984d).

When HT-29 cells are cultured on plastic substrata under standard culture conditions they exhibit a characteristic anaplastic, undifferentiated morphology (Pinto *et al*, 1982). It is therefore quite simple to demonstrate morphological changes representing differentiation in an intestinal epithelial system, where the fully mature phenotypes are so distinct from the undifferentiated ones. It is also essential that subtle transitional changes be recognized in cells that may not be expressing overt signs of intestinal differentiation. For example, undifferentiated tumour cells rarely exhibit markers that represent cell communication and

adherence, such as gap junctions, desmosomes and tight junctions,the latter being associated with the apical membrane of a polarized cell. However, there is some evidence that these appear before the more overt morphological markers, and it is thought that cell-cell or cell-substratum contacts and the construction of tight junctions is essential for polarization of the cell membrane to be successful (Rodriguez-Boulan and Nelson, 1989). The mature enterocytic cell displays an apical membrane consisting of microvilli (MV), which should be of an even size and distribution over the cell surface. It is useful to use the scanning electron microscope (SEM), to study the development of a brush border, as there can be many artefacts related to the sectioning of samples for transmission electron microscopy (TEM).

However, the latter technique does make it possible to show the extent of cytoskeletal development within the MV, and the degree to which the MV core proteins are anchored into the apical cytoplasm (or terminal web). TEM also allows the observer to look at the overall organization of the cells, intracellularly and intercellularly. The careful use of monoclonal antibodies and TEM techniques have enabled the cytoskeletal changes that take place during intestinal differentiation to be demonstrated at an ultrastructural level (Mooseker, 1985; Heintzelman and Mooseker, 1990). The appearance of mature goblet cells is indicated by the accumulation of large mucus-filled vacuoles within the cytoplasm of the cells, and the characteristic basolaterally situated nucleus. The detection of the remaining two cell types is made difficult by their similarity to the enterocytic cells when in their intermediate forms, and their relative scarcity in the normal tissue (Cheng and Leblond, 1974), and in the *in vitro* situation (Huet *et al.*, 1987).

I have used TEM techniques during the course of this research project to demonstrate any morphological changes indicative of intestinal differentiation, and to allow the visualization of alkaline phosphatase activity. Most of the studies into the manipulation of differentiation of colon carcinoma cell lines *in vitro*, by a variety of exogenous agents, have focused their attention on the functional differentiation of the cells. The differentiated, enterocytic cell has a host of brush-border associated hydrolytic enzymes. These enzymes are intergral membrane glycoproteins, consisting of peptidases, saccharidases and phosphatases.

Alkaline phosphatase

One of these marker enzymes, alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, AlP), is a phosphatidylinositol-anchored membrane glycoprotein (Low *et al.*, 1986), which is abundant in bone, liver, kidney, placenta and intestine (McComb *et al.*, 1979). It has been implicated in the maintenance of the intracellular concentration of phosphate, which is essential for the formation of bone and the processes of absorption and transport across membranes such as those in the intestine. AlP is activated by metallic cations, particularly magnesium, and by certain amino acids (Bitensky and Poulter, 1969). It catalyses the hydrolysis of numerous phosphate esters of primary and secondary alcohols, cyclic alcohols, phenols and amines. Activity can be inhibited by inorganic phosphate, metal-chelating agents, cyanide and the amino acids, L-phenylalanine, L-tryptophan and L-cysteine (Bergmeyer, 1974). A biochemical assay for AlP activity was used in which the production of 4-methyl-umbelliferone, a highly fluorescent product in strongly alkaline solutions, from the substrate, 4-methylumbelliferylphosphate was measured (Jakel *et al.*, 1983). Although, AlP activity is generally associated with the brush border membranes of the small intestine (Kenny and Maroux, 1982), an observation which is indicative of its higher absorptive function, AlP has been demonstrated histochemically in the adult colon, but the activity is low (Lev and Griffiths, 1982). Like the other brush-border associated enzymes, the activity of AlP is high in the human foetal colon, with the activity declining from birth onwards (Lacroix *et al.*, 1984). This supports the suggestions that the HT-29 cell line is of foetal origins which retains the ability to differentiate into mature small intestinal cell rather than mature colon epithelial cells.

Aminopeptidase N

Aminopeptidase N (aminopeptidase (microsomal), EC 3.4.11.2, APN), is the most abundant peptidase in the small intestinal microvilli (Kenny and Maroux, 1982). It is thought to serve important physiological functions in the digestion and absorption of small peptides (Kim *et al.*, 1974), which are the major products of gastric and pancreatic digestion of food protein (Adibi and Mercer, 1973). Lacroix *et al.* (1984), demonstrated

high levels of activity in human foetal colonic epithelial cells, and in the same year Zweibaum *et al.*, also showed that activity was associated with brush border-like structures in 7 out of 27 human colorectal tumours of patients. High levels of foetal activity, and the lack of activity in the adult colon, were also confirmed in their study. A biochemical assay, which is based on a method by Roncari and Zuber (1968), utilizes the change in absorbance at 410 nm, which occurs as the substrate, L-leucine-p-nitroanilide is hydrolysed to p-nitroaniline. Immunocytochemical detection of the enzyme in paraffin-embedded tissue sections, is made possible by the development of a monoclonal antibody by Hauri *et al.* (1985), and mab HBB 3/153/63 was used during this study.

Sucrase-isomaltase

Of all the brush border-associated disaccharidases, sucrase-isomaltase (sucrase α-D-glycohydrolase, EC 3.2.1.48, SI), has received the most attention. A major constituent of the intestinal apical membrane (Kenny and Maroux, 1982), it is interesting because it has been found to be composed of two subunits of unequal size, with the sucrase subunit being the smaller of the two. This enzyme is responsible for up to 90 % of microvillar maltase activity (Hauri *et al.*, 1985). It is initially assembled in the microvillar membrane, as a single-chain bifunctional protein, later to be cleaved into two monofunctional subunits by pancreatic proteases (Kenny and Maroux, 1982). It is one of the best characterized marker enzymes of the enterocytic villus cells of the adult small intestine. In the human, it is expressed very early during foetal development (Grand *et al.*, 1976), and along the entire length of the intestine, including the colon (Skovbjerg, 1982; Triadou and Zweibaum, 1985; Mernard and Pothier, 1987). In the developing colon, as the transient foetal villi gradually disappear, SI activity begins to decrease, reaching very low or negligible levels at term (Semenza *et al.*, 1988). Sucrase-isomaltase activity, has also been found to be higher in malignant human colonic epithelial cells, including xenografts of HT-29, than in normal adult colon. The finding that malignant colonic epithelial cells expressed sucrase-isomaltase activity was unexpected because this enzyme was shown to be absent in normal colonic epithelial cells at birth and beyond (Zweibaum *et al*, 1983).

These results are similar to those reported for the other hydrolytic enzymes, and to those for other antigens, enzymes and polpeptide hormones, which are expressed in different malignant cells, and frequently have foetal origins (Weinhouse, 1980). However, it is interesting to note that sucrase-isomaltase was also expressed in 9 out of 11 juxta-tumoural tissue samples, and the answer to whether this expression was due to the inductive effects of the neighbouring tumour cells or was a result of premalignant changes may have important diagnostic and prognostic implications (Zweibaum *et al,* 1984). It was these studies on the expression of small intestinal enzymes by a colonic adenocarcinoma cell line, that lead researchers to believe that either HT-29 cells originated from a very early stem cell tumour which retained foetal characteristics, or that the process of malignant transformation of the normal cell had resulted in a reversion to a foetal pattern of differentiation. Biochemical assays exist , which generally rely on the hydrolytic degradation of the substrates sucrose and maltose (Dahlqvist, 1968), but such assays require large quantities of cells, and were therefore not used in this present study, to demonstrate SI activity. An immunocytochemical technique was used for the demonstration of SI expression, which utilizes mab HBB 2/219/20, one of a panel of antibodies developed by Hauri *et al.*, (1985).

Carcinoembryonic antigen

Carcinoembryonic antigen (Gold and Freedman, 1965; CEA), is a highly glycosylated cell surface, oncofoetal glycoprotein, which is overexpressed in nearly all human colon carcinomas (Shuster *et al.*, 1980). Its precise function remains the centre of much speculation, although its amino acid sequence has been determined, and compared with that of proteins of the immunoglobulin superfamily (Paxton *et al.*, 1987; Oikawa *et al.*, 1987a, 1987b; Beauchemin *et al.*, 1987), which includes not only the immunoglobulin molecules, but also the T-cell receptors, growth factor receptors and cellular adhesion molecules (CAMS) (Williams, 1987). Its membership of this superfamily of proteins and its localization to the cell surface membrane has implicated that it may be involved in intercellular recognition. Its overproduction has recently been shown to enhance colon carcinoma cell-cell aggregation as demonstrated by a suspension assay, and the Fab'

fragments of anti-CEA antibodies were able to block this aggregation. The authors also demonstrated that CEA was able to function independently of Ca^{2+} ,and its role as a CAM, although probably aberrant, was implicated in tumorigenesis and enhancement of metastasis (Benchimol *et al.*, 1989). CEA expression is generally associated with well differentiated colorectal carcinoma (O'Brien *et al.*, 1981; Brattain *et al.*, 1984), and adenomas of increasing malignant potential (Au *et al.*, 1986), but results have been conflicting (Denk *et al.*, 1972; Bordes *et al.*, 1973; Goldenburg *et al.*, 1976; Rognum *et al.*, 1982). However, the results of a more recent study would suggest that CEA expression is greater in well rather than moderately differentiated colorectal cancers. The expression was also higher in tumours from distal rather than proximal sites in the colon, a phenomenon which was not due to site-dependent differences in differentiation status of the tumours (Davidson *et al.*, 1989). Expression in the normal colon is more strongly associated with the undifferentiated basillar crypt cells and microvillar surface of the mature absorptive cells (Ahnen *et al.*, 1987). These authors also demonstrated the variation in expression in a panel of colon carcinomas and found a number of poorly differentiated tumours which did not express CEA at all, a situation which did not have a parallel in the normal colonic tissue. They suggested that although there were some similarities between normal and malignant colon epithelial differentiation, the two processes were not totally analogous. It had already been demonstrated that the undifferentiated HT-29-D4 cell line only expresses a small amount of CEA at their surface membranes, and they release a small amount into the culture medium. However, when induced to differentiate by substitution of galactose for glucose in the medium, there was considerable enhancement in both surface expression and release (over 50 times higher) with a maximum amount of released CEA being reached 10 days after the media change, the levels remaining constant for several months. CEA expression in the differentiated HT-29-D4 cells was localized to the apical membrane domain, and the release of CEA into the media was also, exclusively from the apical side of the monolayer. These findings were determined by culturing the cells on porous-bottomed culture inserts, upon which the glucose-deprived cells produced a leak-proof epithelial monolayer (Fantini *et al.*, 1989). These results showed a correlation between the levels

of CEA located in the membrane and the levels secreted into the media, with both of them, either being low in the undifferentiated cells, or high in the differentiated cells. It was therefore decided during this present study to measure the levels of CEA released into conditioned media using an ELISA .

1.III Physiological inducers of differentiation

There is considerable evidence that individual components of the ECM such as collagen, glycoproteins and glycosaminoglycans are directly involved in the control of development and differentiation. It is also evident that the ECM has continued effects once the tissues are formed, and that the maintenance of tissue specific functions is still to some extent dependent on the extracellular milieu (Bissell *et al.*, 1982). Kedinger *et al.*, (1987) found that culture of rat intestinal endoderm on various extracellular matrix components (collagens I, III, IV, fibronectin and laminin) produced a limited degree of morphological and enzymatic differentiation, but could not support growth beyond one week. However, when these cells were cultured on a confluent lawn of fibroblasts (either embryonic intestinal-derived mesenchymal cells or fetal skin fibroblasts), the cell survival increased up to 2-3 weeks, and there was enhanced expression of enterocytic differentiation characteristics. These cells were also responsive to the glucocorticoid hormone, dexamethasone, which accelerated morphological differentiation producing an increased number of mature cells and enhanced survival. They also showed that it was the actual cell-cell contact that was in some way responsible for the induction of differentiation, as neither dexamethasone-conditioned medium nor fibroblast-derived matrix produced responses similar to coculture with the mesenchyme. It was also apparent that the effects were mediated by mesenchymal elements without tissue specificity. These outwardly disappointing results with regards the inductive effects of the individual ECM components would appear to refute the considerable, and convincing data presented and reviewed by Bissell *et al.*, (1982) in support of the role of the ECM on directing gene expression and differentiation.

However, it must be understood that each system that is studied will have its own control mechanism for differentiation, which will therefore result in subtle differences in the ability of exogenous inducers to bring about differentiation. Not all epithelial cells respond in the same way to fibronectin as do the epidermal cells of the skin, which when no longer in contact with this ECM component, undergo terminal differentiation, into keratinocytes. The tissue specificity of the composition of the ECM probably explains the subtle differences in responsiveness to the various ECM components of different cell types (Adams and Watt, 1989). The majority of the studies involved in determining the inductive properties of ECM on differentiation have been carried out *in vitro*, and it is known that placing cells into the artificial environment of cell culture leads to the loss of differentiation functions (Bissell, 1981). Bissell, in her review (1982), documents the vast amount of data supporting the reversal of certain functional losses that can be brought about by culturing cells on naturally occurring ECM or components of ECM, rather than tissue culture plastic or glass. For example, by culturing lens epithelium on purified collagen, the cells were induced to form lens stroma (Hay and Meier, 1976); and culturing mammary epithelial cells on floating rat tail collagen (type I) gels, first developed by Michalopoulos and Pitot (1975), brought about morphological and biochemical differentiation (Emerman *et al.*, 1981). Parry *et al.*, (1982) went on to induce the synthesis and secretion of the entire pattern of milk-specific proteins by culturing primary mouse epithelial cells on floating rat tail collagen gels.

Chambard *et al.*, (1981) were able show how four conditions of collagen gel culture influenced the orientation of thyroid epithelial cell polarity. They found that thyroid cells formed a monolayer on collagen gel, with the basal membrane being attached to the substratum. If however, they embedded the cells inside the gel, the cells formed lumen, with the basal membrane in contact with the collagen and the apical membrane at the lumenal side. If they allowed the cells to form floating vesicles, with the apical membranes in contact with the medium and then embedded these inside collagen gel, the polarity of the cells was reversed to produce follicles with the apical membranes oriented inwards. They could also induce monolayers on collagen to form follicles by overlaying with collagen or small pieces of glass. It was also possible to induce cells grown on

plastic or glass, by overlaying with collagen, but sandwiching between two pieces of glass did not induce follicle formation. Kubota *et al.*, (1988), have shown that human endothelial cells could differentiate into capillary-like structures on a basement membrane-like matrix as long as laminin was present, and to a lesser extent if collagen type IV was present. Pre-incubation of these endothelial cells with antibodies to laminin or synthetic peptides which bind to the laminin cell surface receptors successfully blocked differentiation. The dependence upon components of the ECM, in particular laminin and fibronectin, has been shown in the culture of human fetal dorsal root ganglion neurons (Yong *et al.*, 1988). Madin-Darby canine kidney epithelial (MDCK) cells have been studied extensively with respect to epithelial cell polarization (Rodriguez-Boulan and Sabatini, 1978; Rodriguez-Boulan *et al.*, 1983; Vega-Salas *et al.*, 1987; Salas *et al.*, 1988), and recently were shown to exhibit morphological and functional differentiation characteristic of cells engaged in transepithelial transport when grown on complete basement membrane extracted from Engelbreth-Holm Swarm tumour cells (EHS). However, when explants of these cells on basement membrane were placed on top of hydrated type I rat tail collagen gels, cells at the edge of the explant migrated into the gel, becoming elongated, bipolar-shaped cells reminiscent of mesenchyme. These fusiform cells did not exhibit the apical-basal polarity seen in the cells of origin, and membrane-associated proteins were redistributed randomly. In spite of these profound changes in phenotype, these cells were not true mesenchymal cells, and it would appear that only part of the gene program necessary for epithelial-mesenchymal transformation could be triggered in MDCK cells (Zuk *et al.*, 1989). However, this data does support previous work which suggests that definitive epithelia can give rise to fibroblast-like cells in response to suspension in type I collagen (Greenberg and Hay, 1988). From such work it is apparent that some cells are capable of responding differently to the ECM components that they have been cultured on, with respect to their programme of differentiation, and that it is not a simple case of differentiating or not differentiating.

Emerman and Pitelka (1977) and others have already shown that not only lactogenic hormones, but also the substrata, cell shape changes and cell-cell interactions play important roles in morphological and functional differentiation of mammary epithelium

(Emerman *et al.*, 1981; Haeuptle *et al.*, 1983; Lee *et al.*, 1984, 1985). Bissell and her coworkers have further developed this work, to look at not only the effects of culture on floating type I collagen gels, but also the effects of culturing primary mammary epithelium on the complete basement membrane EHS. Electron microscopy showed that mammary cells grown on plastic were squamous and undifferentiated, but become polarized and columnar when grown on floating collagen gels (Emerman and Pitelka, 1977). When these cells were grown on EHS, the cells formed spherical structures, which have similarities to *in vivo* mammary alveoli, with the structures containing a layer of highly polarized cells with basal nuclei, apical microvilli and well-developed secretory apparatus. The cells were joined by tight junctions, which act to seal the lumen, forming a separate compartment from the extracellular medium. It has also been shown that these cells actively secrete a full range of milk proteins apically into the lumen, while transferrin and lactoferrin are secreted both basally and apically (Barcellos-Hoff *et al.*, 1989). Primary mammary epithelium cultured on EHS also secretes a unique protein, whey acidic protein (WAP), which was not secreted under any other culture conditions (Chen and Bissell, 1989).

Further studies have shown that mammary epithelium cells grown on floating collagen gels are also sitting on a basement membrane (BM), which is a specialized ECM, and is situated between the cells and the collagen (Streuli *et al.*, 1988). This system has allowed the investigation of whether the appearance of the BM is correlated with the appearance of lactogenic function, which is not seen in cells grown on plastic, which have little or no BM. Cells grown on floating collagen gels undergo shape changes, becoming polarized and deposit a BM. These cells also reacquire the high steady state levels of ß-casein and transferrin mRNA, and it can be seen that the restoration of function correlates with the shape changes and appearance of the BM (Streuli and Bissell, 1990). The authors current model is that "the BM or its components, in conjunction with the shape changes which accompany the establishment of polarity, are the positive modulators of functional differentiation. One or both of these signals feed back negatively on the synthesis of mRNAs for ECM components such as laminin and type iv collagen" (Streuli and Bissell, 1990).

How then, does the ECM regulate gene expression and differentiation ? It has been shown *in vitro* that the components of the ECM have profound effects on cell shape (for review see Watt, 1986). In this review Watt also details the evidence that exists for a direct relationship between cell shape and differentiation. Such evidence would support the idea that if a cell could maintain its *in vivo* morphology by culture on ECM, then it will also tend to maintain its differentiated characteristics (Watt, 1986). This idea that cell shape was some way involved in epithelial differentiation was considered in 1979 by Emerman *et al.*, when they proposed four possible factors that would be provided by culture on floating collagen gels, that were not provided by plastic substrata. These factors were:

1. Free access of nutrients to basolateral surfaces,
2. close proximity to medium surface and gas phases,
3. interaction of epithelial cells with ECM,
4. and substrata flexibility permitting shape change.

They showed that no one factor alone was capable of causing differentiation of mammary epithelial cells, and that differentiation was a consequence of a subtle combination of these factors. The importance of cell shape in differentiation is further supported by the discovery that primary mammary epithelial cells when cultured on attached collagen or floating collagen gels which had been made inflexible by cross-linking with glutaraldehyde, do not differentiate (Lee *et al.*, 1984).

It is logical to assume that if the ECM and cell shape are involved in differentiation, then a possible link between the two would be the cell cytoskeleton. This is the complex network of protein filaments in the cytoplasm which governs the properties such as shape, internal organization and movement (Alberts *et al.*, 1983). The two most important components of the cytoskeleton are the actin filaments (microfilaments) and the microtubules. There are also the more stable intermediate filaments and many accessory, linker proteins. A close association between the ECM and the cytoskeleton has been shown by Woods *et al.*, (1984), where by surface fibronectin and heparan sulphate proteoglycan are aligned with actin microfilament bundles in the cytoplasm of spread fibroblasts.

How do signals get from the ECM to the cytoskeleton ? Although the intracellular and extracellular proteins have been reasonably well characterized, the integral membrane molecules that act as receptors for the ECM, and that possibly act as transmembrane links to the cytoskeleton, were until recently poorly characterized. These molecules, called the integrins, represent a large family of cell surface receptors which interact with the various components of the ECM (Hynes, 1987). Integrins have similar heterodimeric structures, consisting of an α and a ß subunit noncovalently associated in a 1:1 ratio. To date there are 5 classes (two are only minor) of ß subunits and 11 integrin α subunits, which combine to give rise to receptors with different specificity for ECM components (Akiyama *et al.,* 1990). Most of the research into the integrins has concentrated on their isolation and characterization, and very little is known about how they may interact with the intracellular protein network to bring about changes in gene expression. It has been shown that the avian integrin complex binds directly to an intracellular molecule, talin, but this work has been hampered by technical problems (Akiyama *et al.*, 1990). However, there is evidence that they can exert profound effects on cells, playing a role in switching the cells from a proliferation programme to one of differentiation . For example, the binding of a specific integrin, defined by its reaction with the monoclonal antibody CSAT, to components of the ECM, has been implicated in the control of myogenic differentiation in chick embryo breast skeletal muscle cells (Menko and Boettiger, 1987). It was possible to prevent this differentiation by incubating the cells with the CSAT monoclonal antibody, which has been shown to bind to the ß subunit of the receptor complex (Horwitz *et al.*, 1985). The authors interpreted the data to mean that not only do integrins play a vital role in cell attachment to and migration upon components of the ECM, but also act as signal transducers, which in turn leads to alterations in gene expression and differentiation. Therefore, the integrins are thought to act in a similar way as described for other receptor systems that interact with for example specific hormones or growth factors (Menko and Boettiger, 1987). However, much more work is required to elucidate the exact role that the integrins play in transmembrane signal transduction, cytoskeletal organization and differentiation. Also, the regulation of these integral receptors has important implications for cancer biology, particularly in the area of

metastasis, where the cells release themselves from the tumour of origin, travel to a new tissue and interact with ECM of that new tissue via their integrin receptors.

It has been suggested that perturbations in the cells interactions with its environment may be one of the important steps in cancer progression. For example, Kellokumpu *et al* (1985), showed that the expression of laminin, a component of the basement membrane, was related to cellular differentiation in normal, hyperplastic and adenomatous colorectal mucosa. They saw increasing perturbations in the laminin deposition with the shift from normal to malignant tissue. Their results correlated well with previous results that showed that colon carcinomas *in vitro* secrete no detectable laminin (Vlodavsky and Gospodarowicz, 1981). Bodmer (1988) suggested that loss of the ability to synthesize and express receptors for extracellular matrix components could be a key step in colon cancer progression. Also in that year, Pignatelli and Bodmer (1988) proposed that tumour cells may be able to escape the control of proliferation and differentiation exerted by collagen binding, by being unable to synthesize or express collagen receptors. Later, they went on to show that a panel of colorectal carcinoma cell lines, with varying abilities to bind to collagen, expressed various degrees of epithelial differentiation which positively correlated with their binding ability. They also showed that in two of the cell lines studied, the addition of transforming growth factor-ß (TGF-ß) enhanced the differentiation which, the data would suggest, promoted the binding to collagen, probably by increasing the expression of a cell surface integrin-like collagen receptors. Other cell lines studied, which did not express characteristics of epithelial differentiation in response to the collagen, were also unresponsive to the growth inhibitory effects of TGF-ß (Pignatelli and Bodmer, 1989) . These results would seem to support the proposed idea that the loss of responsiveness of tumour cells to inhibitory growth factors like TGF-ß, may be an important determining factor in the uncontrolled growth exhibited by neoplastic cells in tumours (Roberts *et al.*, 1988). Also, it has been shown that several colon carcinoma cells lines can secrete growth factors *in vitro*, which may have autocrine as well as paracrine actions (Wigley *et al.*, 1986; Culouscou *et al.*, 1987; Coffey *et al.*, 1987; Culouscou *et al.*, 1988).

It is therefore apparent from the above review of the studies into the control of differentiation of cells *in vitro* , that the mechanisms are far from simple, and there would appear to be no one mechanism that works for all cells. *In vitro* methods provide a reliable and simple way of assessing the ability of individual components and combinations of these components to induce differentiation. It is, however far more difficult to assess their role *in vivo* and care must be taken when extrapolating results from the *in vitro* to the *in vivo* situation. There is still a considerable amount of work to be done before we will fully understand the intimately linked processes of proliferation and differentiation and how the organism controls the balance between these two processes. As more is elucidated, a better understanding as to how this balance may be upset in disease processes will become clearer, and may even point to possible cures. This is particularly pertinent for the study of malignancy. The ability to culture epithelial cells in an *in vitro* environment that is more representative of their *in vivo* origins by using ECM components and inert porous inserts resulting in a more differentiated state, has enabled the production of more useful data with regards to normal epithelial function, for example transepithelial transport and metabolism. This may also be the case for the *in vitro* screening of novel anticancer agents, which at present uses a range of cell lines, including the HT-29 cell line, cultured on tissue culture plastic to select active agents (Alley *et al.*, 1988). The limitations of these assays will be discussed in the next section (1.IV.i).

1.IV Cancer Chemotherapy

1.IV.i Screening for new anticancer agents

It is essential that the anti-neoplastic activity of novel agents is determined prior to introduction into a clinical trial, which is conducted in human patients, involving considerable planning and financial expenditure. It is therefore desirable to have a screening system that selects for high anti-tumour activity, and preferably, low host toxicity i.e. drugs with high therapeutic indices. Animal based studies can be used to determine any obvious side effects, but the initial anti-neoplastic activity is determined *in vitro*, using tumour cell lines (Alley *et al.*, 1988). Up until 1955 screening systems

included experimental tumour models, microbiological systems and biochemical synthesis systems. The need for a spectrum of tumours lead to the development by the National Cancer Institute (NCI) USA, of a three-tumour system consisting of the leukaemia L1210, sarcoma 180 and carcinoma 755 (Leiter and Schneiderman, 1959). Although, leukaemia L1210 was fairly accurate at detecting active compounds (16 out of 20 clinically active drugs; Goldin *et al.*, 1966), there was concern that its use as a screen may have resulted in a preferential selection of drugs that were active only against rapidly growing tumours (Venditti *et al.*, 1984). Consequently, the greatest improvements in survival rates have been seen in cancers which share the characteristics of rapid growth (Zubrod, 1972). There has been very little improvement in the survival rates for the solid, slow growing tumours, such as those of the colorectum (Griswold and Corbett, 1978). In light of these findings, new tumour models have been developed that more closely resemble human solid tumours in histology and growth kinetics, and these models were tested to determine whether they would identify agents that were missed by the L1210 screen (Venditti *et al*, 1984).

Animal model screening systems can be used more effectively than *in vitro* screens to determine the effects of altered drug structure on anti-neoplastic activity; to develop improved treatment regimes and routes of administration; to determine chemotherapeutic efficacy with regards tumour site and tumour burden; and to test combinations of drugs and therapies. These models may however, exhibit biological differences that make it difficult to extrapolate results directly to the human situation. The NCI has been studying the possibility of using human tumour xenografts in nude mice, as screening systems for drug evaluation (Goldin , 1983). Unfortunately, the use of animals becomes financially prohibitive when there are large numbers of new chemicals and combinations of chemicals to be tested for anti-neoplastic activity, as is the case in large institutions like the NCI. The NCI now uses a panel of human tumour cell lines and the microculture tetrazolium assay, which is rapid, cheap and reproducible (Alley *et al.*, 1988). The use of cell lines in tissue culture assays for anti-cancer activity also has its problems, the most important one being the environmental constraints which are exerted on the cells. It is generally accepted that when solid tumours, particularly those of epithelial origin are

derived from the animal or patient onto tissue culture plastic, there follows a reversal of differentiated characteristics, producing a more anaplastic cell line (see section 1.III). Consequently, this may lead to alterations in gene expression that will effect their chemosensitivity to the drugs under evaluation. It therefore follows that these cells will no longer be representative of the human or animal tumour from which they were derived originally. This can in itself lead to the inappropriate selection of chemotherapeutic agents, which when they reach clinical trials, display disappointing results. As already discussed, the use of animal systems either as transplantable tumour models, such as the mouse adenocarcinoma of the colon (MAC) system (Double *et al.*, 1975), or as human xenografts in nude mice would appear to offer the only alternative to the non-selective *in vitro* models. It would seem appropriate at this point when a large number of drugs need to be screened in order that more useful anti-cancer agents may be discovered, that a more realistic *in vitro* model be developed which is more representative of the tumour *in vivo*. The use of collagen gel culture has been shown to modulate cellular differentiation and restore *in vivo* characteristics that had been lost by derivation onto tissue culture plastic in a number of cell systems (Bissell *et al.*, 1982). Sinha and White (1988) evaluated such a system by comparing the *in vitro* and *in vivo* chemosensitivity of rat mammary tumour cells to a panel of cytotoxic agents. Their study, although only preliminary, suggested that this system could prove useful for chemosensitivity screening as they showed good correlation between the *in vitro* and *in vivo* data. However, they did not determine the chemosensitivity of the cells cultured on plastic substrata, in order that they could compare that with their collagen data. Therefore, it could not be deduced whether the cells had an altered sensitivity when grown on collagen or *in vivo* compared with that on plastic. Despite these experimental inadequacies the system is one that deserves future evaluation with a range of human epithelial tumours, preferably those derived directly onto the collagen gel in order that the detrimental effects of growth on plastic can be avoided. The constraints of time have only allowed a much smaller study to be undertaken during the course of these investigations. The study aimed to test the efficacy of a panel of commonly used anti-neoplastic agents displaying different modes of action, in a collagen gel system, using the human colon adenocarcinoma cell line, HT-29 .

1.IV.ii Differentiation therapy

In 1961 Pierce first proposed the idea that it may be possible to convert malignant cells back into benign cells. His ideas were based on work carried out on murine teratocarcinomas, in which it had been shown that there was spontaneous differentiation of malignant cells into benign, or possibly normal cells (Pierce and Dixon, 1959; Pierce *et al.*, 1960). His work (Pierce and Verney, 1961) and that of many other laboratories since the 1960's have shown that differentiation can be induced *in vitro* as well, with the use of various chemical agents, such as the alkyformamides (for reviews see Spremulli and Dexter, 1984; Langdon and Hickman, 1987), the short-chain fatty acid, sodium butyrate (Leder and Leder, 1975; Chou, 1979; and Kim *et al.*, 1980), and Vitamin A and its analogues, the retinoids (for a review see Lotan, 1980). The studies involved in exploring the differentiation-inducing effects of putative chemotherapeutic agents, use a wide range of cell lines, varying in their tumorigenicity, tumour origin, differentiation status and *in vitro* growth characteristics. The reason for discussing these ideas in this introduction are twofold. Firstly, HT-29 cells have been shown to differentiate in response to sodium butyrate (Augeron and Laboisse, 1984; Wice *et al.*, 1985), and this in turn lead to the second reason, and that is that this agent along with DMF was investigated initially as a possible inducers of differentiation of the strain of HT-29 used in our laboratory (see Results section).

The earlier studies concentrated on the differentiation of several leukaemic cell lines, for example, virus-induced murine Friend erythroleukaemia cells (Scher *et al.*, 1973; Tanaka *et al.*, 1975), and the human promyelocytic leukaemia cell line HL-60 (Collins *et al.*, 1978; Breitman *et al.*, 1980). This focus on leukaemic cell lines, not surprisingly, mimicked the intensive use of haemopoietic cell lines in the more traditional determination of chemotherapeutic efficacy of novel anti-cancer agents, being used at that time by the large cancer institutes, for example the NCI. The limits of such a narrow spectrum of cell lines lead to the broadening of such studies to encompass cell lines of solid tumours, such as the colorectal-derived cell lines. This expansion also occurred in the chemotherapeutic screens, with the aim to improve the clinical success of the drugs selected, which was unfortunately poor in comparison to the initial responses seen in the

in vitro screening models. This aspect of cancer chemotherapy has already been discussed more fully in section 1.IV.i.

Since the use of epithelial-derived cell lines was developed many agents have been demonstrated to induce morphological and functional differentiation *in vitro*. Borenfreund *et al.*, (1975) used several murine cell lines to show the reversible modulation of tumour-associated characteristics to those of more normal cells as a result of exposure to the simple solvent dimethyl sulfoxide (DMSO), and the polar solvent dimethylformamide (DMF). DMSO was also shown to induce the maturation of murine neuroblastoma cells (Kimhi *et al.*, 1976), and DMF induced differentiation in murine rhabdomyosarcoma cells (Dexter, 1977). The use of human epithelial-derived cell lines, a practice which produces more relevant results, has been equally successful in demonstrating chemical-induced differentiation. Melanin production was induced by exposure of a melanoma cell line to DMSO, which also caused irreversible, terminal differentiation in this cell line (Huberman *et al.*, 1979). A number of laboratories have used colorectal-derived cell lines, such as Kim *et al.*, (1980) who demonstrated the effects of sodium butyrate and DMSO on colon cancer cells and Tsao and coworkers, whose studies used the HRT-18 rectal cancer cell line (Tsao *et al.*, 1982). Dexter and coworkers developed several human colon carcinoma cell lines, and studied the effects of several differentiation-inducing agents, such as DMF (Dexter *et al.*, 1979; Hager *et al.*, 1980), and sodium butyrate (Dexter *et al.*, 1981), and later studied the effects of polar solvents on the growth of human colon xenografts (Dexter *et al.*, 1982). DMF has also been shown to have anti-tumour and differentiation-inducing activity in xenografts of head-and-neck cancer and the authors suggested that differentiation induction might play a role in the anti-neoplastic actions of the drug (Van Dongen *et al.*, 1989).

The four-carbon fatty acid, sodium butyrate (NaB) is produced in substantial amounts by the intestinal flora (Prizont *et al.*, 1975). Like the polar solvents, NaB was initially shown to be effective in inducing differentiation in murine leukaemia cells (Leder and Leder, 1975), and then in animal solid tumour cell systems, such as Syrian hamster fibroblasts (Leavitt *et al.*, 1978), and murine embryonal carcinoma cells (McCue *et al.*, 1984). A permanently differentiated clone of the human colon adenocarcinoma cell line HT-29, was

derived from sodium butyrate-treated parental HT-29 cells (Augeron and Laboisse, 1984). Later, Wice *et al.*, (1985) were able to demonstrate reversible enterocytic differentiation in HT-29 cells , cultured in the presence of NaB.

There remains a great deal of speculation with regards the modes of action of the differentiating agents, and it has been suggested that the depletion of cellular glutathione levels in a number of colonic cell lines may be responsible for their maturation in response to the formamides (Cordeiro and Savarese, 1984, 1986). However, there are results that contradict these theories, for example HL-60 cells differentiate in response to N-methylformamide (NMF), but there were no changes in their glutathione levels (Bill *et al.*,1986). The alkylformamides also effect membrane dynamics (Dibner *et al.*, 1985), and the expression of the "stress" proteins (Richards *et al.*, 1988), but exactly how these factors are involved in malignant cell differentiation is unknown, and whether they have a role to play in normal cell differentiation remains to be determined. The mode of action of sodium butyrate-induced differentiation remains equally as obscure, although NaB has been shown to induce some interesting changes at the chromosomal level. It has also been shown to cause histone hyperacetylation in murine embryonal carcinoma cells (McCue *et al.*,1984), a phenomenon that has been seen in other cells (Riggs *et al.*, 1977; Boffa *et al.*, 1978; Candido *et al.*, 1978; Vidali *et al*, 1978; and D'Anna *et al.*, 1980), and which may lead to the initiation of new gene expression, the products of which could lead to differentiation. NaB also induced DNA hypermethylation in normal human embryonic lung fibroblasts and their malignant counterparts (de Haan *et al.*, 1986), a situation which is generally associated with gene inactivation (Parker *et al.*, 1982). However, an earlier study had shown that NaB caused DNA hypomethylation during the differentiation of Friend erythroleukaemia cells (Christman *et al.*, 1980). It would appear that NaB may be able to induce and/or repress gene expression in different cell types, with the overall result being dependent of those genes effected and the cell line studied. There is still a considerable amount of work to be done in order that the precise mode of action of the differentiating agents may be elucidated, and with that knowledge, possible tailoring of their use to fit the needs of particular human malignancies.

Although, there would appear to be a large volume of evidence supporting Pierce's original proposition, the development of clinically-oriented "differentiation therapy" has been slow. Pierce himself suggested in a recent review, that this has been due in part to the fact that the mechanisms of differentiation and induction are poorly understood in normal tissue, and that his work had been based on a very rare tumour, which is not considered by many oncologists to be representative of the majority of human tumours. It may also be associated with the increase in success that clinicians have had with more conventional cytotoxic chemotherapy (Pierce and Speers, 1988). However, cytotoxic therapy has several disadvantages, including its non-specific toxicity, which not only kills the malignant cells, but also severely damages normal tissues such as the gastrointestinal tract and the haemopoeitic system, producing serious side effects, which in themselves can be life-threatening. Another major drawback with present chemotherapy is its relative low success rate, with the most common tumours being unresponsive. A large number of the putative differentiating agents are actually toxic, an undesirable attribute which will severely restrict their use in the clinic.

One group of naturally occurring growth modulators, the growth factors, which are themselves non-toxic, have been the centre of considerable attention with regards their possible roles in the induction of normal and tumour cell differentiation, of which several are presently in clinical trial. For a very good review on the present status of the differentiating agents that have reached the clinical trial stage, the reader is referred to the report of the conference "Differentiation Therapy" which was held in September, 1988 (Lotan *et al.*, 1990). The report also details how differentiating agents may be used as adjuncts to conventional chemotherapy and radiotherapy, as have several recent publications (Milas *et al.*, 1988; Zupi *et al.*, 1988; and Tofilon *et al.*, 1989). The authors of the report (Lotan *et al.*, 1990) concluded, "the favourable responses reported in the trials conducted thus far suggest that differentiation therapy is on its way", but further progress is very much dependent on an increase in the understanding of the molecular mechanisms of differentiation.

1.V Aims

The initial aims of these investigations were to evaluate the differentiation inductive properties of metabolic manipulation (glucose deprivation) and the differentiating agent sodium butyrate on the human adenocarcinoma colon cell line, HT-29. These experiments were performed with the eventual aim of combining the two inducers of differentiation and to then determine if they had a complementary effect on each others inductive properties i.e. could two inducers of intestinal differentiation administered simultaneously bring about a more rapid transition from undifferentiated to differentiated phenotype than each inducer alone.

Later experiments with the second batch of HT-29 cells ($HT\text{-}29_2$) which form the bulk of the work presented in this thesis, aimed to determine the differentiation inductive properties of the extracellular matrix component, collagen type I. Several parameters were to be used to assess the degree of intestinal differentiation achieved by $HT\text{-}29_2$ cells cultured on plastic substrata, attached (non-released) and floating (released) collagen gels. The use of floating (released) collagen gels and inert porous inserts aimed to determine the role of being cultured in an environment which allowed the cells to feed from the basolateral surface in contrast to being forced to derive nutrients from the apical surface, may play in regulating the differentiation of $HT\text{-}29_2$ cells.

The long term culture of $HT\text{-}29_2$ cells on both non-released and released collagen gels aimed to determine the effects that a longer exposure to the collagen would have on their differentiation. Ultimately, the experiments aimed to determine if collagen gel culture could alter the chemosensitivity of $HT\text{-}29_2$ cells to a panel of chemotherapeutic agents with different mechanisms of action, with the hope that this may lead to the production of a more representative *in vitro* screen for novel anticancer agents.

SECTION 2: MATERIALS

SECTION 2: MATERIALS

2.I Chemicals

2.I.i Purchased from Sigma Chemical Company Limited, Poole, Dorset, England.

2-amino-2-methyl-1-propanol hydrochloride (A-5888), n-butyric acid sodium salt (B-5887), citric acid - free acid (C-0759), cobalt chloride (C-2644), collagen type VII rat tail, acid soluble (C-8897), collagenase type 1A (from *Clostridium histolyticum*, C-9891), diethanolamine (D-8885), dimethylsulfoxide (D-5879), dulbecco's modified eagle's medium base w/o glutamine, sodium pyruvate, phenol red and sodium bicarbonate (D-5030), fast-red TR salt (F-1500), 5-fluorouracil (F-6627), hydrogen peroxide 30% solution (H-1009), inosine (I-4125), l-leucine p-nitroanilide hydrochloride (L-2504), leucine-aminopeptidase (L-0632), levamisole (L-9756), D-mannitol (M-4125), magnesium sulphate (M-7506), mem non-essential amino acid supplement powdered mixture (M-2025), 4-methylumbelliferone - free acid (M-1381), 4-methylumbelliferyl phosphate (M-8883), naphthol AS-MX phosphate - free acid (N-4875), phenol red (phenolsulfonphthalein,P-3532), sodium acetate - trihydrate (S-8625), sodium bicarbonate (S-8875), sodium carbonate (S-2127), sodium hydroxide (S-5881), sodium phosphate - dibasic (S-0876), polyoxylethylene-sorbitan monolaurate (Tween 20, P-1379), triton X-100 (T-6878), tris-base (tris (hydroxymethyl)aminomethane) , T-1503), trypan blue (T-6146).

2.I.ii Purchased from BDH Chemicals Limited, Poole, Dorset, England.

Acetone (10003), D.P.X. mountant (36029), ethanol (10107), 25% electron microscopy grade gluteraldehyde (36080), magnesium chloride hexahydrate (29096), methylene blue (34048), sodium cacodylate (30118), potassium dihydrogen orthophosphate (10203).

2.I.iii Purchased from Johnson Matthey, Materials Technology U.K., Royston, Herts., England.

Ruthenium red (code: 193000).

2.I.iv Purchased from Agar Scientific Ltd., Stansted, Essex, England.

Agar 100 resin kit (R1030), osmium tetroxide (R1015).

2.I.v Purchased from Fisons, FSA Laboratory Supplies, Loughborough, England.

Acetic acid (glacial, A/0360), ammonium acetate (A/3400), ammonium sulphide solution (A/6560), barbitone sodium (B/0100), calcium chloride (C/1500), dimethylformamide (D/3841), formaldehyde 37/40% w/v solution (F/1450), hydrochloric acid (H/1150), lead nitrate (L/1500), sodium chloride (S/3120), sodium dihydrogen orthophosphate (S/3720).

2.I.vi Purchased from Dako Ltd., High Wycombe, Bucks., England.

Mouse APAAP (soluble complexes of alkaline phosphatase and monoclonal mouse anti-alkaline phosphatase, D651), glycergel aqueous mountant (C563), pen for immunocytochemistry (S2002), peroxidase-conjugated rabbit immunoglobulins to human CEA (anti-CEA - HRP, P167), 1,2 - phenylenediamine dihydrochloride (OPD, S2000), rabbit anti-mouse immunoglobulins (Z259), rabbit immunoglobulins to human CEA (anti-CEA, A115).

2.I.vii Purchased from Gibco Ltd., PO Box 35, Paisley, Scotland.

Dialysed foetal calf serum (063-06300 M), dulbecco's modified eagle medium w/o sodium pyruvate with 4500 mg/L glucose (041-01965 M), PBS (10x) w/o calcium and magnesium (042-04200 H), penicillin - streptomycin solution (10,000 IU/ml and 10,000 µg/ml respectively, 043-05140 H), trypsin-EDTA solution (10x) (043-05400 H), tryptose phosphate broth (043-08050 M).

2.I.viii Purchased from Imperial Laboratories (Europe) Ltd., Andover, Hants., England.

Foetal calf serum (6000-07), RPMI medium 1640 (10X) (2-540-14).

2.I.ix Purchased from Advanced Protein Products Ltd., Brierley Hill, West Midlands, England.

Foetal calf serum (AS-302-10).

2.I.x Purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England.

Sodium beta-glycerophosphate (75812).

2.I.xi Purchased from Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts., England.

Protein standard (bovine plasma gamma globulin, 500-0005), dye reagent concentrate (500-0006).

2.I.xii Purchased from ICN Flow, Gomm Road, High Wycombe, Bucks., England.

Chicken serum (29-501-49)

2.I.xiii Tissue culture materials

6-,12-, and 96- well tissue culture plates, 135 mm petri dishes, 25 cm^2 and 80 cm^2 tissue culture flasks (Nunc Intermed, Denmark); acrodisc disposable filter assembly 0.2 μm and acrocap filter unit with filling bell 0.2 μm (Gelman Sciences, Ann Arbor, Michigan, USA); millicell TM-HA 12 mm culture plate insert (Millipore (U.K.) Ltd., Watford, Herts., England); Lab-Tek tissue culture chamber slides (Miles Scientific, Division of Miles Laboratories Inc., Naperville, Illinois, USA); petriperm hydrophil flexible 5 cm petri dishes (Heraeus Equipment Ltd., Brentford, Essex, England); plastipak sterile polypropylene syringes 10 ml and 50 ml (Becton Dickinson and Company Ltd., Dun Laoghaire, Co. Dublin, Eire); 30 ml sterile universals, 7 ml bijou bottles, sterile pipettes, polypropylene centrifuge tubes, and polyethylene centrifuge tubes (Sterilin Ltd., Feltham, England); Heraeus CO_2-Auto-Zero gassing incubator (Heraeus Equipment Ltd., Brentford, Essex, England) ; Leec incubator (Leec Ltd., Nottingham, England); peristaltic pump (Watson-Marlow Ltd., Falmouth, Cornwall, England), class II Gelaire

BSB 3 microbiology safety cabinet with unidirectional laminar downflow (ICN Flow, Gomm Road, High Wycombe, Bucks., England).

2.I.xiv Gifts

Mouse anti-human aminopeptidase monoclonal antibody (HBB3/153/63) and mouse anti-human sucrase-isomaltase monoclonal antibody (HBB2/219/20) were kindly donated by Dr. H-P Hauri, Department of Pharmacology, Biocenter of the University of Basel, Switzerland.

2.II Buffers and solutions

2.II.i DMEM w/o glucose

powdered DMEM base (correct amount for 1L)	1 pot
MEM non-essential amino acids (correct amount for 1L)	1 pot
sodium bicarbonate	3.7 g
inosine	0.67 g
phenol red	45 mg
dH_2O	900 ml

The pH was adjusted to 7.2 using 1M hydrochloric acid, and the volume adjusted to 1000 ml using dH_2O.

2.II.ii Phosphate-buffered saline (PBS)

potassium dihydrogen orthophosphate	1.22 g
sodium chloride	8.77 g
dH_2O	800ml

The pH was adjusted to 7.4 using 4 M sodium hydroxide, and the volume adjusted to 1000 ml.

2.II.iii EM visualisation of alkaline phosphatase activity

0.2 M calcium chloride	20 ml
0.1 M barbitone sodium	20 ml
0.1 M sodium ß-glycerophosphate	5 ml
0.05 M magnesium chloride	5 ml

The solution was filtered through a 0.2 μm acrodisc filter unit (Gelman Sciences Inc., USA) prior to use.

2.II.iv 10% Formol saline

formaldehyde 37/40 % (w/v) solution	100 ml
0.9 % sodium chloride solution	900 ml

2.II.v Tris buffered saline (TBS)

stock solution:

0.5 M Tris/HCl pH 7.6

working solution:

stock solution diluted 1/10 in 0.9 % sodium chloride solution (isotonic saline).

2.II.vi Substrate 1 from APAAP immunocytochemistry procedure

naphthol AS-MX phosphate, free acid	2 mg
dimethylformamide	200 μl
0.1 M Tris buffer pH 8.2	9.8 ml
1 M levamisole	10 μl
fast red TR salt	10 mg

The fast red salt was dissolved in the substrate solution immediately prior to use, and the solution filtered through a 0.2 μm acrodisc filter unit (Gelman Sciences Inc., USA), directly onto the slides.

2.II.vii EM fixative 1

25% glutaraldehyde solution (EM grade)	20 ml
sodium cacodylate	2.68 g
dH_2O	up to 250 ml

2.II.viii EM fixative 2

25 % glutaraldehyde solution (EM grade)	20 ml
$Na\ H_2\ PO_4$	3.63 g
sodium hydroxide	0.8 g
dH_2O	up to 250 ml

2.II.ix Fluorescent microassay for alkaline phosphatase

1 M AMP buffer:

2-amino-2-methyl-1-propanol	62.8 g
dH_2O	500 ml

The original pH of this solution was in the range of 4 - 5, and was adjusted to pH 12 using concentrated sodium hydroxide solution.

0.5 M diethanolamine buffer:

diethanolamine	5.25 g
dH_2O	90 ml

The pH was adjusted to 10.4 using 1 M HCl, and the volume adjusted to 100 ml.

incubation medium:

methylumbelliferyl phosphate	56 mg
magnesium chloride	8 mg
0.5 M diethanolamine buffer pH 10.4	40 ml

2.II.x Brush border membrane extraction and aminopeptidase assay

50 mM mannitol- 2 mM Tris, pH 7.1:

Tris	0.0242 g
dH_2O	90 ml

The pH was adjusted to 7.1, the volume increased to 100 ml and 0.911 g of mannitol added.

50 mM phosphate buffers:

1) 50 mM disodium hydrogen phosphate	1.78 g
dH_2O	200 ml
2) 50 mM sodium dihydrogen phosphate	1.56g
dH_2O	200 ml

Solutions 1 and 2 were mixed together to produce solutions with pH values of 7.2 and 6.6.

50 mM phosphate buffer/ 0.1 % TX-100:

TX-100	100 µl
50 mM phosphate buffer pH 7.2	up to 100 ml

substrate solution:

l-leucine p-nitroanilide	71.9 mg
50 mM phosphate buffer pH 6.6	10 ml

2.II.xi ELISA for detection of human CEA

0.2 M acetate buffer, pH 5:

sodium acetate	13.6 g
dH_2O	400 ml

The solution was adjusted to pH 5 using glacial acetic acid, and the volume increased to 500 ml with dH_2O.

wash buffer, pH 7.2:

Tween 20	100 µl
PBS pH 7.2	up to 1000 ml

carbonate-bicarbonate buffer, pH 9.6:

sodium carbonate	1.59 g
sodium bicarbonate	2.92 g
dH_2O	1000 ml

The pH was adjusted to 9.6 by the addition of sodium bicarbonate.

citric acid-phosphate buffer, pH 5:

citric acid	7.3 g
sodium phosphate	23.88 g
dH_2O	1000 ml

If necessary the pH was adjusted using citric acid.

2.II.xii Electron microscopy using Ruthenium red (RR)

RR stock solution:

ruthenium red	50 mg
dH_2O	5 ml

The RR was crushed in a mortar with a few drops of dH_2O, and using the remaining water the mortar was washed repeatedly, and the washings transferred to a centrifuge tube. The solution, with an approximate concentration of 10 mg/ml was heated to 60 °C for 5 min, then centrifuged at 1600 g for 10 min using a minifuge T (Heraeus Equipment Ltd., Brentford, England). The supernatant was diluted 1:100 in 0.1 M NH4OAc, and the absorbance read at 533 nm using a spectrophotometer (Beckman DU-70, Fullerton, CA, USA). The stock concentration in ppm was determined from the fact that 20 ppm at 533 nm gives a density of 1.583, and adjusted to 1500 ppm with dH_2O.

EM fixative 3:

3.6 % glutaraldehyde	0.5 ml
0.2 M sodium cacodylate	0.5 ml
1500 ppm RR stock	0.5 ml

EM fixative 4:

5 % OsO4	0.5 ml
0.2 M sodium cacodylate	0.5 ml
1500 ppm RR stock	0.5 ml

SECTION 3 : METHODS

SECTION 3 : METHODS

3.I IN VITRO

3.I.i Cell culture

The HT-29 colonic adenocarcinoma cell line (Fogh and Trempe,1975), was initially obtained from the MRC Radiobiology Unit, Chilton, Didcot, Oxon. Cells from this culture ($HT\text{-}29_1$) were used for the first 18 months of studies until they were found to be infected with an obligate intracellular bacteria. The infection was detected by the use of a Hoechst Stain Kit for the detection of mycoplasma (ICN Flow, High Wycombe, Bucks., England) and the careful examination of electron micrographs. All samples of these cells that had been stored at -196 °C were discarded immediately and all experimentation on these cells discontinued. Those studies included in this thesis that were done on the infected cells have been described as such, and have only been included because of the inability to repeat those studies due to time limitations.

The majority of studies set out in this thesis were carried out on a new batch of HT-29 cells ($HT\text{-}29_2$) obtained from the Experimental Drug Delivery Research Group, Ciba Geigy, Horsham, West Sussex. The cells obtained were of unknown passage number on arrival and were referred to as x + n, where 'n' was equal to the number of passages since arrival. Cells were used between x + 1 and x + 10 to ensure minimal phenotypic drift that may occur during prolonged cell culture.

Cells were routinely cultured at 37 °C in an atmosphere of 90% air/10% CO_2 in a LEEC incubator (LEEC Ltd., Nottingham, England), in Dulbecco's Modified Eagles Medium supplemented with 10% foetal calf serum (FCS), 1% glutamine , penicillin and streptomycin (89 IU/ml and 89 µg/ml respectively). Cells were grown as a multilayer attached to tissue culture plastic and were passaged weekly by enzymic digestion using a solution of trypsin/EDTA in PBS (without calcium and magnesium, Gibco, Paisley, Scotland). Cells were usually seeded at 2 x 10^5 cells per T25 tissue culture flask (Nunc,Denmark) with 10 ml of media. Regular growth curve determinations were carried out in order to check cell doubling times and initially to ensure that the antibiotics added to the media did not inhibit cell growth (see section 3.I.iii).

For studies involving the growth of HT-29 cells in glucose-free medium, powdered medium was purchased from Sigma (Poole, Dorset, England) which did not contain glucose, glutamine, sodium pyruvate, phenol red or sodium bicarbonate. This medium base was reconstituted according to the manufacturer's instructions, containing 2.5 mM inosine as the glucose substitute, and filter sterilized using an acrocap filter sterilizing unit (Gelman Sciences, USA) with a peristaltic pump (Watson-Marlow Ltd., Falmouth, England). To ensure that there was no microbiological contamination, duplicate aliquots of each filtered bottle of media were incubated with tryptose phosphate broth (Gibco Ltd., Scotland) at 37 °C for a minimum of one week. This media was supplemented with dialysed 10% FCS (Gibco, Paisley, Scotland) and 1% glutamine.

K562 human chronic myelocytic leukaemia cells were generously provided by Dr. R. Hoffman, Charing Cross Hospital, London. The cells were maintained between 1 x 10^5 and 7 x 10^5 cells/ml in RPMI-1640 medium supplemented with 10% FCS and 1% glutamine.

3.I.ii Cell counts

HT-29 cells were routinely counted using a model ZM coulter counter (Coulter Electronics Limited, Luton, Bedfordshire, England). A 200µl aliquot of cell suspension obtained by trypsinization was diluted to 10 ml using Isoton (Coulter Electronics Limited, Luton, Bedfordshire, England). The cell number was counted using predetermined settings for the HT-29 cell line : current = 180, attenuation = 16, lower threshold (TL) = 18.5, upper threshold (TU) = 99.9. Cell counts were also regularly determined using a haemocytometer (Improved Neubauer BS 748, Weber,England) and a Nikon Optiphot light microscope (Nikon,Japan). Using a haemocytometer enabled the degree of cell clumping to be ascertained and also to determine the health status of the cells. This was particularly relevant after a toxicity experiment.

3.I.iii Growth curve determinations

8 - 10 T25 flasks were seeded with 2 x 10^5 cells on day 0. This cell number was determined prior to experimentation to be the optimum cell number for an adequate growth curve which showed a small lag phase followed by a period of exponential growth culminating in the cells reaching confluency by days 7-8. The total cell number was determined each subsequent day until confluency was reached. Cell numbers were determined as described in section 3.I.ii. The doubling time in hours was calculated by plotting the time (hours) versus the log of the total cell number (y axis), and can be described as the time taken for the cells to double their number during their exponential phase of growth.

3.I.iv Trypan blue exclusion

A small aliquot of cell suspension (100 - 200 µl) was mixed with an equal volume of 0.5% trypan blue in 0.9% sodium chloride solution (isotonic saline). The cell suspension was then applied to a haemocytometer and examined under a light microscope. The number of cells that excluded trypan blue were counted and the values expressed as a percentage of the total cell number.

3.I.v Cell centrifugation

When it was necessary to centrifuge cell suspensions, cells were transferred to sterile plastic universal tubes and spun in a Heraeus Christ labofuge 6000 centrifuge (Heraeus Equipment Ltd., Brentford, Essex,England) at a speed of 1,500 rpm for 5 min.

3.I.vi Cell cryostorage

When necessary, cells were stored at - 196 °C in a Linde BF-5 cell bank under liquid nitrogen. Cells were stored in 1ml aliquots containing 10^6 cells in a solution of 10% DMSO/90% complete DMEM. Cells were cooled to +4 °C for 1 hour, mixed with a vortex (Spinmix, Gallenkamp, Fisons Plc., England) and then gradually cooled by 1 °C/minute to - 196 °C, by placing the vials (Cryotubes, Nunc Intermed, Denmark) at the top of the cell bank in a specially designed rack. Cells were stored indefinitely in this manner.

Upon resurrection, frozen cell aliquots were rapidly warmed to 37 °C by placing vials into a water bath. Cells were then diluted with fresh media and spun as described in section 3.I.v. The supernatant media, containing DMSO, was discarded and the cell pellet resuspended in 1 ml of media per vial and the cell suspension seeded into a sterile T25 tissue culture flask (Nunc, Denmark). Estimations of growth rate were carried out on all resurrected cells to check that the doubling time was normal for the cell line.

3.I.vii Mycoplasma screening

After the initial batch of HT-29 ($HT\text{-}29_1$) cells were found to be infected with an obligate intracellular bacteria, a regular screening programme was instigated using a Hoechst stain kit for the detection of mycoplasma (ICN Flow, High Wycombe, Bucks., England) . When the kit became unavailable, Hoechst stain was obtained from Sigma (Poole, Dorset, England) and an identical procedure as outlined with the kit was adopted. A Nikon Optiphot light microscope with fluorescent attachment was used (Nikon,Japan). Samples of the second batch of cells were also sent to Flow Laboratories (Ayrshire, Scotland) for professional screening to confirm the results of our own screening procedures.

3.I.viii Collagen gel preparation

A method of collagen gel preparation was adapted from Elsdale and Bard (1972). 100 mg of acid-soluble Type I rat tail collagen was dissolved in 40 ml of sterile 0.2% acetic acid in PBS in a sterile glass bottle. The collagen, which had the consistency of cotton wool was handled at all times with sterile forceps (20 minutes in 70% IMS). The collagen solution was stirred continuously with a sterile magnetic flee at +4 °C until a homogeneous viscous solution was obtained.

In order to set the collagen into gels, the stock collagen solution was mixed with 10x concentrated RPMI 1640 medium and sterile* 0.33 N sodium hydroxide (* filter sterilized through a 0.2 µm disposable Acrodisc, Gelman Sciences Inc., USA), at a ratio of 8:2:1 respectively. The appropriate volume was then dispensed into the wells of either a 6-welled or 12-welled tissue culture plate (1000 µl and 300 µl respectively). The gels were then incubated at 37 °C for at least 1 hour to set and then stored inside a sealed

plastic box at +4 °C, each gel under a small volume of sterile PBS to prevent them drying out. Gels were found to be more robust if stored for a short period prior to use and were often used several weeks after being set. All gels were checked using a light microscope for microbial contamination prior to use.

3.I.ix Collagen gel culture

Gels were removed from the cold room prior to use and allowed to warm to room temperature. Gels were washed with sterile PBS or media before seeding cells directly onto them. All manipulations were done manually as gels were easily sucked up the pressurized aspiration line. For the smaller gels of a 12-welled plate, 1 ml of media and 1ml of cells containing 5 x 10^4 cells* were added to each gel. For the larger gels of a 6-welled plate, 2 ml of media and 1 ml of cells containing 10^5 cells were added to each gel (* this number was determined to be the optimum for growth in these wells. It was necessary to determine this before experimentation, because the cells were not routinely grown in these tissue culture plates). Cells were allowed to attach to the collagen for at least 24 hours before gently ringing those gels that were to float freely in the medium, using a sterile microspatula. These gels were designated 'released' gels. Those that remained attached to the tissue culture plastic were designated 'non-released' gels.

In order to remove cells from the collagen gels for passaging and assaying purposes, the collagen was digested with a 0.25% solution of collagenase in PBS (containing calcium and magnesium - needed as activaters for the enzyme). The solution of collagenase was filter-sterilized if necessary i.e. when the cells were going to be used for further cell culture. Growth characteristics were determined for cells grown on collagen gels as for cells cultured on plastic (section 3.I.iii).

It was necessary to post-treat cells taken off collagen gels with trypsin/EDTA solution, as there was significant cell-cell adherence, which made accurate number determinations and plating out for clonogenic assays very difficult. This was also necessary when passaging those HT-29 cells that were permanently passaged on collagen gels (section 3.I.x).

3.I.x Permanent passaging on collagen gels

It was decided that the effect of a more prolonged exposure to the collagen should be investigated and compared to the effects seen when cells were seeded onto the collagen gels just for the duration of any particular experiment. In order to do this, cells were passaged repeatedly on either non-released or released collagen gels, with these two groups of cell being kept separate throughout the passaging. Cells were passaged weekly and used for assays between passages P+10 and P+20, including processing for electron microscopy. Samples of these cells were also stored at - 196 °C for future resurrection when necessary.

3.II Procedures for microscopy

3.II.i Light microscopy

Samples of cells grown on various substrata were processed for routine histological purposes and immunocytochemistry.

In order to cross-sectionally visualise cells grown on plastic substrata (grown on flexible petriperm dishes (Heraeus, LKB Ltd., England) to enable sectioning), collagen gels (both non-released and released, non-passaged and passaged) and permeable membrane inserts (Millicell-HA, Millipore (UK) Ltd., Watford, England), samples were fixed in 10% formol saline for a minimum of 7 days. Samples were then processed over night using an automatic tissue processor SE40 (Shandon Scientific Co. Ltd., England) and embedded with the cell layer both parallel and perpendicular to the plane of subsequent sectioning, in wax using a histocentre (Shandon Southern Products Ltd.,Cheshire, England). 4μm sections were then cut using a rotary microtome (Biocut 2030, Reichert-Jung, England) and sections 'floated out' on a paraffin section mounting bath (Electrothermal,England). Sections were mounted onto glass microscope slides (Blue Star, Chance Propper Ltd., Warley, England) and air-dried over night. Sections were stored in a dry, dust-free environment until required for haematoxylin and eosin staining (H&E, Bancroft and Stevens, 1982) or immunocytochemistry. Xenograft samples were also fixed in 10% formol saline for 7 days and processed as described above.

3.II.ii Immunocytochemistry

4 μm sections of wax embedded formalin-fixed tissues were used for the immunocytochemical detection of the brush-border associated enzymes aminopeptidase and sucrase-isomaltase, using primary mabs (HBB3/153/63 and HBB2/219/20 respectively) kindly provided by Dr. H-P Hauri, Department of Pharmacology, Biocenter of the University of Basel, 4056 Basel, Switzerland. A procedure was used as outlined by the Dako method sheet 30015, using the Alkaline phosphatase-anti alkaline phosphatase (APAAP) detection system.

This involved rehydrating the wax sections through a graded series of alcohols, and incubating the samples in primary mab diluted in TBS (pH 7.6) for 30 min at room temperature in a moist chamber. A special Dako pen for immunocytochemistry was used to produce a waterproof ring around each sample. Samples were then washed in TBS, and incubated with the secondary mab (Dako rabbit anti- mouse immunoglobulins, Z 259) diluted in TBS, for 30 min at room temperature, followed by further washes in TBS. The tertiary mab (Dako APAAP-conjugated to mouse anti-rabbit immunoglobulins, D 651) diluted in TBS was also incubated at room temperature for 30 min, followed by washes in TBS and repeat incubations with the secondary and tertiary mab for 10 min each to amplify the staining. The final stage of the procedure was the incubation with substrate solution 1 from the Dako data sheet 30015 (section 2.II.vi), containing naphthol AS-MX phosphate as the substrate and Fast Red TR salt as the chromogen for 20 min. Sections were then washed in TBS, counter-stained with haemotoxylin, and mounted in a water-based mountant (Glycergel, Dako Ltd., High Wycombe, England).

3.II.iii Photography for light microscopy

For phase contrast, micrographs were taken using a Leitz Labovert FS inverted microscope with an Orthomat E automatic camera attachment (E.Leitz (Instruments) Ltd., Luton, England) and black & white film (FP4,Ilford Ltd., England and TMax 100, Kodak Ltd., England). All micrographs were developed by Audio Visual Photographic Services, Aston University, England.

For colour photography of H&E and immunocytochemistry, micrographs were taken using a Photomicroscope III (Carl Zeiss, England) and Kodak Ektachrome 50 colour reversal film (Kodak Ltd., England).

3.II.iv Transmission electron microscopy

Samples for TEM were washed with PBS and then fixed for 1h at room temperature in EM fixative 1 (section 2.II.vii). Samples were then rinsed in 0.18M sodium cacodylate, and then stored in the same buffer at +4 °C until they were sent for processing by Stuart Townsend at the MRC Radiobiology Unit, Chilton, Didcot, Oxen, England. Briefly, this involves post-fixation with 1% osmium tetroxide, dehydration through a graded series of alcohols into propylene oxide, then infiltration and embedding in Epon resin. After polymerization of the resin at 60 °C, the blocks were trimmed and sectioned using an ultramicrotome (Ultracut, Reichert-Jung, England). Sections were mounted onto copper grids, stained with uranyl acetate and lead citrate using an automatic processor, and viewed in a Cora electron microscope.

Representative samples were stained prior to embedding for alkaline phosphatase activity using the method of Reale and Luciano (1967), taken from Lewis and Knight (1977). Briefly, this method involved fixing the tissue in EM fixative 1 for 1 h, followed by incubation at room temperature in the filtered substrate solution for 1h. Samples were then washed in cold dH_2O and then placed in 0.05 lead nitrate solution for a few minutes. Samples were washed, post-fixed in 1% osmium tetroxide and embedded in epon resin. Sections were cut as usual, but the uranyl acetate/lead citrate staining omitted, to allow detection of the electron-dense lead phosphate deposited during the enzyme reaction. Control incubations were carried out using substrate-deficient incubation medium.

The method of Luft (1971) incorporating ruthenium red in the fixation buffer (section 2.II.xii) was used to stain extracellular acidic mucosubstances for electron microscopic visualization.

3.II.v Scanning electron microscopy

For SEM cells were grown on glass coverslips and collagen gels until confluency. Samples were washed in PBS and fixed for 1h at +4 °C in EM fixative 2 (section 2.II.viii). Samples were then transferred to 70% ethanol, and dehydrated through a graded series of alcohols. Samples were critical point dried (Emscope CPD 750) and mounted onto copper rectangular plates with aryldite. Samples were coated with platinum of a fine particle size (20 nm) using an Emscope SC 500 splutter coater. Samples were viewed in a Jeol 100 CX II JEM SCAN operating at 40kv. Electron micrographs were taken using Kodak Plus XPan 120 film, developed in Acutol Developer (1/10 parts water) and fixed in Ilford Hypan fixer (1/4 parts water). A Durst L900 enlarger and Kodachrome II RC paper were used to produce prints. This work was carried out by Lesley Tomkins, Department of Physiology, Medical School, University of Birmingham, Edgbaston, Birmingham, England.

3.III Biochemical assays for markers of colonic cell differentiation

3.III.i Development of an alkaline phosphatase microassay

Initially a spectrophotometric assay was used to measure the activity of alkaline phosphatase using a sodium carbonate/bicarbonate buffer and p-nitrophenyl phosphate as the substrate (Frais, 1972). The substrate being enzymatically hydrolysed to yield p-nitrophenol which at alkaline pH absorbs light at 450 nm. However, this assay required large numbers of cells and for this reason its use was prohibitive.

An ultramicro phosphorimetric assay was then tried (Ohkura *et al.*,1980). This assay was dependent on the phosphorimetric characteristics of p-nitrophenol in ethanolic potassium hydroxide. Unfortunately, the equipment could not be kept at the required temperature (77 °K) for the reading of phophorescence.

Finally, a microassay for measuring the activity of alkaline phosphatase in as few as 10^5 cells was developed from an assay described by Jakel *et al* . (1983). The original assay was designed to measure the enzyme activity in single cells in microtitre-welled plates using a microtitre fluorescence reader. The volumes were scaled up in order that a standard luminescence spectrometer (Perkin-Elmer LS-5, Perkin-Elmer Ltd.,

Beaconsfield, England) with a cuvette volume of 3ml could be used instead of a fluorescence microtitre reader, which was not available.

Cells were removed from the appropriate substrata, washed 3x with PBS to remove contaminating enzyme from the media, and counted using a haemocytometer. 10^5 or 10^6 cells (depending on the number available) were pelleted in polystyrene centrifuge tubes. 200 µl of distiled water added to each pellet to lyse the cells, and pellets mixed using a spinmix (Gallenkamp,England). 200 µl of incubation media was added to the lysed cells, tubes sealed with nescofilm (Bando Chemical Ind.Ltd., Kobe, Japan) and incubated at 37 °C for 4 hours. The substrate 4-methylumbelliferyl phosphate (Sigma, Poole, Dorset, England) was converted by alkaline phosphatase to 4-methylumbelliferone, which was highly fluorescent in a strongly alkaline solution of AMP (pH 12). At termination of the reaction, 5.5ml of ice cold AMP (pH 12) was added to each cell sample and the samples mixed well. Fluorescence was read on a Perkin Elmer LS-5 luminescence spectrometer with settings of : excitation wavelength=365nm; excitation slit=15;emission wavelength=450nm; emission slit=2.5, versus a reagent blank of incubation media, distiled water and AMP alone.

Calibration curves were constructed for each enzyme reaction using 4-methylumbelliferone diluted in AMP (pH12) over a range of 0.05 - 1µm, read against a blank of AMP alone. Enzyme activity was expressed as unit/mg protein, where 1 unit hydrolyses 1µmole of substrate/min.

3.III.ii Aminopeptidase assay - P_2 membrane extraction

In order to assay for aminopeptidase activity, the P_2 membrane pellet was extracted from cells using the method of Schmitz *et al.*, (1973). Cells were removed from their substrata, washed 3x with PBS and counted using a haemocytometer. 1.7×10^7 cells were pelleted and 5ml of ice cold tris-mannitol buffer (pH 7.1) added to the pellet. Cells were then sonicated on ice with 3 x 10 sec bursts using a MSE sonicator (MSE Scientific Instruments, Crawley, England) on settings 26-28, and solid calcium chloride added to a concentration of 10 mM. The cell lysate was then centrifuged at 2000g at +4 °C for 10 minutes using a Heraeus Minifuge T (Heraeus Equipment Ltd., Brentford, Essex,

England) to produce the S_1 supernatant. This was centrifuged at 20,000g at +4 °C for 15 minutes using a Pegusus ultracentrifuge 65 (MSE Scientific Instruments, Crawley, ENGLAND) to produce the P_2 membrane pellet, which was resuspended in phosphate buffer 50 mM / 0.1% TX-100 (pH 7.2) . P_2 membranes were stored at +4 °C until assayed for enzyme activity on the same day as extraction. The protein content in the P_2 samples were also determined using the method of Bradford (1976) (section 3.IV.iii) .

3.III.iii Aminopeptidase assay - demonstration of P_2 membrane activity

The assay for aminopeptidase activity was based on a method by Roncari and Zuber (1968). The enzyme cleaves the substrate L-leucine-p-nitroanilide to form p-nitroanilide which absorbs at a wavelength of 405nm. The reaction was carried out in 96-well microtitre plates with flat bottomed wells (Nunc Intermed, Denmark). Each reaction well contained 150µl of phosphate buffer 50 mM (pH 7.2) and 60µl of sample (either enzyme calibration or P_2 membrane sample). The calibration curve was constructed using standard enzyme leucine aminopeptidase (EC 3.4.11.2, Sigma, England) diluted in phosphate buffer 50 mM / 0.1% TX-100. The zero absorbance reading was taken using an Anthos reader 2001 microtitre plate reader (Anthos Labtec Instruments, Salzberg, Austria), followed by the addition of 20µl of substrate solution (25mM L-leucine p-nitroanilide (Sigma,England) in phosphate buffer 50mM (pH 6.6)). Further readings were taken after 2 hours incubation at 37 °C. The standard curve was determined by substracting the zero values from the 2 hour readings. The unknown sample values were then determined from the standard curve and expressed as units of activity/mg protein. One unit will hydrolyse 1.0 µmole of L-leucinamide to L-leucine and NH_3 per min at pH 8.5 at 25°C (Sigma, England).

3.IV Determination of carcinoembryonic antigen production

3.IV.i Collection and concentration of conditioned media

For the purpose of assaying for CEA, conditioned media was collected and concentrated as described here. Cells were grown to confluency on the appropriate substrata, the media removed, the cells washed with sterile PBS and sera-free media added. Cells were incubated for a further 48 hours and the conditioned media collected and spun to remove any cell debris. Media samples were stored at -20 °C until being vacuum dried using a Buchi rotavapor-RE (Orme Scientific Ltd.,Manchester, England), a 37 °C waterbath (Rikakikai Co Ltd, Tokyo) and a high vacuum pump E2M2 (Edwards, England). Dried samples were resuspended in PBS and stored at -20 °C until being assayed for CEA concentration using an ELISA (see section 3.IV.ii). Cells were counted as described is section and samples assayed for protein content using the Bradford assay (see section 3.IV.iii). This was carried out in order to standardize the results.

3.IV.ii Enzyme-linked immunosorbent assay for CEA

The amount of CEA secreted into the media by cells grown on various substrata was determined by using an ELISA with mabs provided by Dako (Dako Ltd., High Wycombe, England). A method was used as previously described by Smith and MacDonald (1983). Briefly, this involved coating the required number of wells of a 96-well sterile flat bottomed tissue culture plate (Nunc Intermed, Denmark) with 200 µl of primary mab (Anti-CEA,Product No. A115, Dako Ltd.,High Wycombe,England) diluted 1: 600 in carbonate/bicarbonate buffer (pH 9.6). The plate was covered and incubated at room temperature overnight. Just prior to use the plate was washed 4 x with wash buffer (0.01% Tween 20 in PBS) and drained by inversion.

A calibration curve was constructed using CEA standard (coded 73/601) obtained from the National Institute for Biological Standards and Control, P.O. box 1193, Potters Bar, EN6 3QH,England. The contents of the ampoule were dissolved in 100ml of chicken serum (ICN Flow Laboratories,England), divided into 1ml aliquots and stored at -20 °C.

To produce the calibration curve each aliquot which contained 100 µg/L of CEA was re-diluted in chicken serum to give a range of dilutions 3.1 - 100 µg/L.

In order to extract the CEA from the serum or media samples, 0.5 ml was added to 1 ml of acetate buffer pH 5.0, heated to 70 °C for 15 min, cooled and spun at 12,000 rpm in a minifuge (Heraeus Biofuge, Heraeus Equipment Ltd., Brentford, England) at +4 °C. 200 µl of supernatant from each sample was added in triplicate to wells of the primary mab-coated plate, covered and incubated at 37 °C for 1h. The plate was then washed 4 x with wash buffer and drained by inversion. The secondary mab (peroxidase-conjugated anti-CEA, Code No. P 167, Dako Ltd., High Wycombe, England) was diluted 1:550 in 1:1 mixture of heat inactivated chicken serum and PBS (this solution was centrifuged in a minifuge at 12,000 rpm in order to precipitate out the debris found in chicken serum, which was found to interfere with the assay. The supernatant was used to dilute the mab prior to use). 200 µl of the diluted secondary mab was added to each well, the plate covered and incubated at 37 °C for 1h.

Substrate solution was prepared immediately prior to use by dissolving 4 tablets of o-phenylenediamine (Dako Ltd., High Wycombe, England) in 12 ml of citric acid-phosphate buffer (pH 6). The plates were washed 4x with wash buffer and drained by inversion. 5 µl of hydrogen peroxide (30%) was added to the substrate solution and 100 µl of this added to each well. The plate was wrapped in tin-foil to exclude the light and incubated at room temperature for 30 min. 50 µl of 4M sulphuric acid was added to each well to terminate the enzyme reaction and the plate read on an Anthos reader 2001 microtitre plate reader (Anthos Labtec Instruments, Salzberg, Austria) at 492 nm. Levels of CEA in the conditioned media were determined by reading values off of the calibration curve. Chicken serum was used as the blank for the calibration curve and fresh DMEM media for the conditioned media samples.

3.IV.iii Protein determination

The concentration of protein in samples from all biochemical assays were determined using the method of Bradford (1976) and reagents from Bio-rad Laboratories Ltd. (Hemel Hempstead, England). A micro-calibration curve was constructed for each assay using protein standard 1 (500-0005) over a range of 1 - 75 μg/ml. 200 μl of dye reagent (500-0006) was added to 800 μl of sample or standard, mixed with a spinmix (Gallenkamp, England) and incubated for a minimum of 5 minutes at room temperature. The OD_{595} was then read on a spectrophotometer (Beckman DU-70, Fullerton, CA, USA), versus a reagent blank of PBS and dye alone. OD_{595} was plotted against protein concentration and unknown concentrations read from the standard curve.

3.V Procedures for toxicity testing

3.V.i Toxicity analysis

Various experiments with different protocols and dosing regimes were carried out on the parent HT-29 cells grown on plastic substrata and on cells grown on collagen gels, either non-passaged or passaged. Initially, experiments were carried out in triplicate on cells grown in tissue culture flasks and this data was then used to select three dose levels to test on cells growing on collagen gels. For these experiments cells that had not previously seen collagen were used and their sensitivity compared to those cells growing on plastic substrata. In addition, cells that had been passaged continuously on collagen for at least 10 passages as described in section 3.I.x were also assayed for their drug sensitivity compared to cells grown on plastic substrata.

The general procedure for these experiments was as follows. On day 0 cells were seeded onto the designated substrata at a predetermined cell density (2 $x10^5$/T25 flask; 5 x 10^4/well or gel of a 12-well plate for a substrata comparison experiment). On day 1, after microscopic examination to ensure attachment to the substrata and noncontamination, the cells were dosed with the appropriate test agent diluted in media (see section 3.V). Also on day 1, the collagen gels designated 'released gels' were detached gently from the

plastic well bottom as described in section 3.I.ix. Cells were incubated for a further 6 days and counted as described in section 3.I.ii. The cell suspensions were then diluted in media and seeded at 5 $x10^2$ cells per well of a 6-well plate for clonogenic analysis as described in section 3.V.ii. All toxicity experiments were performed in triplicate, and for each clonogenic assay, 6 wells were seeded per treatment group.

3.V.ii Clonogenic analysis

Cells were plated at 5 $x10^2$ cells per well of a 6-welled tissue culture plate, and incubated for 14 days at 37 °C and 5% O_2 (to promote colony formation) in a gassing incubator (Heraeus CO_2-AUTO-ZERO, Heraeus Equipment Ltd., Brentford, England). The colonies were then fixed in 70% IMS for 10 minutes, followed by staining with a saturated solution of methylene blue in 70% IMS for 10 minutes. Plating efficiencies were determined by counting colonies of greater than 50 cells and using the formula below:

$$\text{Plating efficiency \%} = \frac{\text{number of colonies}}{\text{number of cells seeded}} \times 100$$

For toxicity experiments were the results were being compared between cells grown on different substrata it was necessary to compare the plating efficiencies treated cells to those of the untreated control cells for each of the substrata. The data was therefore expressed as % of control plating efficiency, and this enabled direct comparisons to be made between cells grown on different substrata, where there may have been an altered plating efficiency due to the substrata alone.

3.V.iii Preparation of drugs for toxicity analysis

DMF was stored at room temperature. Sodium butyrate and 5-FU were stored at +4 °C. TCNU and SRI 62-834 were stored under nitrogen at -20 °C. Sodium butyrate, N,N,-dimethylformamide, 5-FU, and TCNU were dissolved directly into tissue culture media as concentrated stock solutions from which the required dose concentrations were prepared. Each stock solution was prepared immediately prior to use and sterilized using

a 0.2 μm filter unit (Gelman Sciences Inc.,USA). SRI 62-834 was also dissolved directly into tissue culture media to produce a stock solution, but it was not filter sterilized because of the possibility of significant binding to the filter and the consequent loss of drug. SRI 62-834 was also prepare immediately prior to use. All drugs were weighed out on a Mettler AE163 electronic balance (supplied by Gallenkamp, England).

3.V.iv Studies of the long term exposure to sodium butyrate

The original batch of HT-29 ($HT\text{-}29_1$) cells, which were later discarded, were used in this experiment. Initial experiments with sodium butyrate showed that a concentration of 2mM caused minimal growth inhibition and enhanced the expression of the brush-border associated enzyme alkaline phosphatase. Cells were continuously exposed to the differentiation-inducing agent sodium butyrate at a concentration of 2mM for a period of 6 months and at each passaging (every 10 days), samples of cells were prepared for cryostorage as described in section 3.I.vi. Cells were also regularly fixed and prepared for electron microscopy as described in section 3.II.iv, to check for any morphological changes indicative of cellular differentiation and the expression of alkaline phosphatase. Black and white photographs were taken using phase contrast microscopy on a Leitz Labovert FS microscope with Orthomat E automatic camera attachment (E. Leitz (Instruments) Ltd., Luton, England).

To demonstrate the reversibility of those changes seen in the presence of sodium butyrate previously shown in preliminary short term experiments, cells were seeded back into control media after 9 passages (=90 days) in the presence of sodium butyrate. Changes were observed using a light and electron microscope.

3.VI IN VIVO

3.VI.i Growth of HT-29_2 xenografts in nude mice

These studies were carried out by Dr. Simon Langdon, Imperial Cancer Research Fund, Medical Oncology Unit, Western General Hospital, Edinburgh, Scotland. Initially, 10^7 cells were injected subcutaneously into the flank regions of 5 nude mice of mixed background and allowed to develop into tumours for 16 days. Tumours were excised and treated as described in section 3.VI.ii. A second study was carried out in which 10^7 cells were injected via the following routes of administration (3 mice each) :

i) sc - tumours were excised after 14 days and treated as described in section 3.VI.ii.

ii) ip,iv, and ih - mice were sacrificed after 28 days and post mortem examinations carried out. Any tumours found were excised and treated as in section 3.VI.ii.

3.VI.ii Preparation of xenograft tissue for microscopy

Each tumour was excised and cut in half; one half was fixed in formol saline for light microscopy as described in section 3.II.i. and the other half was fixed in EM fixative 1 and processed for electron microscopy as described in section 3.II.iv.

3.VI.iii Analysis of percentage of morphological differentiation

1 μm semi-thin, toluidine blue-stained sections and 4 μm wax-embedded sections were used to determine the percentage of differentiated cells in the xenograft tissue. Samples were viewed using a light microscope (Carl Zeiss, England) and the number of cells displaying morphological features of colonic cell differentiation, for example nuclear and organelle polarization, goblet cells and enterocyte-like cells arranged around intercellular lumen, determined. A minimum of 300 cells were counted for each xenograft sample. Comparable studies were also carried out on 4 μm sections of HT-29_2 cells grown on various substrata in order to determine the degree of morphological differentiation, but the features were not as easy to visualize as in the xenograft samples, and it was only possible to determine the number of goblet cells in the samples.

PART ONE Studies on the differentiation of the second batch of HT-29 ($HT\text{-}29_2$) *in vivo*.

SECTION 4 : RESULTS AND DISCUSSION

SECTION 4 : RESULTS AND DISCUSSION

4.I The effects of the site of implantation on the differentiation status of the HT-29_2 xenografts

4.I.i Introduction

Human tumour cell lines originally derived from solid tumours, can be grown *in vivo* in immunodeficient 'nude' mice (Fogh and Giovanella, 1978), and they will produce solid tumours which display different states of differentiation dependent on the cell line used. As these tumour xenograft cells are exposed to many of the typical control mechanisms of cell proliferation and differentiation which are found *in vivo*, the differentiation seen within a xenograft is thought to represent many features of differentiation which are achievable by any particular cell line. They are also considered to be reasonably representative of the primary tumour from which the cell line was derived. *In vivo* human tumour xenografts are usually produced by a subcutaneous (sc) injection of a cell suspension into the flank of the host nude mouse. At this site they will produce a solid, palpable tumour, which can be easily visualized and measured. This is a distinct advantage when performing chemotherapeutic assays with novel agents which may cause tumour regression. In this context, the human xenograft systems have many uses including, 1) testing the efficacy of novel chemotherapeutic agents, 2) determining novel mechanisms of actions of such agents, and 3) assessing the important pharmacokinetic parameters and drug host interactions that determine drug efficacy. However, they also provide the cell biologist with a very useful tool with which to look at the differentiation potential of cells that in culture have lost many of their original tumour characteristics and are typically anaplastic.

These systems have also been useful in the study of the factors that govern metastasis from primary tumours and the eventual production of distant metastases in secondary tissues and organs. Paget (1889) proposed the 'seed and soil' theory, which aimed to explain why primary neoplasms metastasise preferentially to specific secondary tissues. This dispelled the notion that this was an accidental occurrence and it was proposed that it was a controlled action on the part of the tumour cells. This theory is supported in the

clinic by numerous observations that certain tumours preferentially spread to particular tissues, for example, the preferential spread of colorectal carcinoma to the liver (Ramming and Haskell, 1985). It is therefore clear that, although the exact reasons for this selection have not been elucidated, the site of secondary growth is not random and obviously confers distinct advantages on the cells growing there. If tumours can choose preferentially, by unknown mechanisms, where they will invade and grow as metastases, then it must follow that this is due to a range of advantages conferred upon the cells by the preferred secondary site. It may be the composition of the extracellular matrix, the degree of vascularization, supply of nutrients and growth factors, or oxygenation of the tissue, or a combination of these factors, which promote the interaction of the metastasising tumour cells and the secondary tissue.

It was considered to be essential to determine the differentiation potential of HT-29_2 cells by growing them as xenografts in nude mice, in order that I could compare their morphology with those that might undergo differentiation *in vitro*. In view of the evidence that tumours metastasise preferentially to specific secondary tissues, the hypothesis that the site of implantation may effect the cellular and structural differentiation achieved by the HT-29_2 cell line xenografts was also tested.

4.I.ii Results

This work was performed in collaboration with Dr. Simon Langdon, Imperial Cancer Research Fund, Medical Oncology Unit, Western General Hospital, Edinburgh.

A preliminary study was performed, in which 5 female nude mice of mixed background, were injected with 10^7 HT-29_2 cells, subcutaneously (sc) into the flank. After 16 days of growth, the mice were sacrificed by cervical dislocation and the tumours excised. Each tumour was cut into two, one half being fixed in 10% formol saline for LM, and the other in 2% glutaraldehyde (EM fixative 1) for TEM. A sample of formal fixed tissue was sent to Dr. M. A. McIntyre, Consultant Pathologist, Department of Pathology, Western General Hospital, Edinburgh, for histological evaluation. The report dated 25.7.89 stated:

" The tumour is a poorly differentiated adenocarcinoma. The malignant cells form sheets and groups separated by a little fibro-connective tissue. The nuclei are pleomorphic with large nucleoli and frequent mitotic figures. Extracellular and neutral mucopolysaccharide is present. Only a minimal inflammatory cell infiltrate is seen in the adjacent fibro fatty tissue. The appearances are those of a poorly differentiated mucus secreting adenocarcinoma".

Further LM studies were performed by myself and samples were prepared for TEM by Mr. Stuart Townsend, MRC Radiobiology Unit, Didcot, Oxon. The LM sections show little evidence of structural differentiation, characterized by a lack of glandular formation and epithelial-like organization (figure 5). The round, undifferentiated cells are grouped into homogeneous masses, separated by narrow sheets of fibroblast-like cells and connective tissue. Our results support the report presented by the pathologist. These results were in contrast to earlier findings which showed that HT-29 cells grew as a well differentiated xenograft tumour (Hajdu and Fogh, 1978). Figure 6 has been included to show the normal LM morphological appearance of human small intestinal epithelium for comparison.

The TEM studies revealed more detail of the cellular and structural differentiation of the sc xenografts. The normal ultrastructural appearance of HT-29_2 cells grown *in vitro* will be discussed in this section as it will then provide a base line to which the xenograft morphology can be compared. Figure 7 shows HT-29_2 cells grown on tissue culture plastic and it can be seen that they display a characteristically anaplastic morphology, with little evidence of intestinal differentiation. The cells are roughly spherical with centrally located nuclei, which have prominent nucleoli. They have only a few desmosomes between adjacent cells. There are no polarized cells exhibiting organized microvilli- lined apical membranes or tight junctions, as seen in the normal differentiated intestinal epithelial cells. There are tucks or clefts seen in the nuclear membranes, which are thought to be due to the over-production of nuclear membrane by malignant cells (personal communication with Dr. T. Allen, Paterson Institute, Manchester.) An interesting feature of the HT-29_2 cells is the association of some mitochondria with these nuclear clefts (figure 7 (b)). This was seen repeatedly in electronmicrographs of HT-29_2 cells grown on a variety of substrata (results to be discussed in Part Three). The apparent damage to the mitochondria resulting in their swollen appearance, often with a lack of

intact cristae, is most certainly artifactual damage occurring during fixation and processing. However, this does not explain their association with the nuclear clefts and the reason for this association is at present unknown. Other than this observation, the cells appear to be normal and healthy with respect their organelles, nuclei and membranes.

The ultrastructural appearance of the HT-29_2 sc xenograft tumour was extremely heterogeneous, with areas of both undifferentiated cells and highly differentiated cells. Figure 8 shows cells from an anaplastic region of the sc xenograft tumour. These cells are very similar to those grown in tissue culture (figure 7), but they do appear to have better developed desmosomes. Within the same tumour there were areas of well differentiated, polarized cells arranged around an intercelluar lumen (figure 9). These cells were bound to adjacent polarized cells by tight junctions at the apical or luminal surfaces and had well developed microvilli with terminal webs penetrating deeply into the cytoplasm. The cells also displayed numerous desmosomes along their basolateral surfaces. There were numerous cytoskeletal filaments, most likely actin. There was also artifactual damage to mitochondria similar to that already seen in the *in vitro* cells. Their cytoplasm contained large numbers of polyribosomes.

In order to determine whether or not the site of implantation has any effect on the differentiation achieved by the xenografts produced, a second experiment was performed in collaboration with Dr. Simon Langdon. 10^7 HT-29_2 cells were injected into groups of 3 nude mice of mixed background using the following routes:

1) subcutaneously (sc) into the flank,

2) intravenously (iv) into the tail vein,

3) intraperitoneally (ip) with repeats for mice which were accidentally injected:

4) intrahepatically (ih).

The mice were sacrificed by cervical dislocation and the tumours excised after 14 days (sc), and 28 days (iv, ip and ih). As for the preliminary experiment, the tumours were cut into two and fixed to LM and TEM (actually used for 1μm toluidine blue sections only). In addition to H&E staining, the LM samples were also used for the immunocytochemical

detection of the brush-border associated enzymes, sucrase-isomaltase (SI) and aminopeptidase (APN).

The sc tumours grew as in the original experiment, with groups of undifferentiated cells separated by bands of fibroblast-like cells and connective tissue (figure 10 (a)). The iv route proved to be unsuccessful and did not result in the production of tumours. The mice were thoroughly examined and no obvious tumour deposits were found in the liver, lungs, kidneys or other major organs. Microscopic analysis of the lungs, liver and colon supported the macroscopic findings. Injection of the tumour cells via the ip route did not result in the production of ascites, however solid tumour deposits were found around the injection site and on other parts of the peritoneal wall. The structural arrangement within these tumours was quite distinct from that seen in the sc tumours, with the cells arranged in gland or acini-like structures surrounded by fibroblast-like cells (figure 10 (c) and 12 (c)). These observations are suggestive of a higher degree of structural organization and these acini-like structures contained numerous goblet-like cells. Unfortunately, errors made during ip injection of cells resulted in intra-hepatic tumours, which provided us with an opportunity to study the differentiation achieved within this tissue. These tumours appeared at post-mortem as white masses adjacent to and within the surface of the liver, which otherwise appeared normal. Extra solid tumours were also found attached to the peritoneal wall, which almost certainly arose from the injection site and were not thought to be metastases from the liver tumours (personal communication with Dr. Simon Langdon, Edinburgh). The malignant cells grew as islands within the normal liver cells and displayed a fairly anaplastic appearance with some goblet-like cells at the LM level (figure 10 (b)). However, due to the difficulty encountered when sectioning the liver and tumour samples there was only enough sections to stain for SI. The lack of viable sections also made it impossible to say definitely that the micrograph shown was representative of the ih tumours. The extra tumours which were found on the peritoneal wall were also processed for microscopy and they were seen to grow with a structural morphology the same as the true ip samples, as would be expected.

The presence of both SI and APN was demonstrated by a method which resulted in the deposition of Fast Red, which gives a dark pink/red colour against the blue counter-

staining of haematoxylin. Both of the enzymes were located in the cytoplasms of the malignant cells and the intensity of staining was heterogenous throughout all the tumour samples analysed. This heterogeneity within each tumour sample has made it impossible to compare tumours from different sites, with respect the presence of either enzymes. Figures 11 and 12 (a) have been included to show the normal staining pattern for SI and APN in human small intestinal epithelium.

In addition to processing for LM, samples of the xenograft tumours were prepared for electron microscopy by myself and Mrs Lesley Tomkins, EM Unit, Department of Biochemistry, School of Biological Sciences, University of Birmingham, Edgbaston, Birmingham. Due to the limits of time, the samples were only used to cut 1 μm sections, which were stained with toluidine blue, rather than ultrathin sections. Figures 13 to 15, show examples of the morphological appearance of the sc, ip and ih tumours when processed and stained in this manner. An interesting observation made was the higher number of cytoplasmic vacuoles seen in the cells of the ip and ih tumours. Their association with nuclear clefts in several of the cells, may indicate that these are actually, artifactually damaged mitochondria, like those already seen in the TEM micrographs of xenograft tissue. This theory is supported by the fact that the vacuoles are not seen in the more mildly, formol saline fixed samples used for immunocytochemistry. However, the vacuoles may also be mucus-filled and without performing further studies using monoclonal antibodies and em immunocytochemistry, it was not possible to state exactly what they were.

The 1 μm sections were also used to determine the extent of structural and cellular differentiation, using a light microscope with a 100x oil immersion objective lens. The following observations were made on a minimum of 5 fields for each sample, counting a minimum of 300 cells in total :

1) the number of cells displaying evidence of polarization,

2) the number of cells arranged around an intracellular lumen,

3) the number of cells displaying a brush-border,

4) the number of goblet cells.

The results of these analyses are shown in Table 1. They add further support to the differences in the structural differentiation of the tumours grown in different sites already observed in the LM sections. It would appear from these results that the site which produced the greatest cellular differentiation was the liver, with a larger proportion of cells being arranged around an intercellular lumen, showing evidence of microvilli and expressing goblet cell-like features. The poor sample quality of the LM sections and the small area covered by the 1 μm sections of ih tumours made it very difficult to determine if these tumours grew with an acini-like structural organization, like that seen in the ip tumours. There was very little difference in those parameters measured, between the sc and ip tumours, although the organization of the ip tumours into acini-like structures, was not seen in the sc tumours. Unfortunately, the time limitations prevented the analysis of more samples of HT-29_2 xenograft tumours for structural and cellular differentiation and this has prevented statistical evaluation of this work. Although the results were very interesting, it would be necessary to carry out many more analyses of tissue sections before definite conclusions could be drawn.

4.I.iii Discussion

The micrographs and limited quantitative data presented in this section suggest that the site of implantation has profound effects of the structural and cellular differentiation of HT-29_2 xenografts. The original sc tumours were classified as being poorly differentiated, which was true at the structural level, but the TEM results revealed that the cellular differentiation was in fact well advanced, although heterogenous. The ip site produced a significantly different structural differentiation, with the cells being organized into acini-like structures, but the cellular differentiation remained similar to the sc tumours. The site which offered the best environment for cellular differentiation was the liver, although poor sample processing prevented a thorough investigation of the structural differentiation achieved at this site. All tumours expressed SI and APN, although the intensity of staining varied within each individual tumour and thus made site to site comparisons impossible. In general, the staining intensity was not as high as was seen in the normal human small intestinal epithelium.

It is therefore evident that although the HT-29_2 cells form a multilayer of undifferentiated cells when grown on tissue culture plastic, they do retain the potential for enterocytic differentiation, which they re-express when grown in a suitable environment which provides them with the necessary signals for differentiation. In these experiments that suitable environment was the tissues of a nude mouse. The results, although not statistically validated, also suggest that the site at which the xenografts were grown in the nude mice influenced the extent of structural and cellular differentiation achieved by the cells. It is interesting to note that the site which produced the highest level of cellular differentiation (table 1) was the liver, which is also coincidentally the preferred site of colon carcinoma metastases (Ramming and Haskell, 1985). More exhaustive studies on the HT-29_2 cells, coupled with studies on the site-dependent differentiation of a variety of tumour cell lines would be needed before the significance of this result could be determined.

Sample type	Percentage of cells displaying the following markers (%) *: polarized nucleus	polarized organelles	organized around a lumen	microvilli	goblet -cell like
sc 1	3.3	2.0	11.5	4.4	0.7
sc 2	1.7	1.1	11.1	4.2	2.8
ip	1.5	0.9	7.7	3.4	1.5
ih	4.9	2.3	15.5	12.6	12.6
ih +	4.0	1.1	8.5	3.1	6.2
ih ++	1.3	0	3.9	2.9	3.9

Table 1 : Analysis of the morphological markers of xenograft cellular differentiation.
* Minimum of 300 cells counted over a minimum of 5 fields at a magnification of x100 oil immersion, using 1 µm toluidine blue stained sections.
+ & ++ Tumours were recovered from mice with ih tumours, but were actually growing on the peritoneal wall.

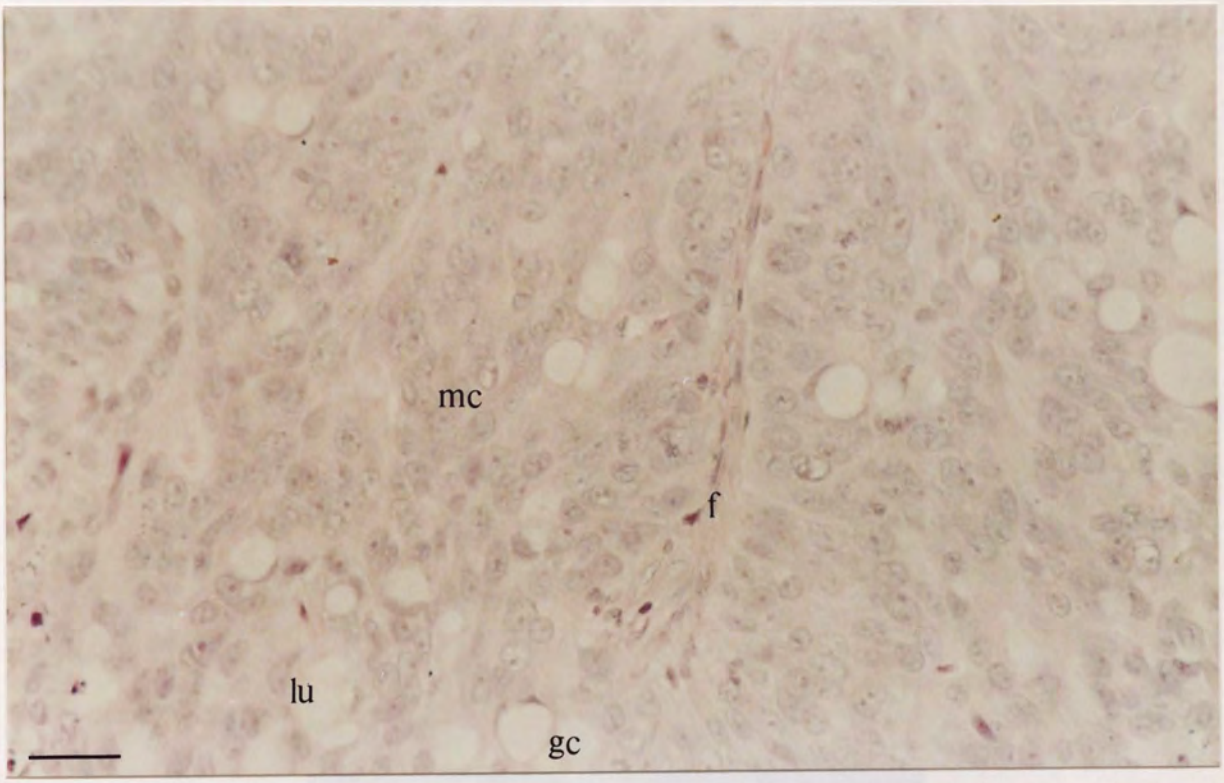


Figure 5 : Light micrograph of a HT-29$_2$ sc xenograft grown for 16 days.
The malignant cells (mc) appear as a homogeneous mass interspersed with fibroblast-like cells and connective tissue (f), and intercellular lumen (lu). The vacuolated cells are probably goblet-like cells (gc). H & E. *Scale bar,* 10 μm.

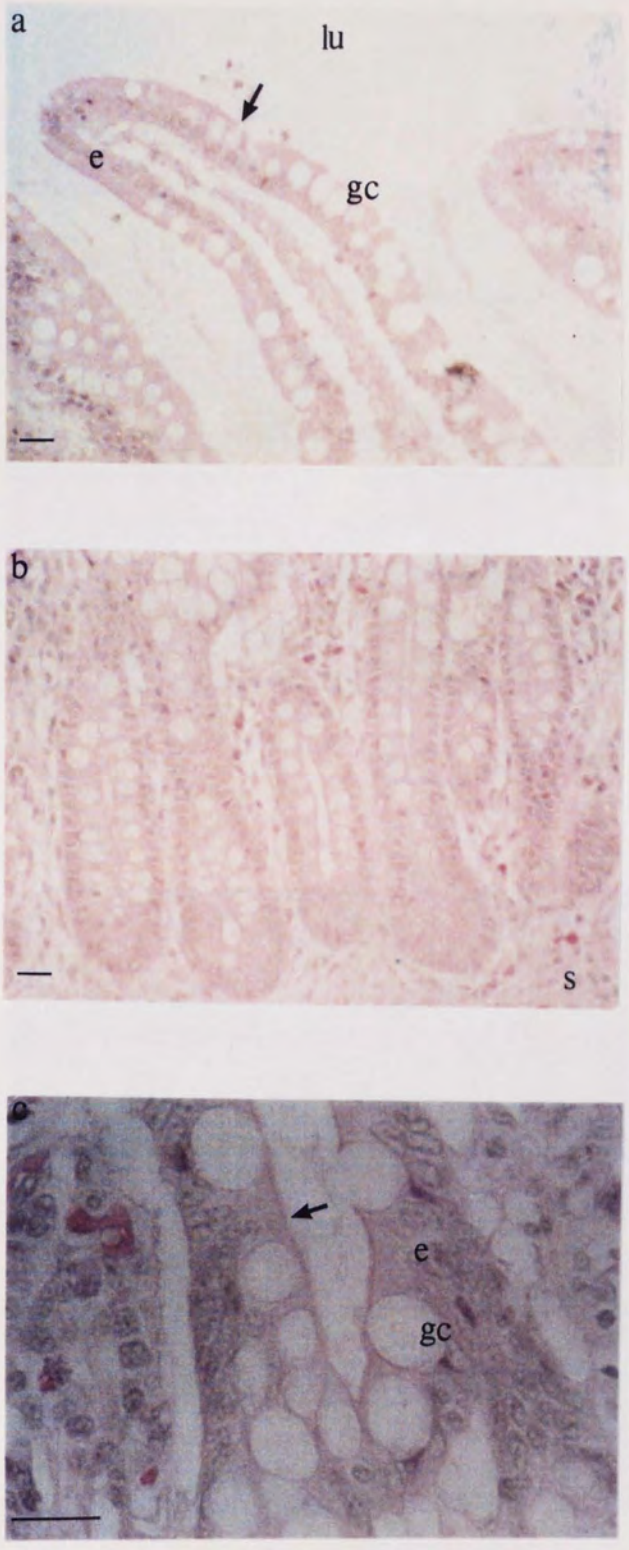
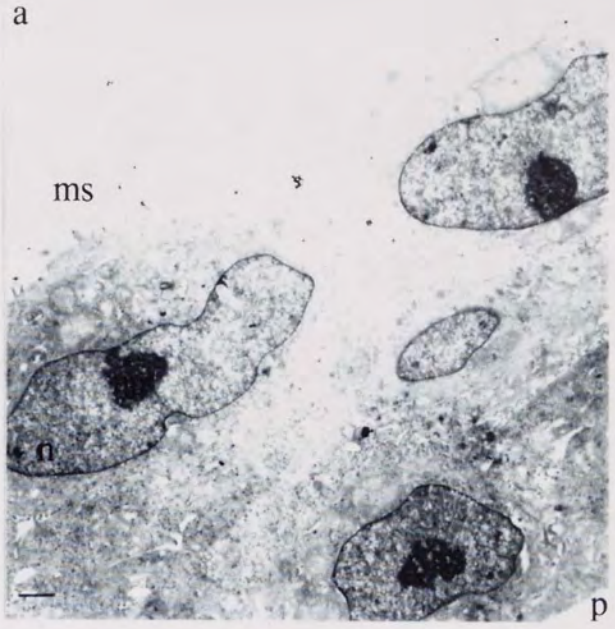


Figure 6 : Light micrographs of human small intestinal epithelium showing a) a villus tip, b) crypts and c) individual cells lining a crypt. The micrographs show the lumen (lu), goblet cells (gc), absorptive enterocytes (e), the microvillus brush border (arrow), and the underlying stromal cell layer (s). H & E. *Scale bars*, 10μm.



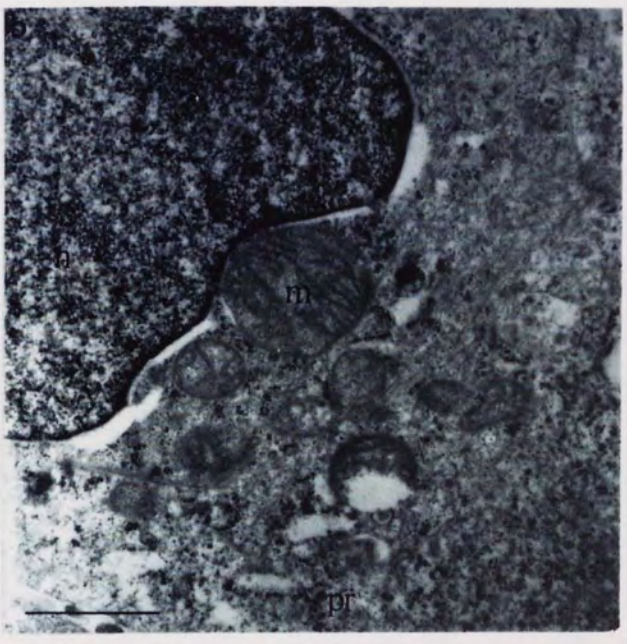


Figure 7 : Transmission electron micrographs of HT-29$_2$ cells grown on tissue culture plastic showing a) a multilayer of cells, and b) a single cell, with nuclear clefts and the associated mitochondria (m). Nucleus (n), nucleolus (nu), polyribosomes (pr), media surface (ms) and the plastic (p). *Scale bars,* 1μm.

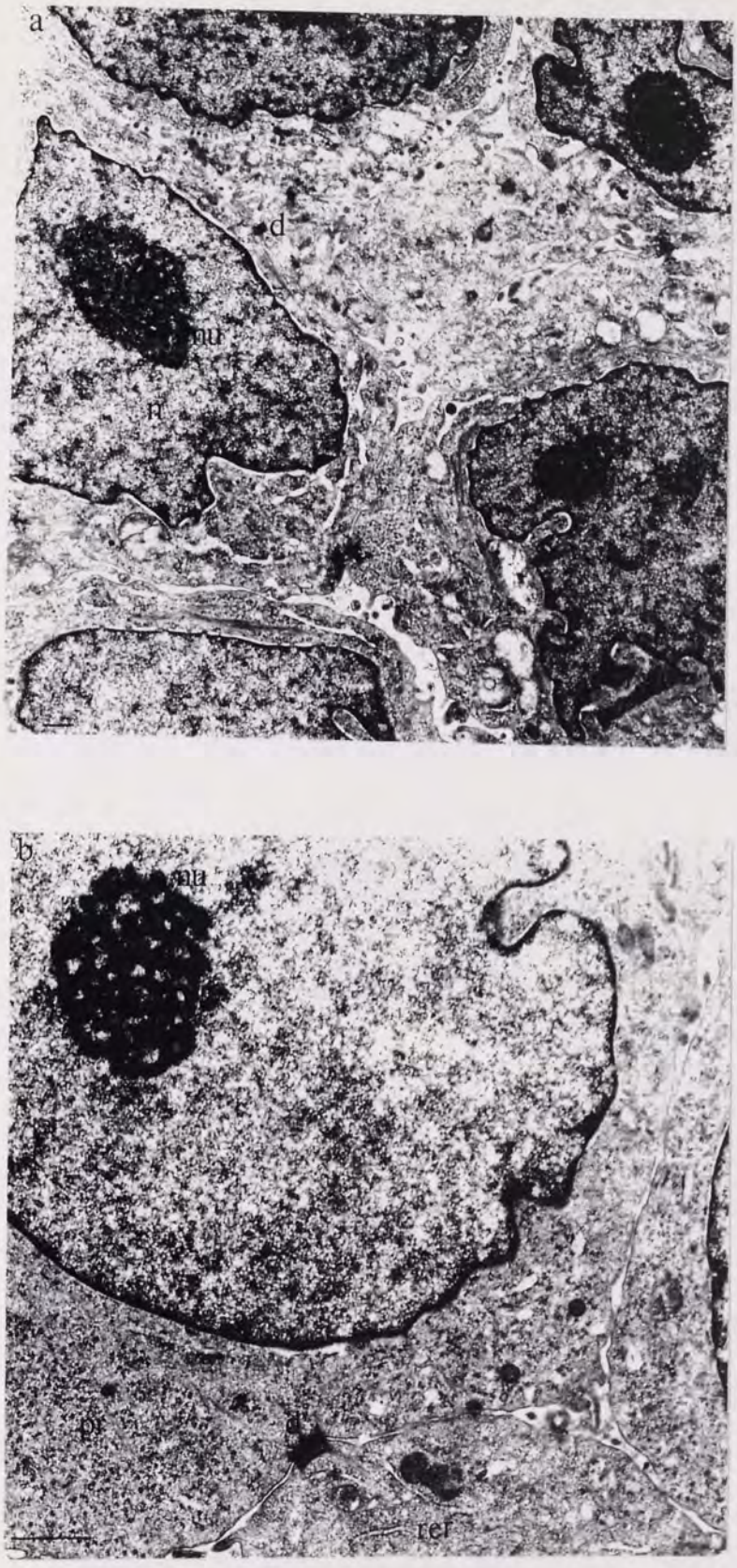


Figure 8 : Transmission electron micrographs of HT-29$_2$ xenograft showing a) an area of undifferentiated cells with centrally located nuclei (n) and b) a cell displaying a clefted nuclear membrane. Nucleoli (nu), desmosomes (d), polyribosomes (pr), and rough endoplasmic reticulum (rer). *Scale bars*, 1µm.

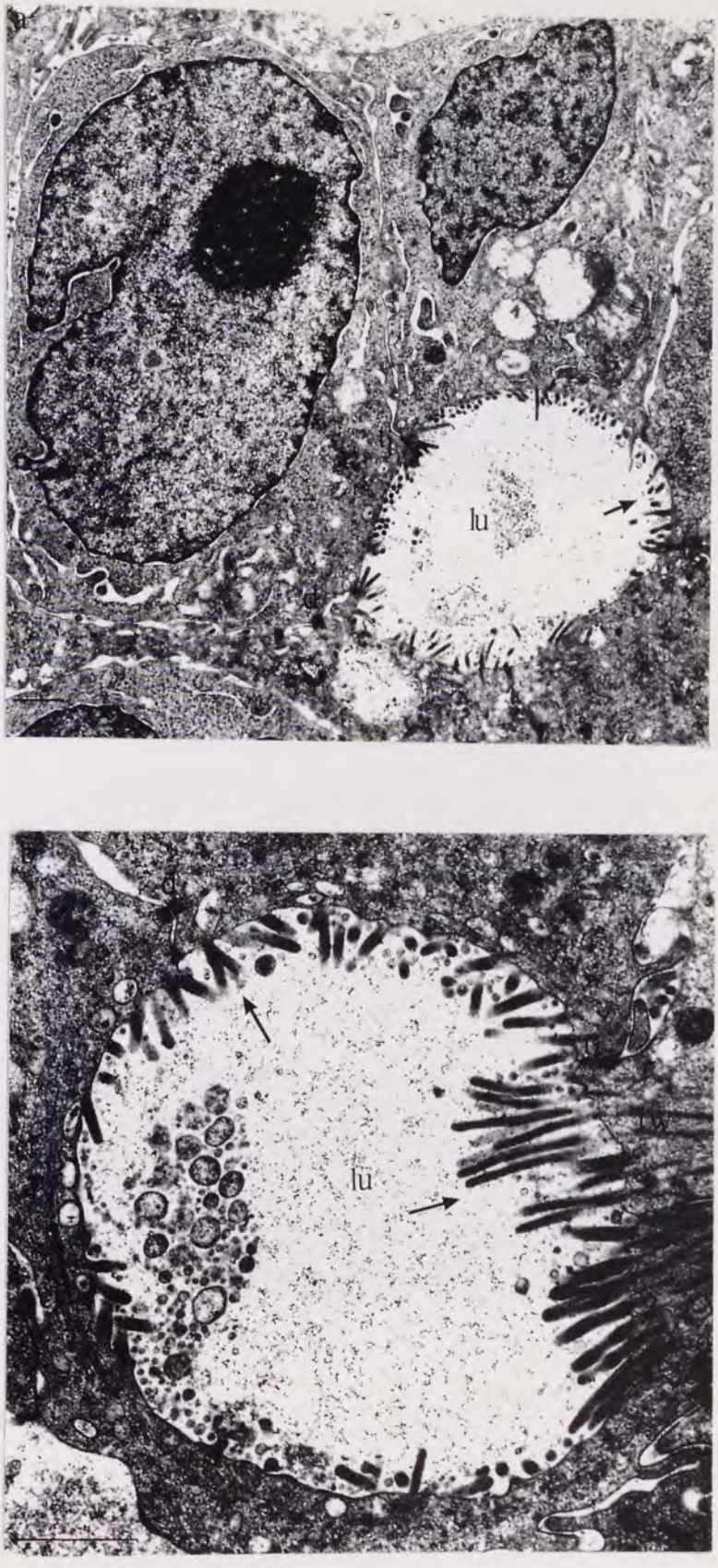


Figure 9 : Transmission electron micrographs of HT-29_2 xenograft showing a) an area of well differentiated, polarized cells arranged around an intercellular lumen (lu), and b) an intercellular lumen with the apical microvillus-lined surfaces (arrows) of the cells bordering the lumen, with well developed terminal webs (tw), tight junctions (tj), desmosomes (d) and cytoskeletal filaments (cf). *Scale bars*, 0.5μm.

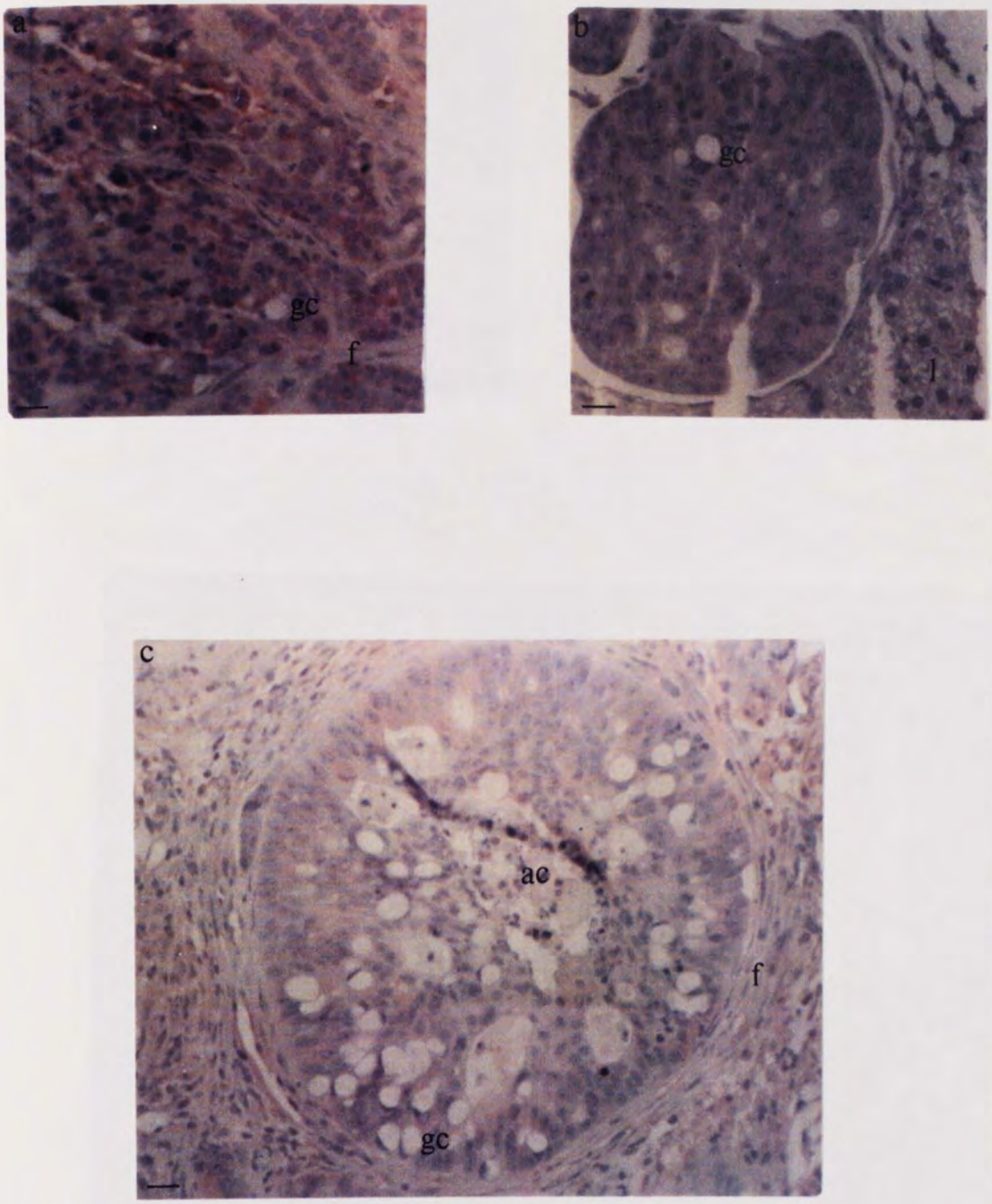


Figure 10 : Light micrographs of sections stained immunocytochemically for sucrase-isomaltase. HT-29_2 xenografts implanted a) sc, b) ih, and c) ip. Bands of fibroblast-like cells and connective tissue (f), goblet-like cells (gc), normal liver cell (l), and acini-like structure (ac). *Scale bars*, 10μm.

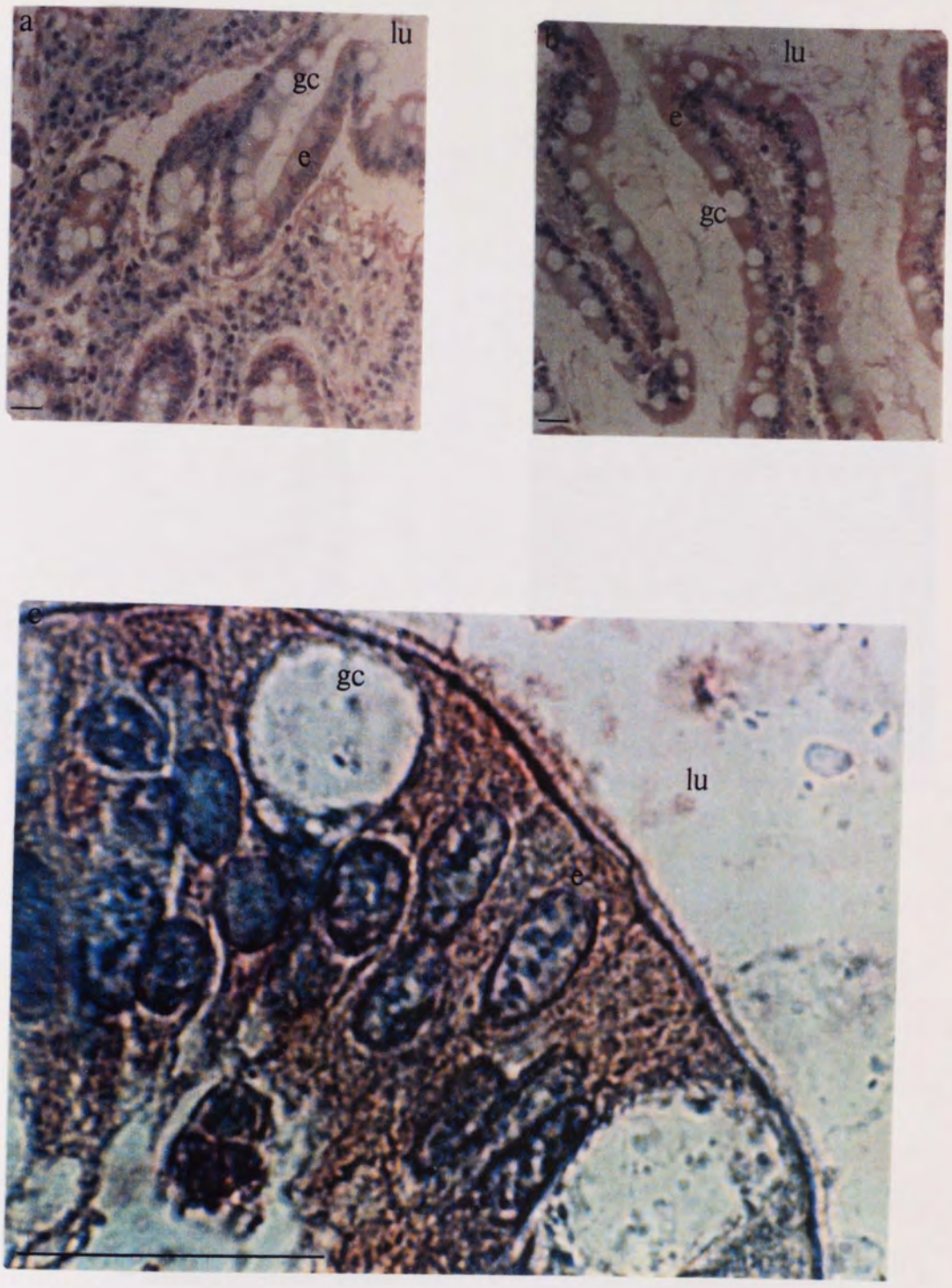


Figure 11 : Light micrographs of human small intestinal epithelium stained immunocytochemically for sucrase-isomaltase. a) crypt cells b) villus cells, and c) crypt cells at a higher magnification , showing goblet cells (gc), enterocytes (e) and the intestinal lumen (lu). *Scale bars*, 10μm.

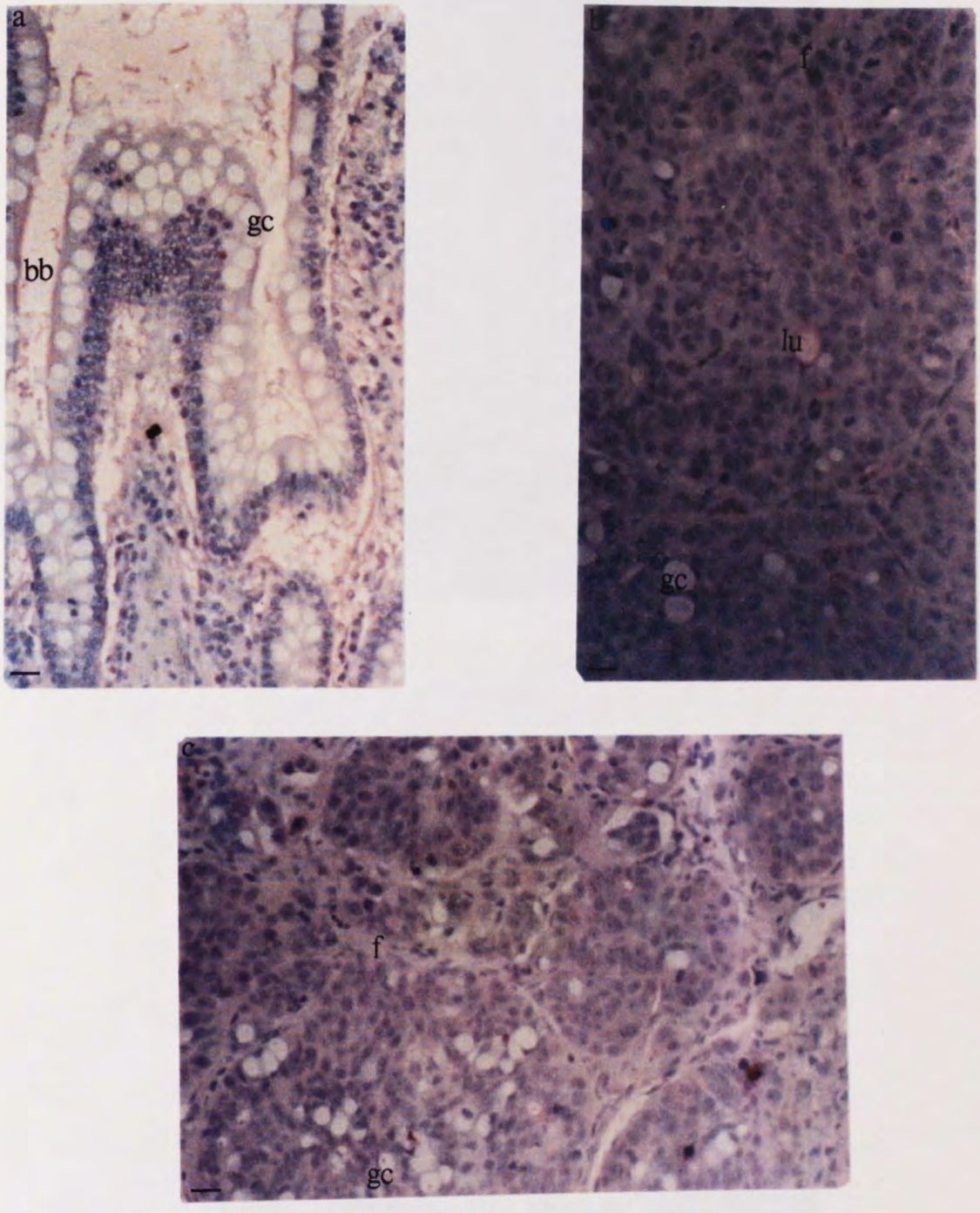


Figure 12 : Light micrographs of sections stained immunocytochemically for aminopeptidase. a) human small intestinal epithelium, b) HT-29_2 sc xenograft, and c) HT-29_2 ip xenograft. Intestinal brush border (bb), intercellular lumen (lu), goblet cells (gc), and fibrous connective tissue (f). *Scale bars*, 10μm.

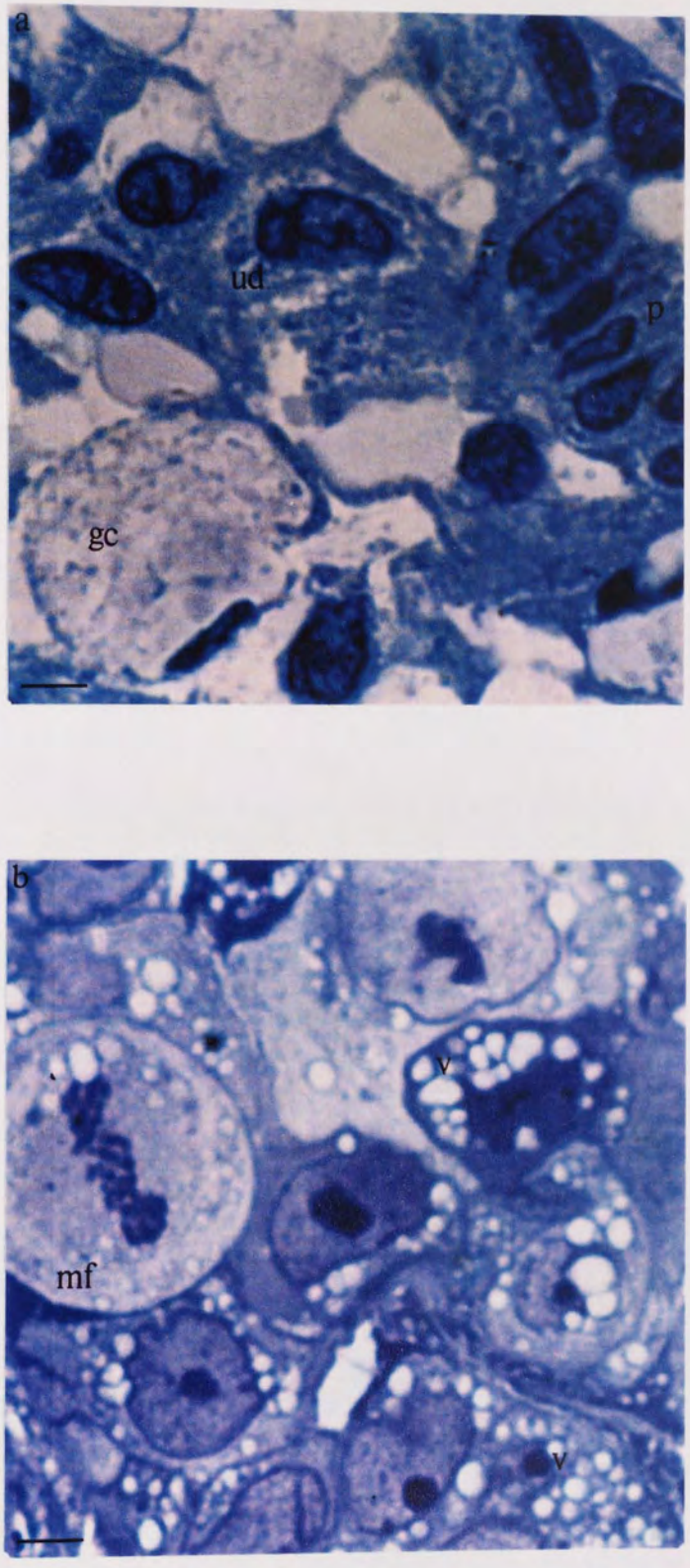


Figure 13 : Light micrographs of 1 µm sections of HT-29_2 xenografts implanted a) sc, b) ip. Undifferentiated cells (ud), polarized cells (p), goblet-like cells (gc), mitotic figure (mf), and vacuolated cells (v). toluidine blue. *Scale bars*, 2µm.

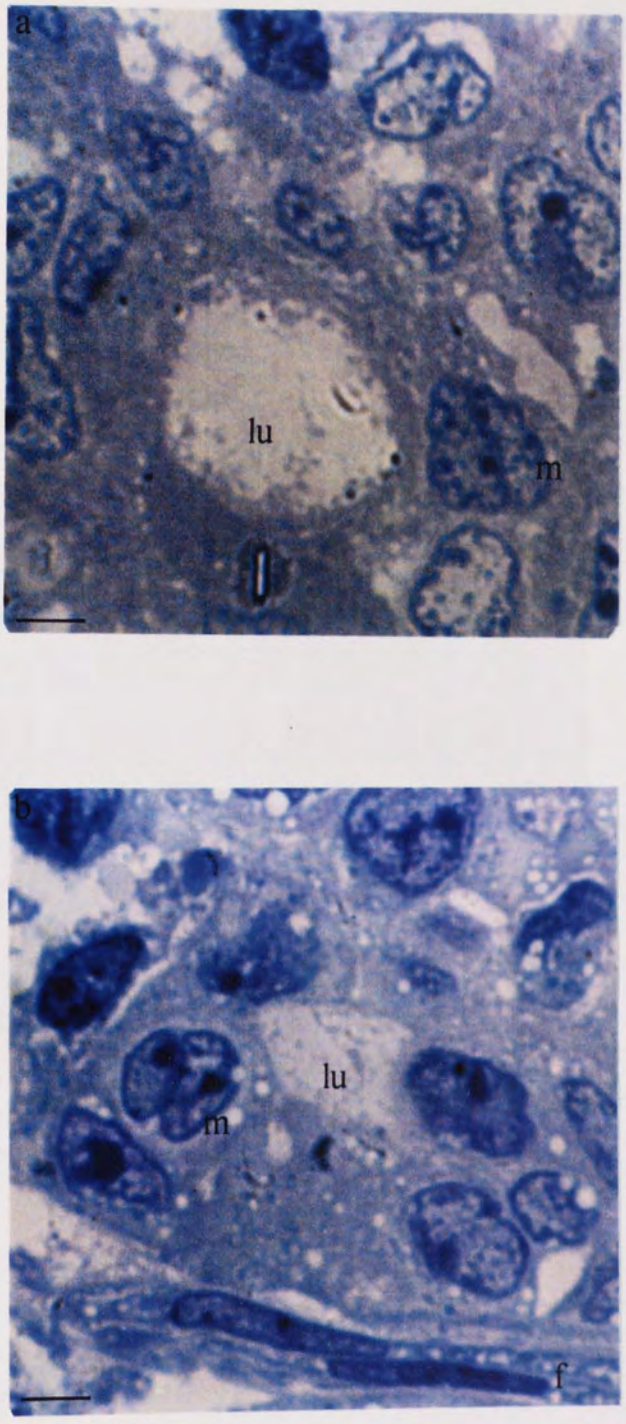


Figure 14 : Light micrographs of 1 μm sections of HT-29_2 ip xenografts. Intercellular lumen (lu), HT-29_2 cells (m), and fibroblast-like cells (f). toluidine blue. *Scale bars*, 2μm.

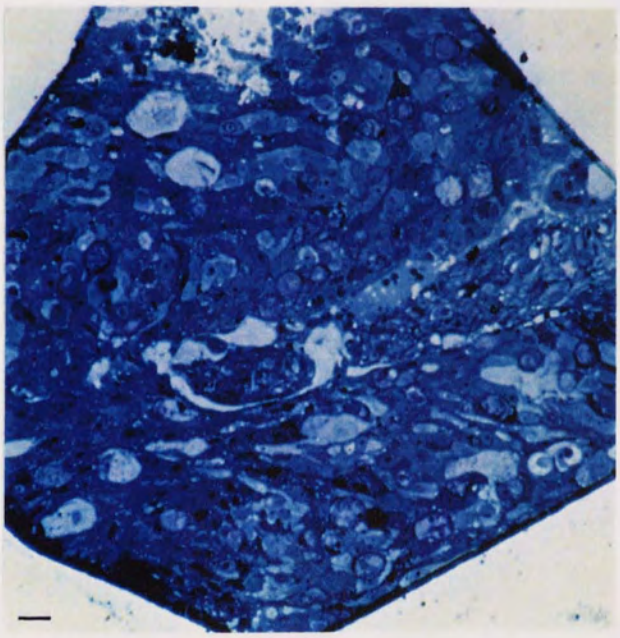


Figure 15 : Light micrograph of 1 μm section of HT-29_2 ih xenograft, toluidine blue. *Scale bar*, 10μm.

PART TWO Studies on the induction of differentiation of the original batch of HT-29 ($HT\text{-}29_1$) cells *in vitro*.

SECTION 5 : RESULTS AND DISCUSSION

SECTION 5 : RESULTS AND DISCUSSION

5.I The effects of sodium butyrate on HT-29$_1$ cells

5.I.i Introduction

Butyric acid is a four-carbon fatty acid, naturally occurring in the human intestine, being the product of intestinal flora (Prizont *et al.*, 1975). The sodium salt, sodium butyrate (NaB) has been described as a 'differentiating agent' because of the differentiation-inducing properties it exhibits with a number of cell lines *in vitro*. These include murine leukaemia cells (Leder and Leder, 1975), hamster fibroblasts (Leavitt *et al.*, 1978), and also the subject of my research, HT-29 cells (Augeron and Laboisse, 1984). The latter authors reported on the production of permanently differentiated clones of HT-29 cells, by following a protocol of exposure which first involved culturing the parental cells for 9 days in 5 mM NaB. The cells were then trypsinized and subcultured in NaB for a further 14 days, during which multinucleation occurred producing foci of giant cells. The monolayer of cells began to detach and on day 25 the cells were returned to control NaB-free media, upon which the multinucleation disappeared. They then reported that 10-12 days later, whilst still growing in control media, morphological changes occurred, which produced foci of flat cells amongst densely packed undifferentiated cells. It was from these foci that they derived their clones. The altered phenotype persisted for more than 24 months in culture and was thus considered permanent. The clonal cell lines exhibited morphological polarization with cells having distinct apical surfaces separated from the basolateral surfaces by tight junctions. Some of the clonal cell lines formed domes, evidence of transepithelial transport, and therefore functional differentiation. NaB treatment also induced a higher proportion of mucus-secreting cells, a reduced plating efficiency, and a retardation in growth. The changes in the later two parameters would be expected as the cells were treated with a mildly toxic concentration of NaB. Biochemical assays of brush border associated enzyme activities, before and after treatment, were not performed during their experiments, and the rationale for the exposure protocol was not clearly elucidated. It would appear that their finding may have been entirely fortuitous. However, the profound morphological changes were of great interest,

because prior to their publication only glucose-deprivation had been successful in causing such differentiation in HT-29 cells (Pinto *et al.*, 1982) and also this method would appear to be easier.

A later publication by Wice and his coworkers (1985) described the structural and functional enterocytic differentiation of HT-29 cells upon reaching confluency in the presence of 2 mM NaB. The cells were passaged for a minimum of four times before the cells were used for experimentation. Their results suggested that the accumulation of UDP-N-acetylhexosamines in undifferentiated HT-29 cells at confluency was preventing their differentiation and they showed that the levels of these nucleotide sugars were very low in HT-29 cells differentiated by glucose-deprivation and NaB. The markers of differentiation used in this study were limited to morphological changes and there were no biochemical changes measured, for example the activity of brush border associated enzymes. However, it was clear from their study that the cells had to be continuously exposed to the NaB and indeed they were passaging the cells in its presence.

An earlier study which shed some light on the biochemical changes that could be induced by NaB was performed by Herz and coworkers (1981). They demonstrated that exposure to 2 mM NaB caused an elevation in the activity of alkaline phosphatase in HT-29 cells. The baseline activity of alkaline phosphatase was found to be very low, but this could be increased 30 fold following a 48 hour incubation with NaB. Other interesting findings, were 1) the increased activity of the enzyme caused by placing the cells in hyperosmolar media (4 fold increase) and 2) the synergistic effects of combining NaB and hyperosmolality, which resulted in a 1,300 fold increase in enzyme activity. However, they did not undertake any morphological studies so the effects of NaB and hyperosmolality on morphological differentiation were not determined.

In view of the published data, it was hoped that treatment with NaB would provide us with a method of differentiating HT-29_1 cells in a controlled manner. This would enable us to study the effects that the use of combinations of differentiation inducers may have upon HT-29_1 differentiation. To this end the hypothesis that NaB could induce the enterocytic differentiation of HT-29_1 cells was tested.

5.I.ii Results

Control HT-29_1 cells grew readily on tissue culture plastic with a population doubling time of 24 hours (figure 16). The toxicity of NaB to these cells was determined by exposing the cells to a range of concentrations (1 - 20 mM). Cells were seeded on day 0 and allowed to attach for 24 hours. On day 1, exposure to the appropriate concentration of NaB began and was continued for 6 days. The period of exposure was set at 6 days because this covered the period of exponential growth of these cells and it was thought that the agent would have its maximal toxic effects during the phase of rapid cell growth. The toxicity was assessed by determining the number of cells that remained at the termination of the experiment. Figure 17 shows the concentration-response curve of HT-29_1 cells to NaB. The results show that there was a concentration-dependent reduction in cell number after the 6 days of treatment with the IC_{50} = 2 mM.

A second assay for assessing toxicity was also used. This was a clonogenic assay which aimed to determine the proliferative potential of the cells that remained after treatment. It was therefore important to determine whether or not HT-29_1 cells would grow as colonies when seeded into 6-welled plastic plates and the optimal conditions for this growth. These preliminary experiments yielded a mean plating efficiency (PE) for control HT-29_1 cells of 50.3 % (n = 6 ; sd = 12.4), and an optimal plating density of 2 x 10^2 cells/well, and 14 days incubation. However, when clonogenic assays were performed on cells treated with NaB, the control PE fell drastically to 6.9 % (n = 6 ; sd = 4.4). The values for the cells treated with NaB were as follows : 2 mM = 10.5 % (sd = 8.0); 5 mM = 12.5 % (sd = 1.2); and 10 mM = 15.8 % (sd = 4.6). These results would suggest that concentrations of NaB which cause 50 %, 79 %, and 95 % cell reductions (respectively), actually enhance cell clonogenicity to 152 %, 181 %, and 222 % (respectively), when compared to a standardized control PE of 100 %. This apparent enhancement of clonogenic potential by a cytotoxic agent was highly irregular and although an explanation for these results can not be offered it was a phenomenon that was seen throughout the study. A possible explanation may be that the process of cloning the cells was very stressful and only those cells already stressed by exposure to NaB were able to survive the cloning procedures. The control cells, having not been exposed to NaB, were still

highly sensitive to the stresses of cloning. However, Augeron and Laboisse (1984) had already found that 5 mM NaB could reduce the PE from the control value of 75 % , to 10.6 %, which was a more realistic result for a clonogenic assay. In addition, this explanation did not account for the more worrying finding of the sudden decline in control PE from the preliminary to the actual toxicity experiments. Later studies on the chemosensitivity of HT-29_2 cells also suffered from poor and variable control PEs and a possible reason for this was found which will be discussed fully in Part Three.

In order to determine the effects that NaB may have on cell growth, duplicate flasks of cells were incubated with and without 2mM NaB and cell numbers recorded at regular intervals during the period of cell growth. The concentration of 2 mM was selected because it caused only approximately 50 % cell death and was the concentration used by other investigators (Herz *et al.*, 1981; and Wice *et al.*, 1985). Also, the cells looked healthy after treatment with this concentration when observed at the LM and TEM levels (figure 18), which was not the case for cells treated with the higher concentrations. Figure 19 shows the results of this experiment, with the growth of cells being inhibited by exposure to 2 mM NaB increasing their population doubling time to 36 hours. These inhibitory effects were reversible as growth resumed at the control rate upon removal of the agent on day 7 and the cells reached a confluent cell density comparable to that of the controls.

A fluorescent microassay for alkaline phosphatase was used to determine whether or not 2 mM NaB could enhance the activity of this enzyme in treated HT-29_1 cells. Cells were incubated with the agent for 6, 10, 14 and 20 days and then assayed in triplicate for alkaline phosphatase activity. Figure 20 shows the enhancement of activity expressed as µmoles of product/mg of protein/min induced by 2 mM NaB. The enzyme activity in both control and treated cells remained relatively constant up until day 14, after which there was a considerable increase in activity in both groups. The differences in enzyme activity were increasingly significant as incubation time increased, with t-test analyses giving p values for each time point, as follows : day 6 = 0.138 ; day 10 = 0.007 ; day 14 = 0.000 ; and day 20 = 0.000. Unfortunately, this experiment was never repeated due

to the premature termination of work on these cells as a result of the discovery that they were infected (see section 3.I.vii).

Although NaB was seen to increase the population doubling time and enhance alkaline phosphatase activity in HT-29_1 cells, microscopical observations were not indicative of enterocytic differentiation of these cells. Intracellular lumen were observed in treated HT-29_1 cells (figure 18 (b)), but these were lined with poorly developed microvilli and the cells were not polarized. It was thought that these disappointing observations may have been due to the length of exposure to the agent, which although was long enough to cause growth inhibition and increased alkaline phosphatase activity, was not sufficiently long enough to produce morphological differentiation. Therefore, a final experiment was performed, in which the cells were cultured and passaged continuously in the presence of 2 mM NaB for 6 months. Cells were passaged, samples were processed for TEM and cells prepared for cryostorage every 10 days during the exposure period. Very little evidence of differentiation was seen at the LM level, with the only difference being that the treated cells were at a lower cell density on day 7 due to the growth inhibitory effects of NaB (figure 21). Following 9 passages in the presence of NaB, the effects on growth were rapidly reversed by the removal of the agent resulting in the cell density returning to almost control levels after 3 passages in control media, evidence of which can also be seen in figure 21. At the TEM level, changes in morphology were very similar to those seen during the shorter exposures to NaB, with the presence of intracellular lumen (figure 22 (b)) being the only significant change observed. There was still evidence of these lumen in those cells exposed for 9 passages followed by 3 passages in control media (figure 22 (c)). Figure 22 also shows the enhancement in alkaline phosphatase activity in the NaB treated cells, and the reduction in activity to control levels which occurred following removal of the agent. This qualitative data shows the same trends in alkaline phosphatase activity as the quantitative data for the shorter exposure experiments already shown in figure 20. It is therefore apparent that the increased exposure time to NaB has not affected the response of the cells with respect to growth retardation, alkaline phosphatase activity and morphological differentiation.

5.I.iii Discussion

This series of experiments, which were performed on the original batch of HT-29 cells ($HT\text{-}29_1$), aimed to determine whether of not NaB could induce enterocytic differentiation. If this had been the case I would have then had a way of studying the cellular changes that occur when these cells are differentiated in a controlled manner *in vitro* and also the effects of combinations of differentiation inducers on the differentiation observed.

The data has shown that NaB produced a typical concentration-response curve with an IC_{50} of 2 mM (figure 17). A concentration of 2 mM was selected for further study and was found to have a reversible, growth inhibitory effect on the cells. These cells retained a morphological appearance similar to the control cells at both the LM and TEM levels, even after passaging in the presence of NaB (figures 18, 21, and 22), with the appearance of intracellular lumen being the only significant observation. The agent was however successful in enhancing the activity of the brush border associated enzyme, alkaline phosphatase, as shown by preliminary biochemical data (figure 20) and by TEM histochemistry (figure 22).

Although, the elevation of alkaline phosphatase was significant in the butyrate-treated cells, the lack of morphological evidence of polarization and differentiation would suggest that this enzyme may be a very early marker of differentiation and thus it may be easily induced. This idea is supported by the earlier work of Herz and coworkers (1981), which showed that not only NaB, but also hyperosmolar media could increase its activity. However, these authors did not make any morphological observations so it can not be stated categorically that they did not achieve morphological differentiation with NaB and hyperosmolality. The fact that hyperosmolality could induce alkaline phosphatase activity may suggest that this enhancement was actually a response to the stress caused by a mildly toxic concentration of NaB and not a step in the pathway of differentiation. Considerable experimentation would be necessary to determine if that was in fact the case in HT-29 cells and was beyond the scope and aims of this research.

It was clear however, that the $HT\text{-}29_1$ cells did not respond to NaB in the same way as those used by Augeron and Laboisse (1984) or Wice *et al.* (1985). This variability of

results between different research groups was not entirely surprising and has also been seen in studies involving the measurement of metabolic changes during HT-29 differentiation, a subject discussed fully in the general introduction. An explanation for such variability may be the fact that the prolonged length of time that this cell line has been growing in tissue culture, since its original derivation in 1964, may have lead to the production of different 'strains' of HT-29 cells in individual research establishments. The production of these strains may have been the result of 1) deliberate subcloning which selected for specific subpopulations or 2) accidental selection by the pressures of tissue culture procedures which can vary from laboratory to laboratory.

The disappointing morphological results lead to the decision only to pursue this avenue of research as a means of showing the cell batch differences, when work commenced with the second batch of HT-29 cells ($HT\text{-}29_2$), after work on the $HT\text{-}29_1$ cells was prematurely terminated by the finding that they were infected. The work to be carried out on the $HT\text{-}29_2$ cells was to be of a limited nature and was not to involve a repeat of the 6 month continuous exposure experiment. The results of those studies will be discussed fully in section 6.I. The unusual results of the clonogenic assays will be discussed more fully in section 6.VIII where they will be put into context with further clonogenic results.

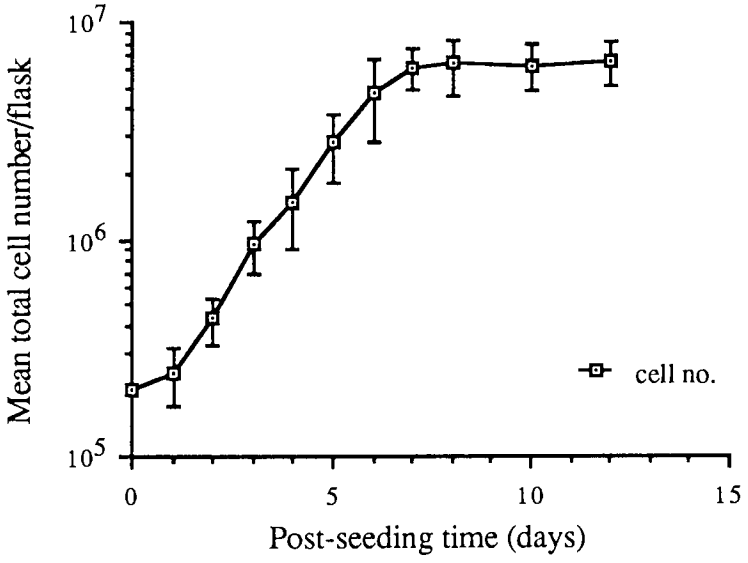


Figure 16 : Growth curve of HT-29_1 cells grown on tissue culture plastic. Values are the means from several experiments (n = 4 - 11 ; ± sd).

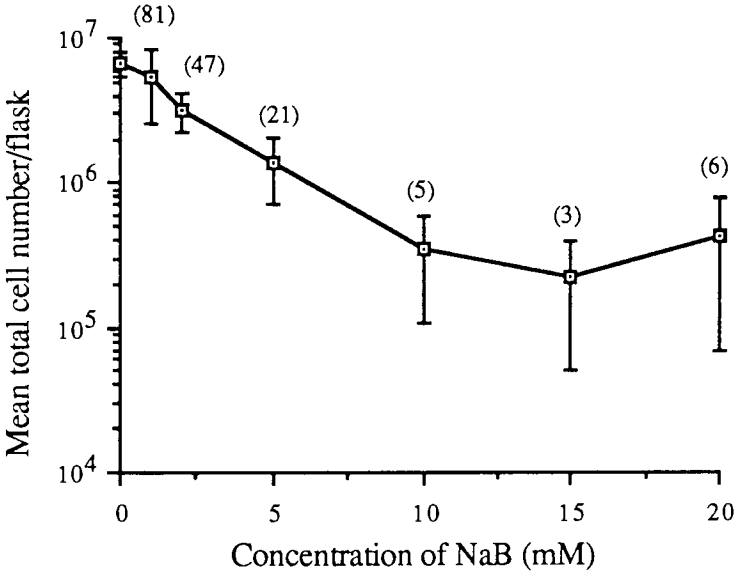


Figure 17 : Concentration-response curve of NaB toxicity to HT-29_1 cells for an exposure time of 6 days. Values are the means from several experiments (n = 4 - 6 ; ± sd). The numbers in parentheses are the values expressed as percentage of control (%).

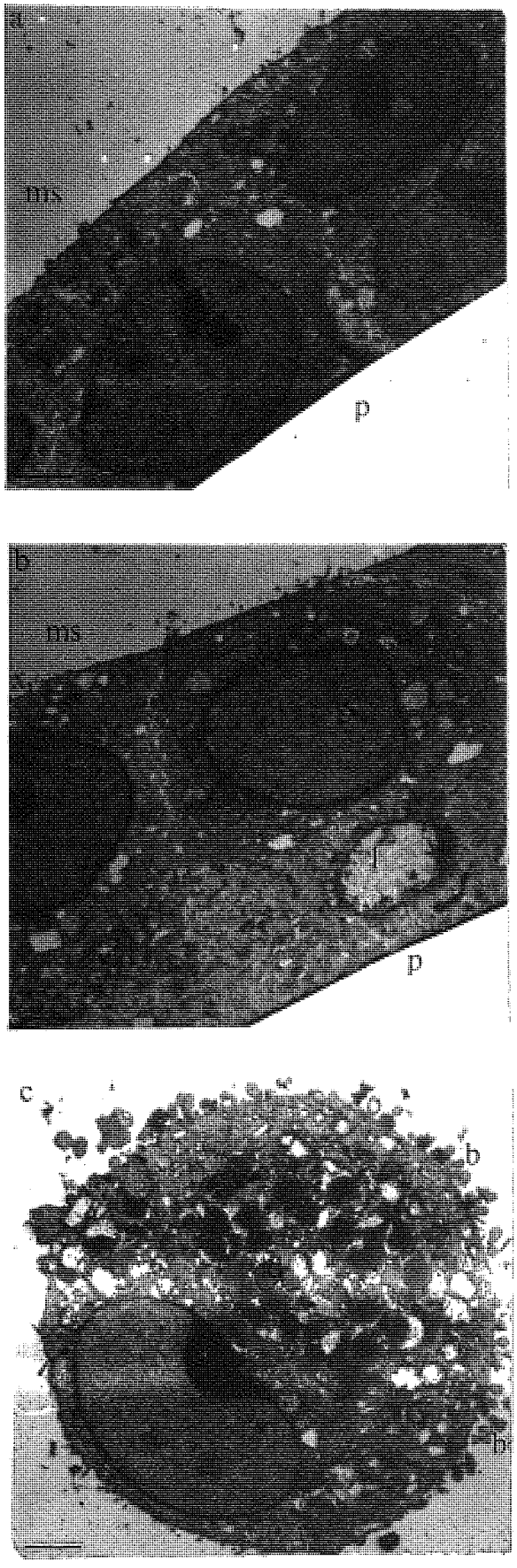


Figure 18 : Transmission electron micrographs of HT-29$_1$ cells treated with a) control media, b) 2 mM NaB, and c) 20 mM NaB for 6 days.
Plastic surface (p), media surface (ms), intracellular lumen (l), lipid droplets (ld), and blebs (b). *Scale bars*, 2μm.

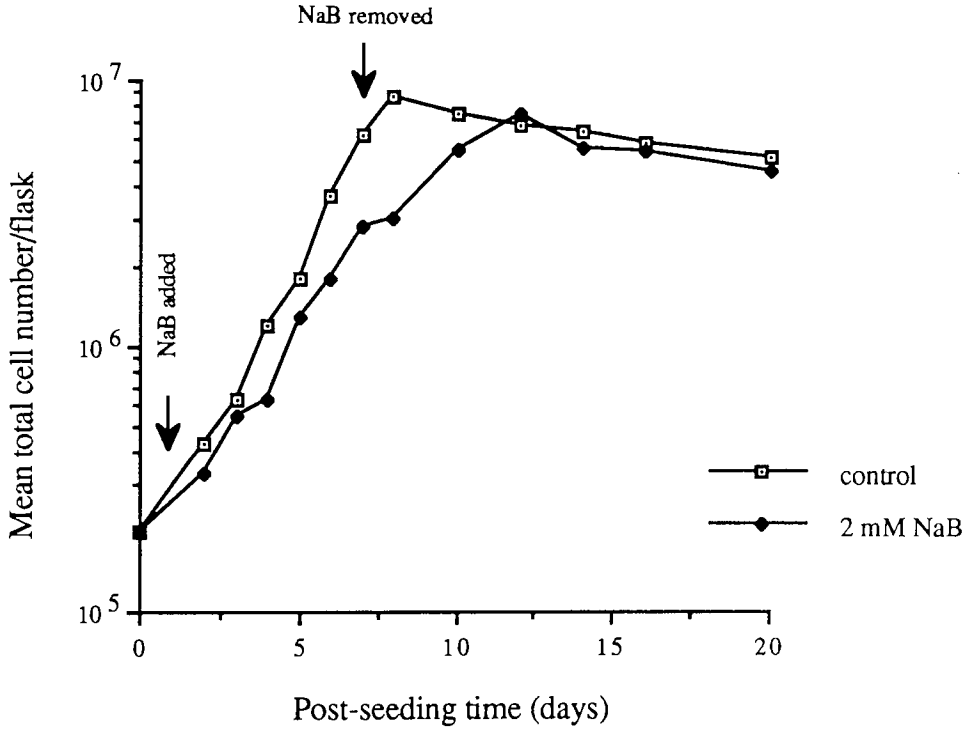


Figure 19 : Growth inhibitory effects of exposure of HT-29_1 cells to 2 mM NaB. Values are the means of 2 experiments only, as work was terminated on these cells before additional experiments could be performed. (n = 2)

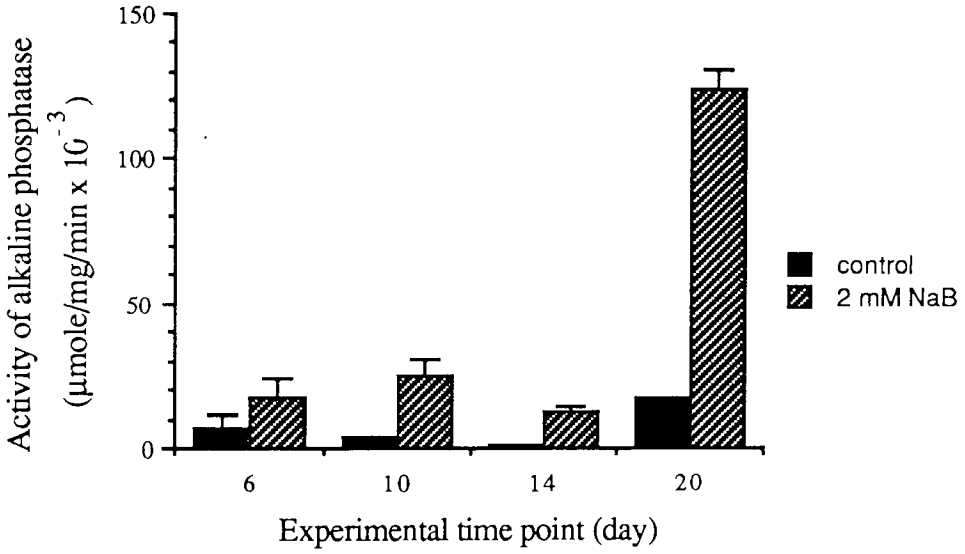


Figure 20 : NaB induced elevation in alkaline phosphatase activity of HT-29$_1$ cells. Values are the means of triplicate samples obtained during one experiment only, as work was terminated on these cells before additional experiments could be performed. Significance values from a t-test were : day 6 = 0.138; day 10 = 0.007; day 14 = 0.000; and day 20 = 0.000. (n = 3 ; ± sd).

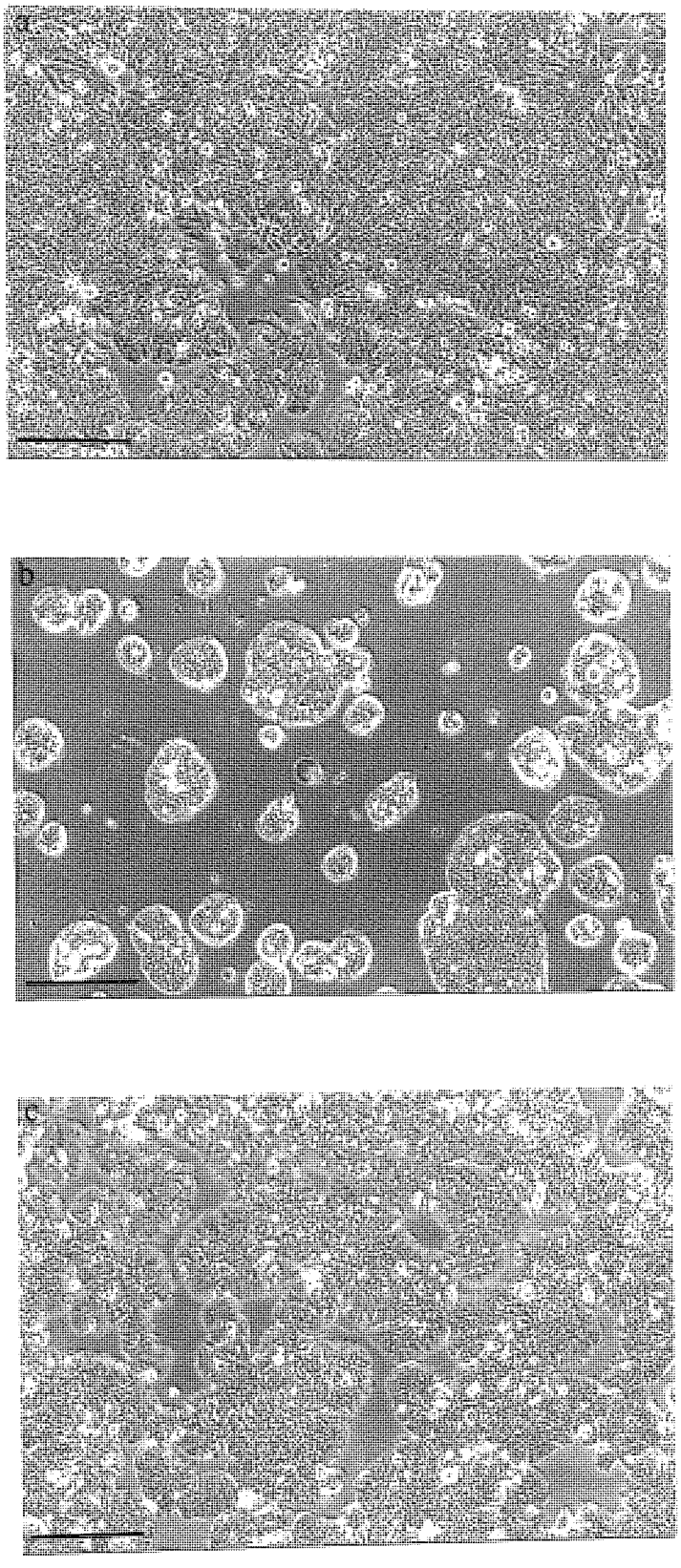


Figure 21 : Light micrographs of HT-29$_1$ cells grown in a) control media, b) 2 mM NaB for 120 days, and c) 2 mM NaB for 90 days followed by 30 days in control media. Photographs were taken 7 days post-seeding, and show the reversible, growth inhibitory effects of NaB exposure. *Scale bars*, 100μm.

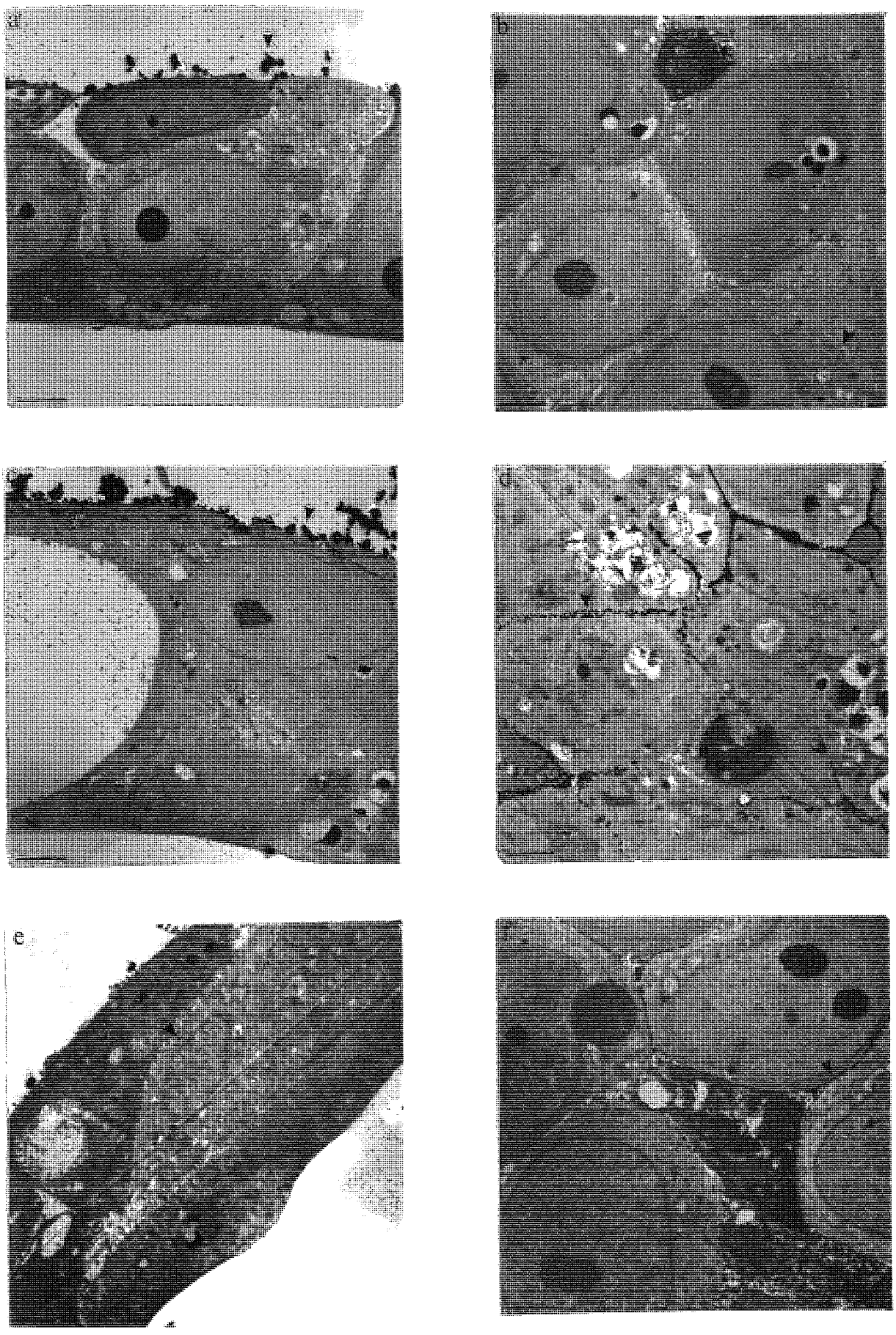


Figure 22 : Transmission electron micrographs of HT-29$_1$ cells stained histochemically for alkaline phosphatase activity.►a & b) control cells, c & d) cells exposed to 2 mM NaB for 120 days, and e & f) cells exposed to 2 mM NaB for 90 days, followed by 30 days in control media. a,c, & e = sectioned perpendicular to plastic surface, and b,d, & f = sectioned parallel to the plastic surface. *Scale bars*, 2μm.

5.II The effects of DMF on HT-29_1 cells

5.II.i Introduction

The closely related alkylformamides, N-methylformamide (NMF) and N,N-dimethylformamide (DMF), have both been shown to induce differentiation *in vitro*. NMF induces the granulocytic differentiation of the human promyelocytic cell line HL-60 (Richards *et al.*, 1988) and DMF has been shown to induce differentiation in a number of solid tumour cell lines, for example murine rhabdomyosarcoma cells (Dexter, 1977) and human colon carcinoma cells (Dexter *et al.*, 1979 ; Hager *et al.*, 1980). The studies of Dexter and his coworkers were extended into the *in vivo* situation with the study of the effects of polar solvents on the growth of human colon tumour xenografts (Dexter *et al.*, 1982). More recent work has suggested that differentiation induction by DMF may play a role in the anti-neoplastic actions of the drug (Van Dongen *et al.*, 1989).

In view of the success that DMF has had in differentiating solid tumour cell lines its effects on HT-29_1 cells were investigated, although only in the form of preliminary experiments due to the premature termination of work on these cells. The data has been included because, not only were the morphological results quite different to those obtained for NaB, but also because the toxicity data could be used to compare the first and second batch of HT-29 cells.

The aims of these experiments were to test the hypothesis that DMF could induce the enterocytic differentiation of HT-29_1 cells, a situation which has not been reported previously for these cells with this agent.

5.II.ii Results

Experiments were performed in order to determine the toxicity of DMF to HT-29_1 cells by exposing the cells to a range of concentrations of the drug for a period of 6 days. Figure 23 shows the concentration-response curve of cells to DMF and it gives an IC_{50} value = 72.5 mM. The cells were therefore more resistant to the toxic effects of DMF than NaB. Clonogenic assays were also performed, but because of the large inter-experimental variations in PEs and poor cloning of the control cells, this data was not of any value and will not be presented.

Light microscopical observations revealed interesting morphological changes in response to mild DMF treatment, with the cells becoming flattened and elongated. They had the appearance of fibroblast-like cells, which was indicative of a lack of enterocytic differentiation. Enzyme histochemistry for alkaline phosphatase at the LM level demonstrated little or no change in activity following exposure to DMF (data not shown). Unfortunately, biochemical assays and extensive TEM studies were not performed before the work on these cells was terminated.

5.II.iii Discussion

The data, although very preliminary would not appear to support the hypothesis that DMF could induce the enterocytic differentiation of HT-29_1 cells *in vitro*. DMF produced a typical dose response curve with the cells being more resistant to its toxic effects, when compared to NaB. The difference in response to DMF between the two batches of cells were minimal and will be discussed in section 6.I.

These studies provided us with enough information to make the decision not to pursue the work on the differentiation-inductive effects of DMF, when work commenced on the second batch of cells (HT-29_2). However, DMF like NaB was to be used as part of a panel of chemotherapeutic agents, which were to be used later to test chemosensitivity of HT-29_2 cells grown on different substrata. The assessment of toxicity was therefore necessary in the later studies, although its use as a potential differentiating agent was not pursued.

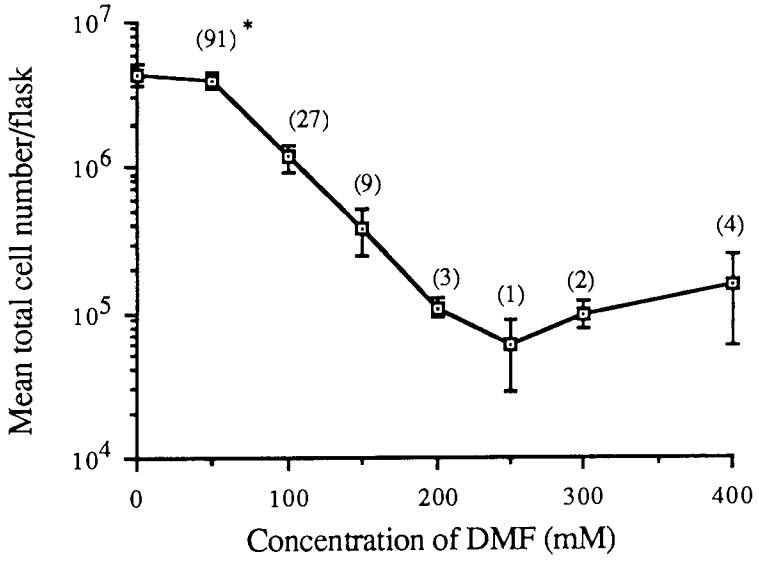


Figure 23 : Concentration-response curve of DMF toxicity to HT-29_1 cells.
Values are the means of several experiments (n = 2 - 6 ; ± sd). Numbers in parentheses are the values expressed as percentage of control (%). * n = 2.

5.III The effects of glucose deprivation on HT-29_1 cells

5.III.i Introduction

At the same time that the evaluation to the differentiation-inductive properties of the chemical agents were being assessed we were also exploring the possibilities of using metabolic manipulation as a driving force for differentiation induction. Since Pinto and her coworkers (1982) discovered that by simple manipulation of the media to replace glucose with galactose induced the enterocytic differentiation of HT-29 cells, this method has been used widely by several laboratories studying the various aspects of cell proliferation and differentiation. The research into why the source of hexose for these cells should have such profound effects on their ability to differentiate *in vitro* has been extensive and in some cases has resulted in the production of conflicting data from different laboratories. Such variations are assumed to result from the strain differences between the HT-29 cells used by different laboratories. However, in summary the data would suggest that a reduced glycolytic activity resulting from a reduction in hexose consumption and consequently a reduction in lactic acid production may be involved in the induction of differentiation. The data also indicates that an accumulation of UDP-N-acetylhexosamines in post-confluent HT-29 cells grown in the presence of glucose may be preventing their differentiation (Wice *et al.*, 1985). The important role that these nucleotide sugars play in protein glycosylation may implicate the involvement of aberrant protein processing in the prevention of HT-29 differentiation. Although the exact mechanism by which metabolic manipulation triggers enterocytic differentiation and its ultimate relevancy to the physiological situation in intestinal differentiation remain to be elucidated, it is still a valuable tool for controlling the differentiation status of HT-29 cells *in vitro*.

In view of the large collection of published data pertaining to glucose deprivation as an inducer of enterocytic differentiation several protocols were followed in order to test whether or not glucose deprivation could be used to induce the differentiation of HT-29_1 cells.

5.III.ii Results

Initially, a protocol for glucose deprivation was followed which involved the gradual replacement of glucose by galactose in the media above the HT-29_1 cells . This protocol had previously been shown to cause minimal cell death (Huet *et al.*, 1987). This method was repeated several times and each time the final transfer from media with a low concentration of glucose to that which contained only galactose (5 mM) resulted in almost total cell death and was completely unsuccessful.

A second method was developed which involved the use of the nucleoside inosine at a concentration of 2.5 mM as the replacement for glucose instead of galactose. The rationale for this change in carbon source was that the lack of success with galactose was probably due to the inability of this particular culture of HT-29 cells to utilize this hexose. As previous work had shown that nucleosides like inosine and uridine could support vertebrate cell growth *in vitro* (Wice et *al.*, 1981), it was thought that inosine may prove to be a usable carbon source for the HT-29_1 cells and thus warranted investigation.

The introduction of confluent cells either gradually, or in one step into the glucose-free media resulted in almost total cell death and proved to be as unsuccessful as the galactose protocol. However, the few remaining attached cells were not left long enough to determine their eventual fate which in light of the results to follow may have proved very interesting.

It was discovered that by seeding cells directly into the glucose-free media (2×10^5/flask) (which still resulted in extensive cell death) individual raised (domed) colonies which were visible to the naked eye were produced. However, the production of colonies took at least 3 -4 weeks and although they were raised in the centre as seen by phase contrast microscopy (figure 24), these were not true domes representing transepithelial transport, because TEM observations showed that the raised areas were multilayers of cells. Also, only approximately 25 colonies grew in each of 10 flasks and if it is assumed that each colony arose from a single cell, it follows that there was only 0.013 % cell survival as a result of glucose deprivation. Typical colonies are shown in figure 24.

Samples of these colonies were processed for TEM after 52 days in culture and the morphological differentiation of the cells was found to depend upon their position within

the colony. Cells which were growing within the central, raised area were generally undifferentiated and showed evidence of necrotic cell death. This necrosis was probably due to the reduction in nutrient transfer from the media to the inner cells. The central areas also contained intercellular lumen, some extremely large, surrounded by polarized epithelial cells. The raised areas were therefore not true 'domes' which are characteristically produced by differentiated cells lines as a result of transepithelial transport. The outer rims of the colonies produced cells with the most advanced morphological differentiation (figure 25). These cells exhibited well-developed features of enterocytic differentiation, being polarized with distinct apical micro-villus lined membranes. Adjacent cells in these polarized cell sheets were separated by tight junctions and attached by well formed desmosomes (figure 26). These results compared favourably with previous work on the differentiation of the HT-29 cell line in response to glucose deprivation (Pinto *et al.*, 1982 ; Zweibaum *et al.*, 1985 ; and Huet *et al.*, 1987).

In order to study these cells more fully it was important to have a population of cells that not only grew in the glucose-free media, but also could be passaged successfully without too much cell loss. It was hoped that once this had been achieved the cells would have been able to be switched from one media to the other and thus from an undifferentiated state to a differentiated state, and vice versa, a situation which had been achieved by the other workers in this field. However, when an attempt was made to passage the cells from these colonies, it proved to be unsuccessful and the cells seeded into the new flasks did not grow. The cells may have had a reduced clonogenic potential because of their differentiated state, or there may not have been a large enough number of cells seeded to support cell growth. Therefore, it was not possible to develop a passaging population of HT-29_1 cells whose differentiation status could be manipulated by altering the nutrient supply. Repeated attempts were made to produce the colonies of differentiated cells by seeding cells directly into glucose-free media, but these were also unsuccessful.

5.III.iii Discussion

It is apparent from the data presented in this section that glucose deprivation was an effective inducer of enterocytic differentiation in HT-29_1 cells. However, the practical problems also discussed in the results section would suggest that this method of inducing differentiation is by no means straight forward or as simple as the existing publications imply. Although the success of this method was rather erratic the cause of this was thought to be more likely a manifestation of the cells themselves and not the method. In light of the later finding that these cells were infected during the entire course of experimentation, the former would seem the most likely cause of the variability in response to glucose deprivation. Glucose deprivation was therefore still considered a potential method for differentiating HT-29 cells when work on the new batch of infection free cells (HT-29_2) was commenced.

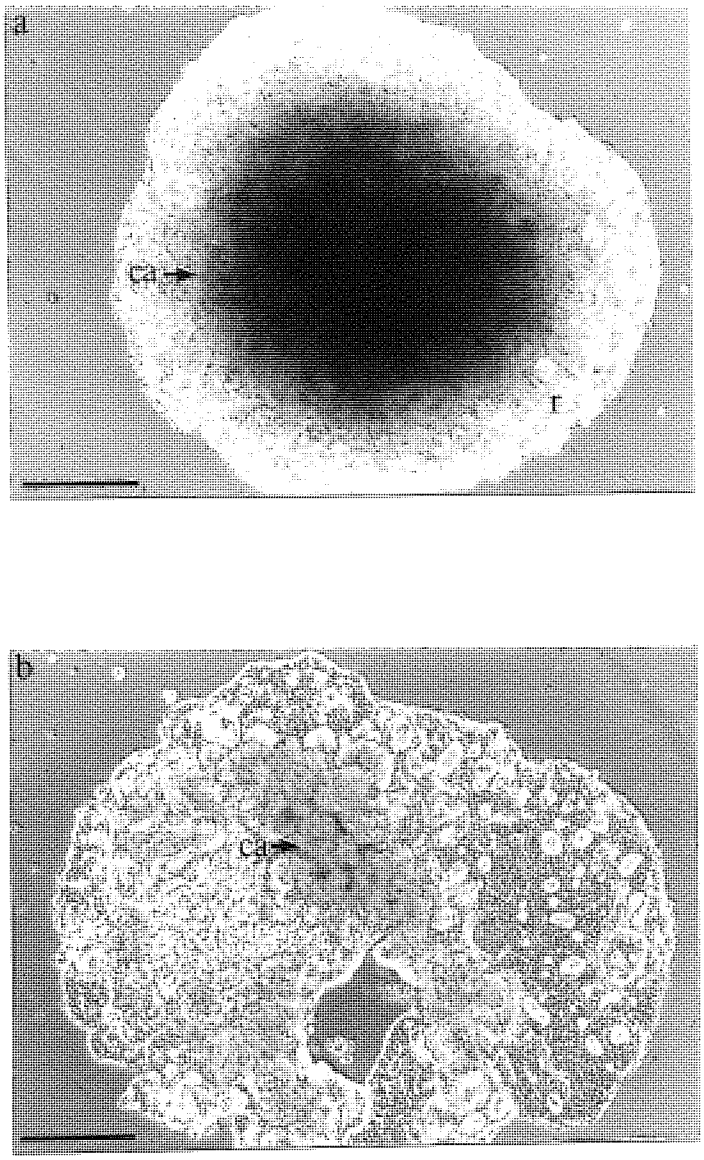


Figure 24 : Light micrographs of colonies of HT-29_1 cells produced by glucose deprivation. a) a typical 'domed' colony , with its raised central area (ca) and outer rim of cells (r), b) a less typical colony showing mixed cellularity and raised areas (ca). *Scale bars*, 100µm.

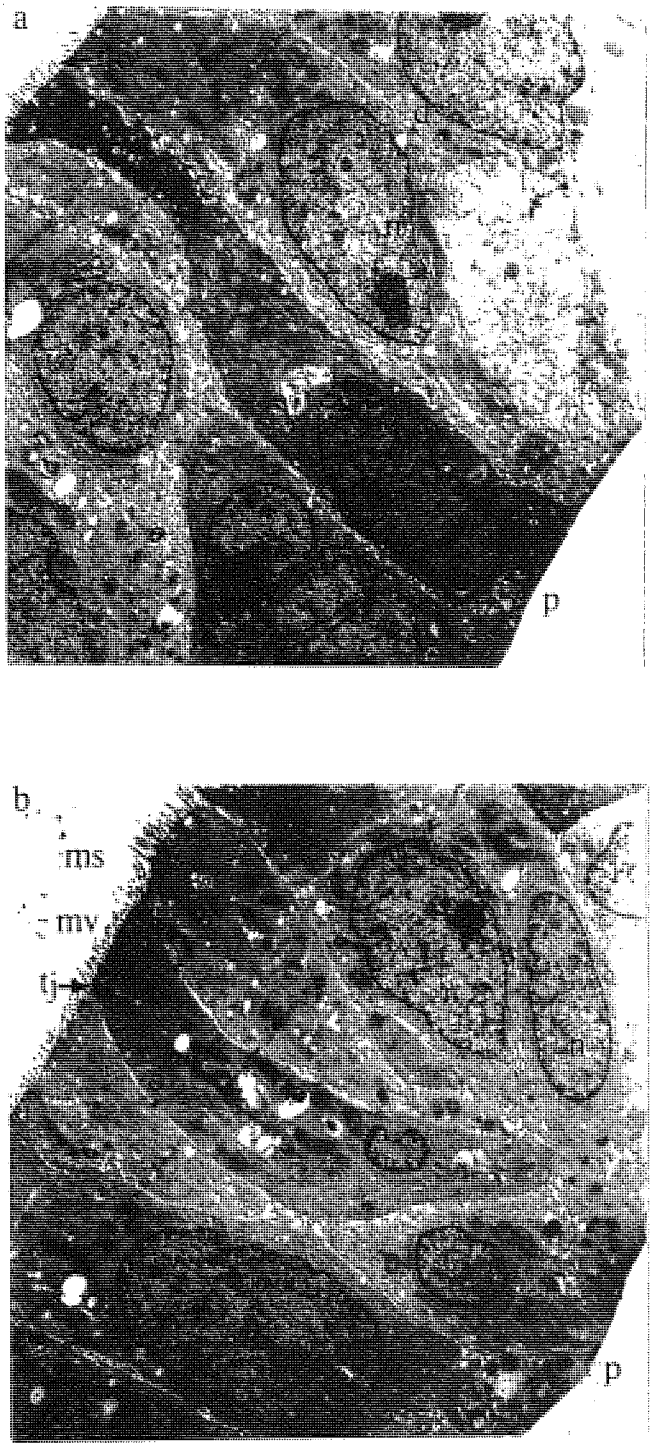


Figure 25 : Transmission electron micrographs of differentiated HT-29$_1$ cells.
a and b) From the outer rim of a typical 'domed' colony grown in glucose-free media for 52 days, showing well polarized cells, with basolaterally situated nuclei (n), apical microvilli (mv), tight junctions (tj), and desmosomes (d). Plastic surface (p) and media surface (ms). *Scale bars*, 2μm.

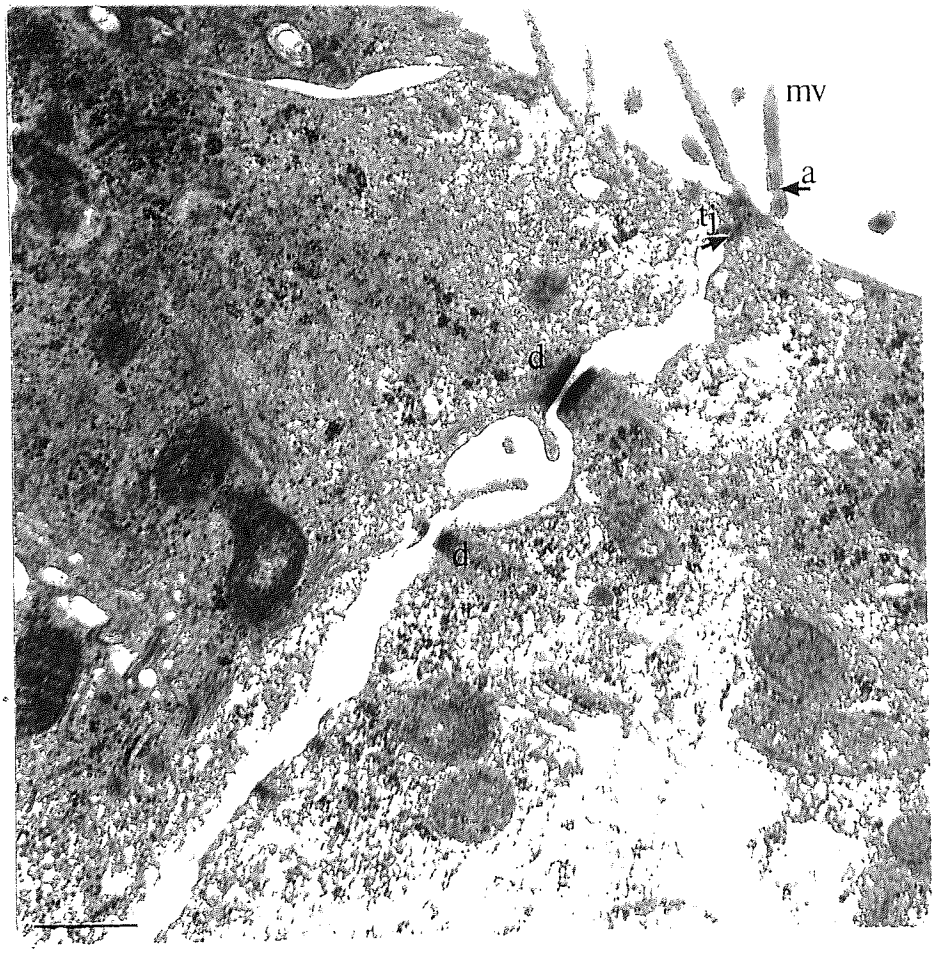


Figure 26 : Transmission electron micrograph of two adjacent HT-29_1 cells.
Cells are from the outer rim of a typical 'domed' colony grown in glucose-free media for 52 days. Their apical surfaces display well-developed microvilli (mv), containing actin filaments (a) which have reached deep into the cytoplasm of the cells. A tight junction (tj), and desmosomes (d) can be seen between the two cells. There has been some artifactual shrinkage of the cells resulting in a separation of the desmosomes. *Scale bar,* 0.25μm.

PART THREE Studies on the induction of differentiation of the second batch of HT-29 (HT-29_2) cells *in vitro*.

SECTION 6 : RESULTS AND DISCUSSION

SECTION 6 : RESULTS AND DISCUSSION

6.I The effects of sodium butyrate and DMF on HT-29_2 cells

6.I.i Introduction

Although the decision was made not to pursue the differentiation inductive effects of NaB and DMF on the second batch of infection-free cells these agents were to be used as part of a panel of chemotherapeutic agents for chemosensitivity screening. It was therefore necessary to determine the toxicity of these two agents on the HT-29_2 cells. In order to achieve this initial experiments were performed which determined the toxicity of these agents by producing concentration-response curves over a range of concentrations. Similar experiments for the other agents used will be discussed in section 6.VIII.

The growth inhibitory effects and the induction of alkaline phosphatase by NaB were also determined in order that the responses of the new cells could be compared with those of the original cells (section 5.I). These experiments would be able to show any differences that may have existed between the two batches of HT-29 cells (HT-29_1 and HT-29_2).

6.I.ii Results

The population doubling time of the HT-29_2 cells was regularly checked by carrying out growth curve determinations each time cells were resurrected from the cell bank. Cells were used for a maximum of 10 passages and then a new supply of early passage cells were resurrected. Figure 27 shows the mean results of all of the growth curves performed on the HT-29_2 cells during the period of experimentation which is described in Part Three. Figure 27 was used to determine the population doubling time which was 28 hours. This value was very similar to that observed for the HT-29_1 cells and to that already published (Pinto *et al.*, 1982).

The toxicities of NaB and DMF were determined as for the HT-29_1 cells and figures 28 and 29 show the concentration-response curves for each agent, respectively. The IC_{50} values were : NaB = 1.9 mM and DMF = 80.8 mM. Differences were observed between the sensitivities of the two batches of HT-29 cells. In response to NaB HT-29_2 cells were generally more sensitive than HT-29_1 cells, particularly at the concentrations of 5,

10, and 15 mM (table 2). However, in response to DMF there was very little difference between the two batches until the higher concentrations of 200 mM and above were reached. At these concentrations the actual percentage survival was slightly lower in the HT-29_2 cells (table 3).

Clonogenic assays were more successful with this second batch of cells and the effects of NaB on the clonogenicity are illustrated in figure 30 and those for DMF are in figure 31. The results show that the mean control plating efficiencies were relatively high at 46 % and 44 % respectively. NaB caused a gradual decline in PE with the increasing concentrations used, although the toxic effects on clonogenicity were not as significant as they were on the actual cell survival (table 2 column 4). For example, a concentration of 15 mM NaB caused a reduction in cell survival of 99.5 %, but the remaining 0.5 % of original cells had a 46 % PE when compared to the controls (standardized to 100 %). The results with DMF were even more interesting because at a concentration of 150 mM, which caused a 92 % reduction in cell survival (table 3 column 4), the PE was still 98 % of the control value (figure 31). It was only at the extremely toxic concentration of 250 mM (99.7% reduction in cell survival) was the PE reduced to 5 % of the control. However, problems with the cloning of HT-29_2 cells were encountered later in the project and the possible cause and procedures for rectification will be discussed fully in section 6.VIII. The outcome of such troubleshooting with the HT-29_2 cells will shed light on the problems encountered previously with the often poor and variable cloning of HT-29_1 cells which were at the time unexplained.

The growth inhibitory effects of exposure to 2 mM NaB were determined as for the HT-29_1 cells and are presented in figure 32. This concentration was selected for investigation to maintain consistency between this experiment and that which was performed on the HT-29_1 cells. NaB reduced the population doubling time to 40 hours, a value which compares favourably with that determined for the first batch of cells. Again, it was seen that the effects were reversible upon removal of the agent on day 7 with the cells reaching a confluent cell density comparable to that of the control cells.

The inductive effects of NaB on the activity of the brush border associated enzyme, alkaline phosphatase are presented in figure 33. The levels remained relatively constant

over the time period assayed, with the activity of the treated cells being consistently greater than the control cells. The results were significantly different for each time point, giving significance values in t-test analyses as follows : day 6, P = 0.038 ; day 8, P = 0.000; day 11, P = 0.046. The changes in activity observed in response to NaB were very similar to those seen in the preliminary experiments carried out on the HT-29_1 cells with the control activity being comparable for both batches of cells. However, the activities of treated cells although significantly elevated in both batches were 10 fold greater in the HT-29_2 cells. It would appear from these results, combined with those of the toxicity experiments, that an increased sensitivity to the toxic effects of NaB correlates positively with an increase in its inductive effects on alkaline phosphatase activity.

6.I.iii Discussion

Both agents were shown to be toxic to the HT-29_2 cells although the clonogenic assays indicated that the effects on clonogenicity were minimal, particularly in the case of DMF. Even though toxic concentrations were used (10 mM NaB and 150 mM DMF) the remaining cells (2 % and 17 % of control, respectively) were still highly clonogenic (46 % and 98 % of control plating efficiency, respectively). Obviously these results are particularly pertinent when relating *in vitro* data to situations experienced in the clinic and when developing novel chemotherapeutic agents. A drug is useless if after it has killed a large number of the malignant cells, it leaves untouched, the resistant, highly clonogenic cells, which can then go onto repopulate the tumour and possibly metastasise with greater efficiency than the untreated cells. Poor advancements in the therapeutic efficacy of chemotherapeutic agents used in the clinic over the last 40 years despite the improvements made in both hospital care and early detection may be explained by the idea that some chemotherapeutic agents can promote malignant behaviour of the residual population of cells following treatment (Kerbel and Davies, 1982; McMillan and Hart, 1987). Drug-induced enhancements of malignant properties have been observed in both *in vivo* models (Lazo *et al*, 1978; McMillan *et al*, 1986; Takenaga, 1986) and *in vitro* clonogenicity models (Vichi and Tritton, 1989; Jones and Taylor, 1980). These ideas

will be discussed more fully together with the results of the chemosensitivity experiments presented in section 6.VIII.

This section has also illustrated the differences between the HT-29_1 cells and the HT-29_2 cells with regards their sensitivity to NaB and the induction of alkaline phosphatase activity. An increased chemosensitivity to NaB seemed to correlate positively with an enhancement in the increased alkaline phosphatase activity of the HT-29_2 cells. As the results for the HT-29_1 cells suggested that the increase in alkaline phosphatase activity was not associated with morphological differentiation, although it is found in the brush border of mature enterocytes and is used routinely as a functional marker, the increase in activity may be a simple response to a chemical stress. This idea may also explain the earlier findings of Herz *et al.*, (1981) which showed that hyperosmolality, a stressful event in itself could also cause an elevation in alkaline phosphatase activity in HT-29 cells. Unfortunately it was not within the scope or aims of this research to investigate the effects of stress on alkaline phosphatase activity in HT-29 cells. However, it would seem to be an area worth investigating in the future because the stress effects caused by the exposure of malignant cells to chemotherapeutic agents may have profound effects on their subsequent properties. If a toxic stress event can induce the enhanced expression of a simple housekeeping protein like alkaline phosphatase, what may it do to the expression of other proteins which are more important in that they may enhance the malignant properties of those treated tumour cells ? This may explain our clonogenic data and also data produced by other laboratories which suggest that treatment with some chemotherapeutic agents seem to enhance the metastatic ability of the primary tumour (Kerbel and Davies, 1982; McMillan *et al.*, 1986). Of course this is not a situation which is either desired or expected in the clinic, but it may explain the poor advancements made in cancer chemotherapy to date (McMillan and Hart, 1987). Further research into the cellular response to chemotherapeutic agents and the consequences of those responses with regard to cell survival and malignant potential are therefore very important.

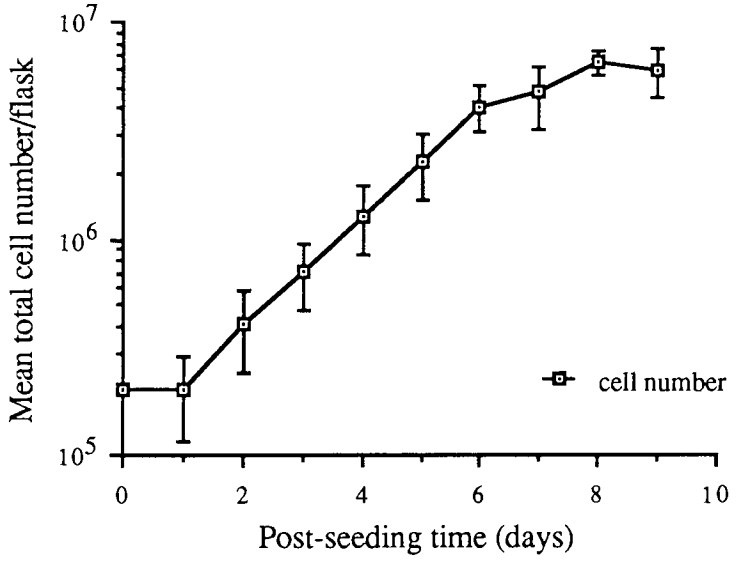


Figure 27 : Growth curve of HT-29_2 cells grown on tissue culture plastic. Values are the means from several experiments (n = 3 - 10 ; $\pm$ sd).

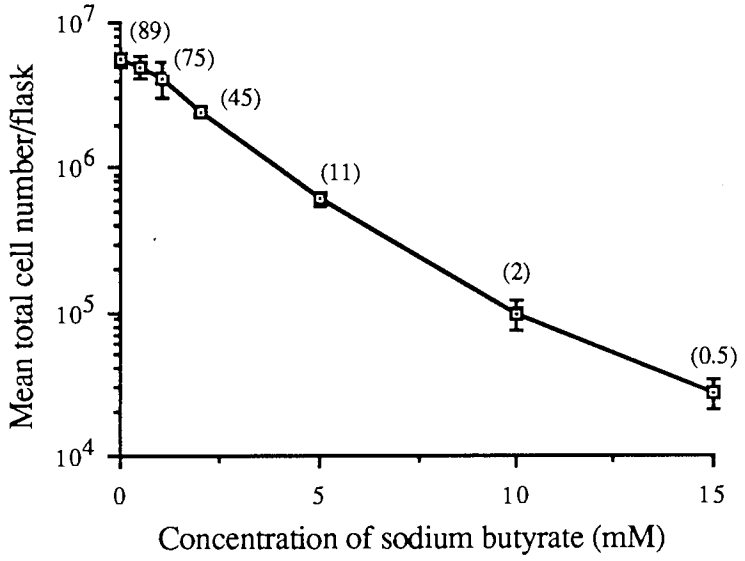


Figure 28 : Concentration-response curve of NaB toxicity to HT-29_2 cells for an exposure time of 6 days. Values are the means from several experiments (n = 9 ; $\pm$ sd). The numbers in parentheses are the values expressed as percentage of control (%).

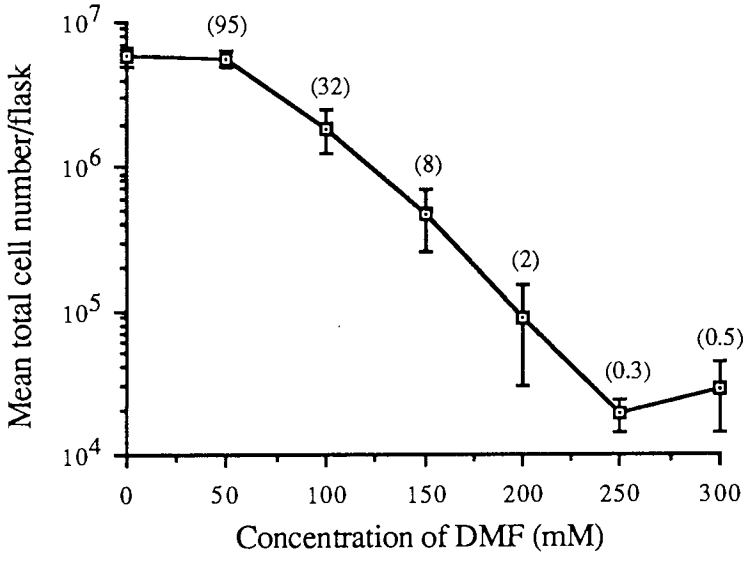


Figure 29 : Concentration-response curve of DMF toxicity to HT-29_2 cells for an exposure time of 6 days. Values are the means from several experiments (n = 9 ; ± sd). The numbers in parentheses are the values expressed as percentage of control (%).

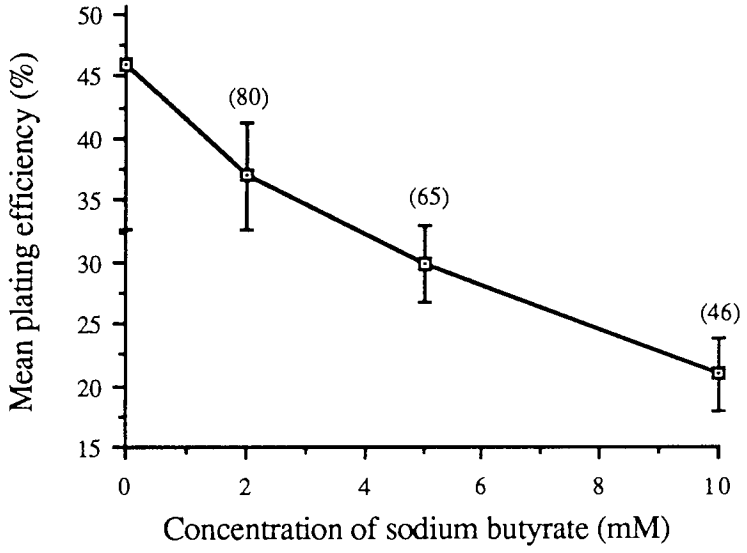


Figure 30 : Clonogenic analysis for HT-29_2 cells treated with NaB for 6 days.
The experiment was performed in triplicate, with five or six separate values for the plating efficiency of each treatment group (n = 15 - 18 ; ± sd).
The numbers in parentheses are the values expressed as percentage of control (%).

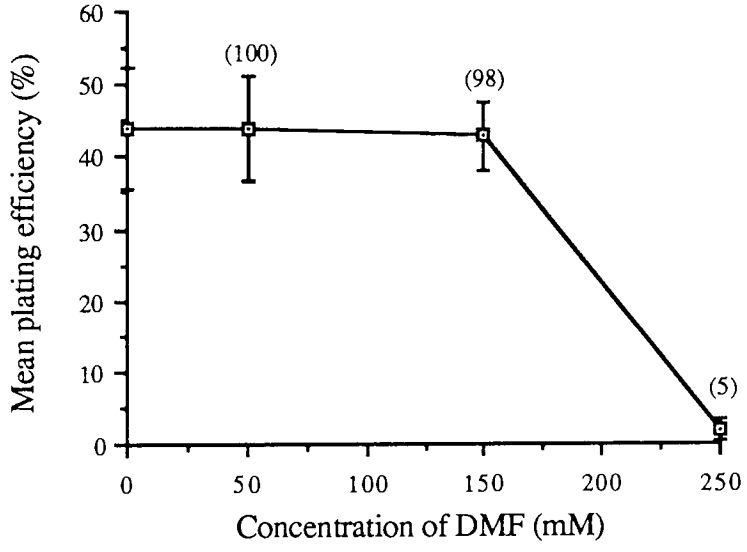


Figure 31 : Clonogenic analysis for HT-29_2 cells treated with DMF for 6 days.
The experiment was performed in triplicate, with four or five separate values for the plating efficiency of each treatment group (n = 12 = 15 ; $\pm$ sd).
The numbers in parentheses are the values expressed as percentage of control (%).

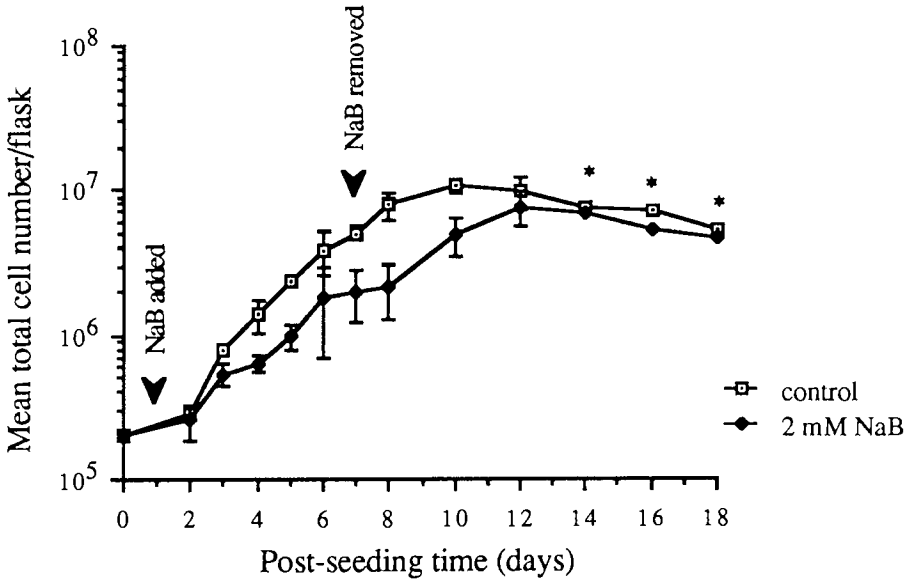


Figure 32 : Growth inhibitory effects of exposure of HT-29_2 cells to 2 mM NaB. Values are the means of several experiments (n = 4 : $\pm$ sd). * n = 1 value only.

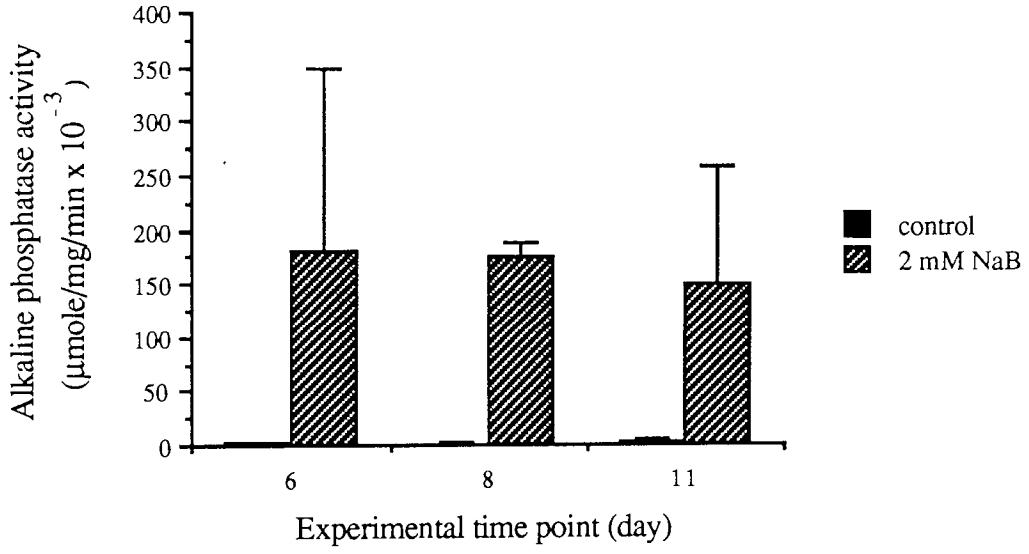


Figure 33 : NaB induced elevation in alkaline phosphatase activity of HT-29$_2$ cells. Values are the means of 2 experiments, each being performed in triplicate (n = 6; ± sd). Significance values from a t-test analysis were : day 6 = 0.038 ; day 8 = 0.000 ; and day 11 = 0.046.

Concentration of NaB (mM)	HT-29_1 cells * Mean cell no.	HT-29_2 cells ** Mean cell no.	HT-29_1 cells % of control	HT-29_2 cells % of control
0	6.8×10^6	5.6×10^6	100	100
1	5.5×10^6	4.2×10^6	81	75
2	3.2×10^6	2.5×10^6	47	45
5	1.4×10^6	6.2×10^5	21	11
10	3.5×10^5	1.0×10^5	5	2
15	2.3 x 105	2.8 x 104	3	0.5

Table 2 : Toxicity of NaB to the two batches of HT-29 cells.
Mean cell no. = mean total cell number/flask after treatment ; * n = 4 - 6 ; ** n = 9.

Concentration of DMF (mM)	HT-29_1 cells * Mean cell no.	HT-29_2 cells ** Mean cell no.	HT-29_1 cells % of control	HT-29_2 cells % of control
0	4.4×10^6	5.9×10^6	100	100
50	4.0×10^6	5.6×10^6	91	95
100	1.2×10^6	1.9×10^6	27	32
150	3.9×10^5	1.0×10^6	9	8
200	1.1×10^5	9.1×10^4	2.5	1.5
250	6.0×10^4	1.9×10^4	1.4	0.3
300	1.0×10^5	2.9×10^4	2.3	0.5

Table 3 : Toxicity of DMF to the two batches of HT-29 cells.
Mean cell no. = mean total cell number/flask after treatment ; * n = 2 - 6 ; ** n = 9.

6.II The effects of glucose deprivation on HT-29_2 cells

6.II.i Introduction

Although the results from the work on the HT-29_1 cells were rather erratic, when cell survival and growth did occur after glucose deprivation the TEM data revealed very profound morphological differentiation (see section 5.III.). It was thought that the erratic nature of the success of glucose deprivation with the HT-29_1 cells was probably a manifestation of their infection. Glucose deprivation therefore still remained a potentially viable method of differentiating HT-29 cells when work commenced on the second batch of cells HT-29_2. In light of the earlier finding of the failure of galactose to support the growth of HT-29_1 cells, a decision was made to use inosine as the substitute for glucose, at a concentration of 2.5 mM. Extra non-essential amino acids were also added to the media, with the aim of helping to support cell growth.

The aim of the experiments detailed in this section, was to determine whether or not glucose deprivation was a feasible, and practical method of differentiating HT-29_2 cells *in vitro*.

6.II.ii Results

A successful protocol was followed which involved the switching of confluent HT-29_2 cells grown in glucose containing media (25 mM) by a single step into glucose-free media (inosine 2.5 mM). Cell death did occur in the first few weeks following transfer, but eventually this slowed down leaving a population of healthy cells. Cells were re-fed every 2 days to ensure that the media did not become exhausted which may have resulted in extra cell death. When the cell population stabilized cells were passaged, a procedure which was repeated approximately every 2 weeks. The cells grew at a much slower rate with a population doubling time of 36 hours (figure 34) than cells grown in glucose containing media. When the cells reached confluency cell death was very rapid. It was also necessary to seed the cells at a higher density (two times higher than controls) when they were passaged as not all of the cells would attach and survive. Figure 34 shows the initial lag phase caused by the considerable cell death which occurred when cells were seeded at 2 x 10^5 cells/flask. The plating efficiency of these cells was also drastically

reduced to 1.8 % of control PE (n = 12) and these results are presented in figure 35. The LM observations of these cells revealed that their appearance was very similar to that produced by the successful glucose deprivation of HT-29_1 cells, with them forming 'domed' colonies (figure 36 (a)). As the cells grew in culture they displayed an unusual morphology, with the appearance of glassy-like vacuoles (figure 36 (b)) not seen in cells grown in control media.

Samples of cells were regularly fixed and processed for TEM and the ultrastructural changes in morphology were initially suggestive of enterocytic differentiation (figure 37), but it became evident as passaging continued that overt enterocytic differentiation was not occurring (figure 38). The pre-passage and early passage cells exhibited numerous features of enterocytic differentiation, displaying microvilli lined intercellular lumen, tight junctions and desmosomes (figure 37). The later passages (P+8 onwards) still displayed very well developed desmosomes, but the occurrence of intercellular microvilli lined lumen was less frequent. The cells also became extremely elongated, lying parallel to the plastic surface (figure 38 (a)). These cells showed little evidence of enterocytic differentiation, and the activity of alkaline phosphatase was very low and was associated with sporadic microvilli and occasionally with nuclei (figure 38 (b)). It was thought that the staining seen in the nuclei was due to the presence of phosphatases which are thought to work in conjunction with the phosphokinases in the nucleus.

The general low level of alkaline phosphatase activity was confirmed by the use of a biochemical assay on cells from passage 10 onwards. Cells were assayed at similar phases in their growth, which was early confluency rather than on a particular post-seeding day to take into account the different growth rates. This was important because the expression may be dependent upon the phase of growth, although earlier assays had shown that the activity of control HT-29_2 cells remained fairly constant over 11 days (see section 6.I). The data is shown in figure 39 and shows that the glucose deprived cells had a significantly lower activity of alkaline phosphatase when compared with cells grown in glucose containing media, with a t-test analysis giving a significance value of 0.000.

6.II.iii Discussion

This work has shown that it was possible to not only to grow HT-29_2 cells under conditions of glucose deprivation but also to passage them. It was necessary to seed the cells at higher starting densities and the cells had severely reduced plating efficiencies. However, the cells maintained their clonogenic potential, as shown in figure 35 when plated in glucose-containing media. There had not therefore been a permanent reduction in their clonogenic potential caused by glucose deprivation. The lack of glucose either directly reduced their proliferative potential by decreasing their glycolytic activity, or indirectly by reducing their production of autocrine growth factors or their corresponding receptors, possibly by interfering with protein glycosylation as already suggested by Wice *et al* (1985). The growth changes observed in HT-29_2 cells were obviously readily reversible and their low proliferative potential was unlikely to be due to terminal differentiation.

The early TEM data was encouraging (figure 37) as it clearly demonstrated morphological evidence of enterocytic differentiation of the HT-29_2 cells. However, the morphological appearance of these cells has drastically changed by the later passages resulting in elongated cells which grew with an orientation that was parallel to the plastic surface (figure 38). They were considerably different to the cells grown in glucose containing media (see section 4.I, figure 7) and were definitely not enterocyte-like. Biochemical and histochemical analyses of these later passage cells demonstrated very low levels of alkaline phosphatase activity compared with the already low levels of control HT-29_2 cells (figures 38 (b) and 39).

In summary, the method was successful in developing a population of HT-29_2 cells which could survive glucose deprivation and not only grow, but also could withstand passaging continuously in glucose-free media. Unfortunately, although these cells underwent a morphological transformation it was not that of enterocytic differentiation. In light of the results, a decision was made not to proceed with work on these cells using this method of inducing differentiation. It had become clear during the course of this work and during that involving the HT-29_1 cells that the successful outcome of differentiation studies depended as much on the strain of HT-29 cells used as on the

inducing agent used. An attempt was made to secure a batch of HT-29 cells from a laboratory which had been successful in inducing differentiation by glucose deprivation but unfortunately this was unproductive. Therefore, after extensively reviewing the existing literature regarding the induction of differentiation *in vitro* work commenced on the HT-29_2 cells which involved studying the effects of growth on type I collagen gels. The rationale behind these studies and the results that they yielded are the subject of the remaining results sections of this thesis.

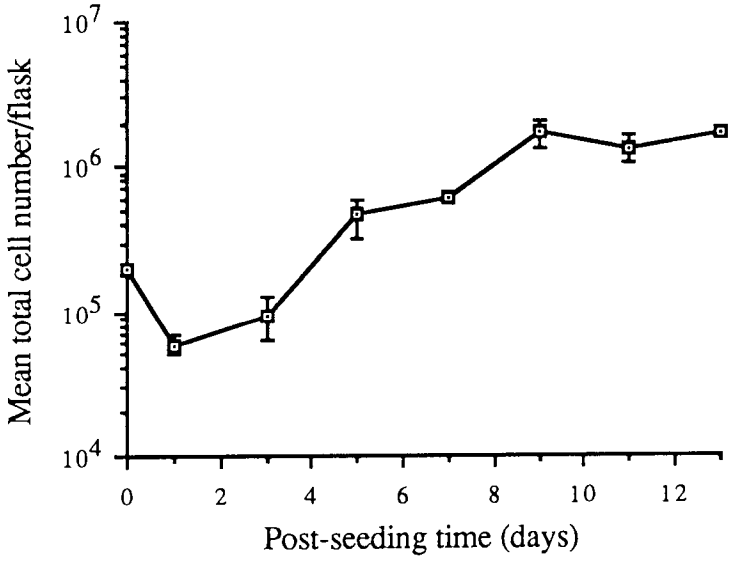
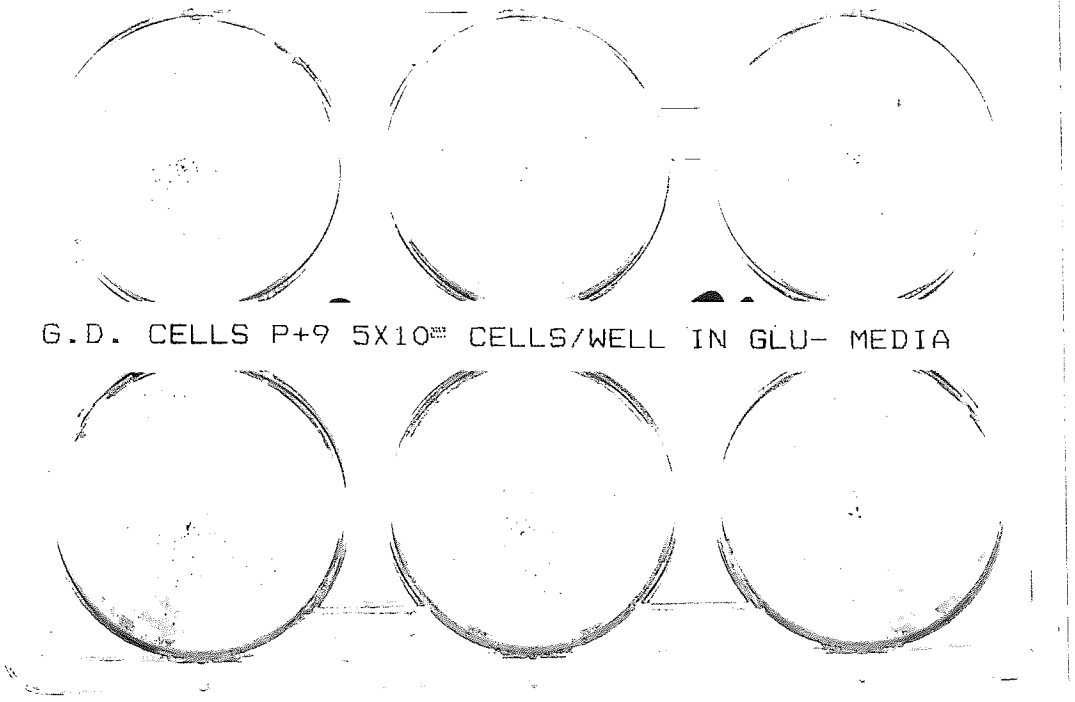


Figure 34 : Growth curve of HT-29_2 cells grown in glucose-free media. Values are the means of three experiments (n = 3 ; ± sd).

a



b

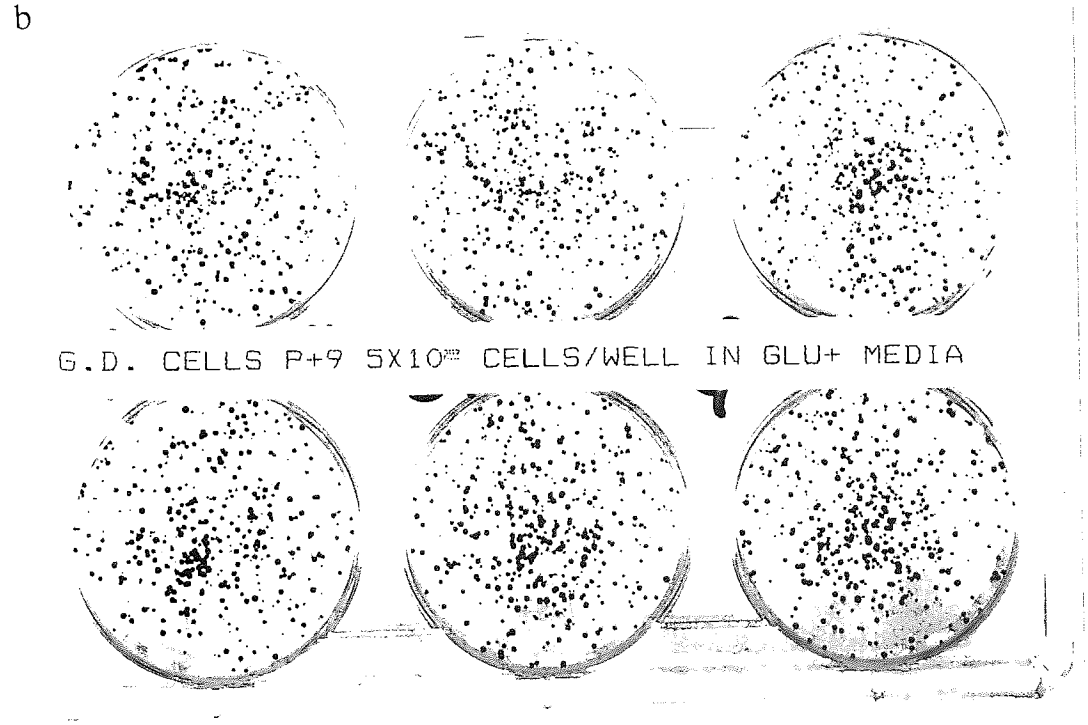
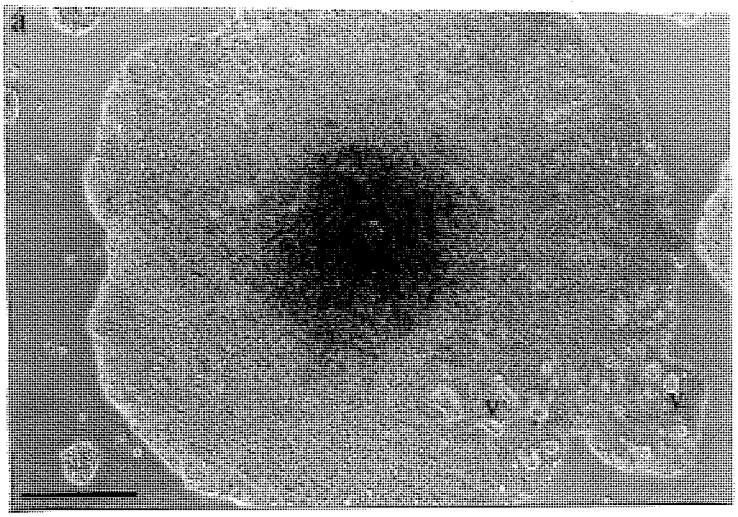


Figure 35 : Photographs of fixed and stained colonies of HT-29$_2$ cells.
Colonies were derived from cells grown in glucose-free media for 9 passages (P+9), seeded at 5 x 10^2 cells/well in a) glucose-free media, and b) glucose containing media, for 14 days. (actual size).



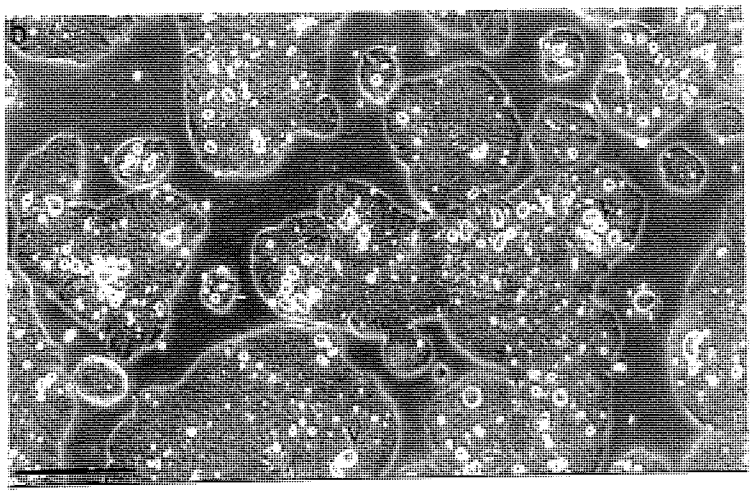


Figure 36 : Light micrographs of HT-29_2 cells grown in glucose-free media. a) 19 days after the first passage, showing a 'domed' colony of cells, and b) 9 days after the 9th passage, showing healthy cells with the glassy-like vacuoles (v). *Scale bars*, 100μm.

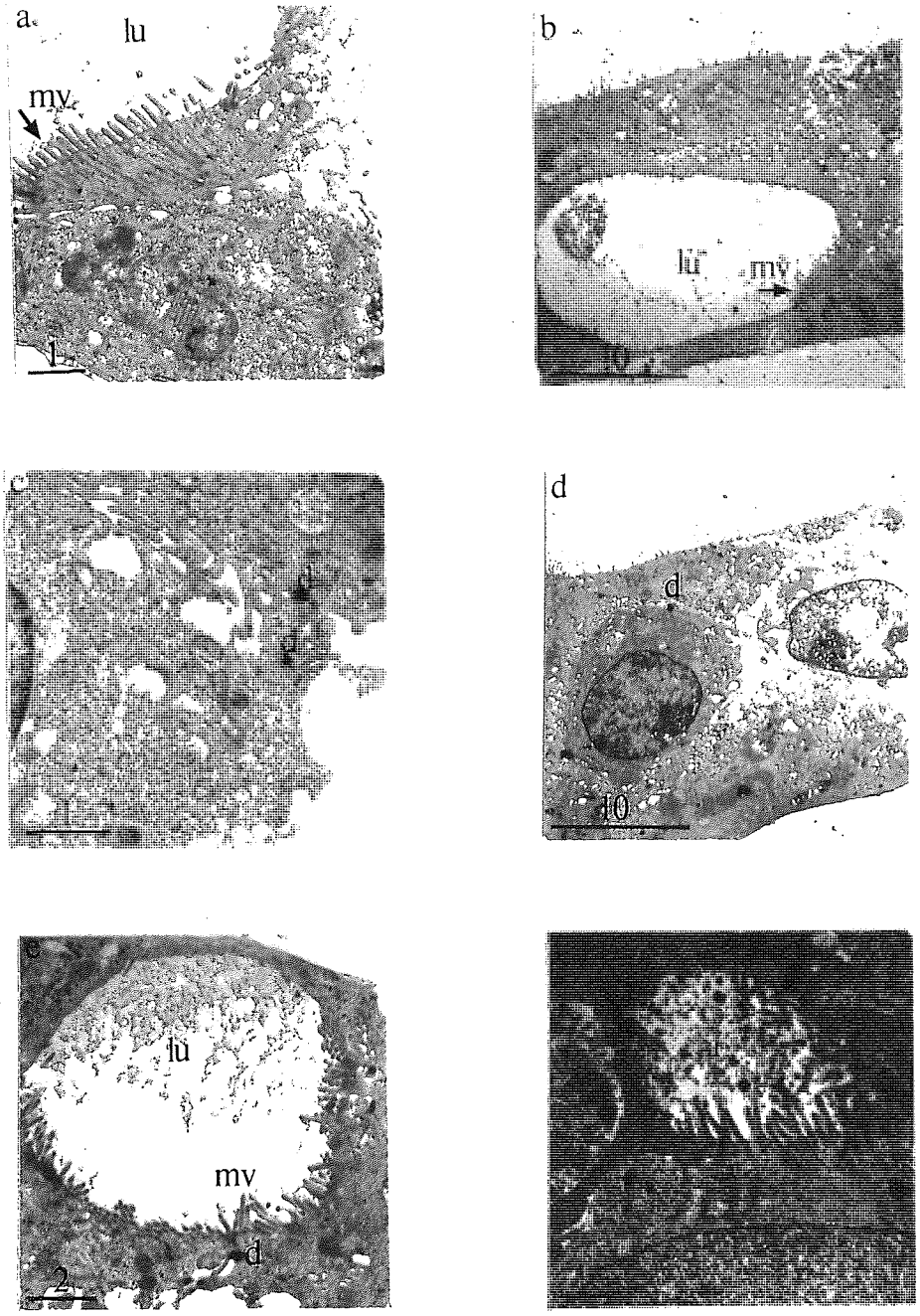


Figure 37 : Transmission electron micrographs of HT-29$_2$ cells grown in glucose-free media. a,b, & c) 20 days after the initial transfer into glucose-free medias, d) 50 days after the first passage, e) 25 days after the 5th passage, and f) 14 days after the 6th passage. Samples show evidence of intercellular lumen (lu), microvilli (mv), tight junctions (tj), and desmosomes (d). *Scale bars*, as labeled in μm.

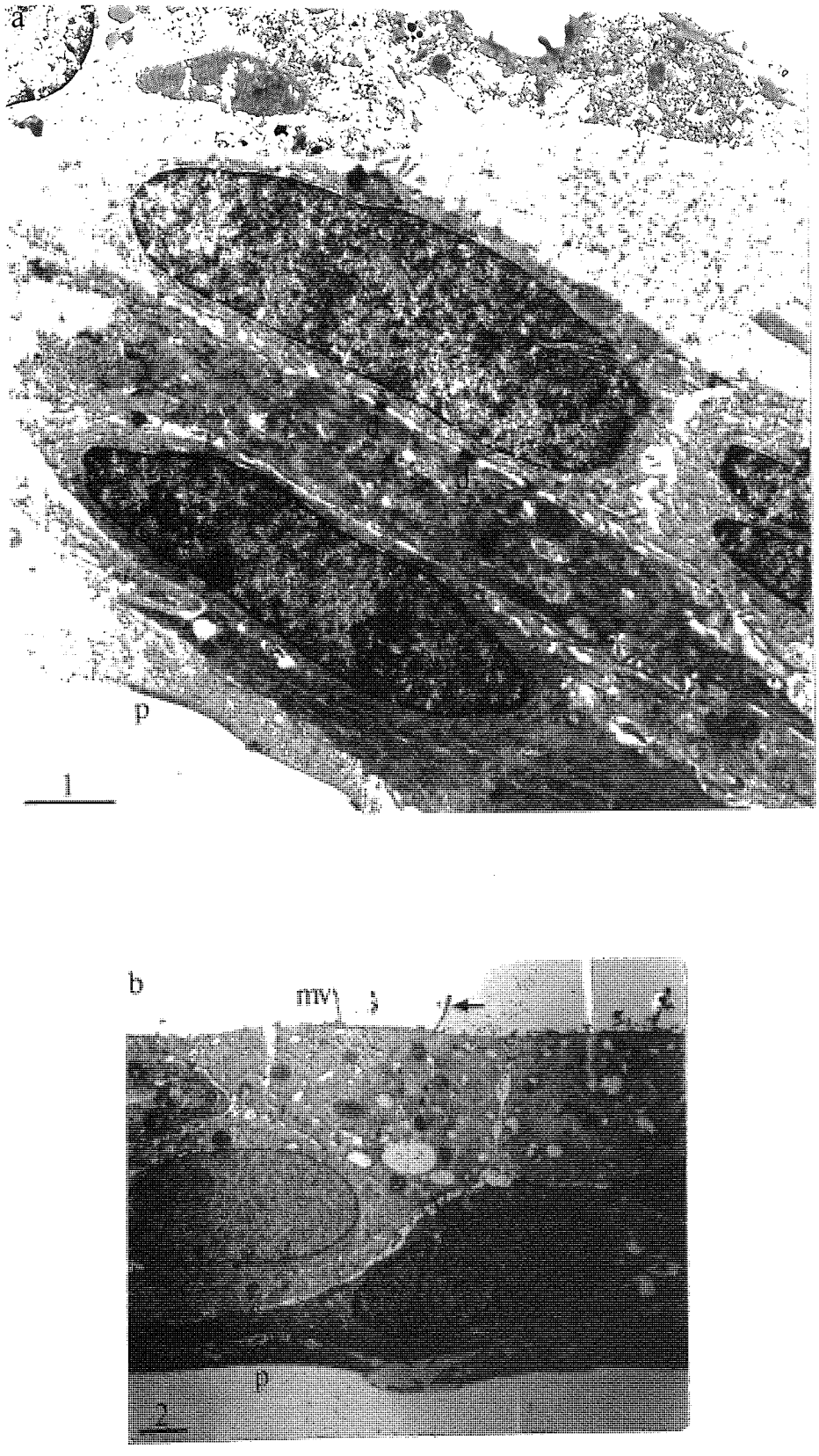


Figure 38 : Transmission electron micrographs of HT-29_2 cells grown in glucose-free media. a) 16 days after the 8th passage, and b) 7 days after the 10th passage and stained histochemically for alkaline phosphatase activity (arrows). Cells show evidence of desmosomes (d) and sporadic microvilli (mv). The plastic surface is labeled (p). *Scale bars*, as labeled in μm.

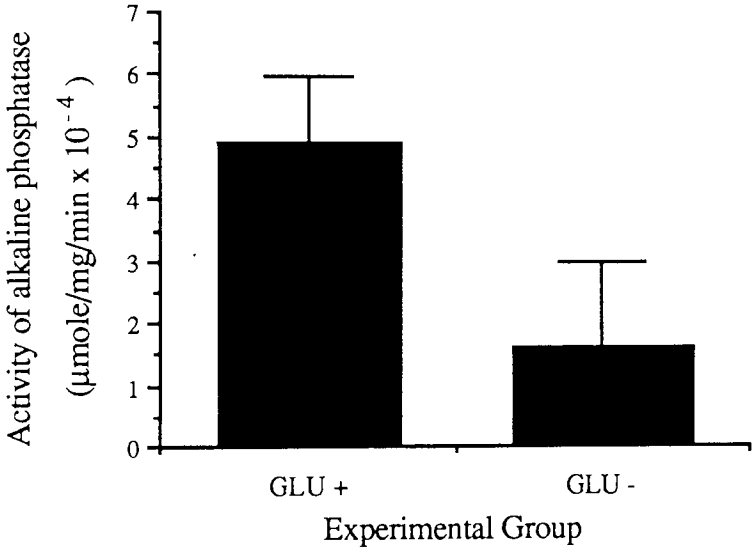


Figure 39 : Alkaline phosphatase activity of HT-29_2 cells grown in glucose-free media. Values are the means of 3 experiments in which each sample was assayed in triplicate. The significance value from a t-test evaluation was 0.000 (n = 9 ; ± sd).
GLU + = cells grown in glucose supplemented media.
GLU - = cells grown in glucose-free media.

6.III The effects of collagen gel culture on growth and morphological markers of differentiation of HT-29_2 cells

6.III.i Introduction

Considerable research in recent years has shown that individual components of the ECM such as collagen, glycoproteins and glycosaminoglycans are directly involved in the control of cellular development and in the maintenance of the differentiated tissues once formed (Bissell *et al.*, 1982). In her review (1982) Bissell presented an impressive collection of data which supported the theory that the ECM, or its individual components can reverse the loss of functional and morphological differentiation caused by the culturing of cells on tissue culture plastic. This loss of differentiation features was particularly evident in epithelial cells whose distinct morphological polarization, which is essential for their correct functional differentiation, was not maintained on tissue culture plastic in the majority of cases. There are a few epithelial cell lines which can polarize on plastic by forming domes produced by transepithelial transport of solutes across the cells. These domes are more like an *in vitro* 'blister' and are formed by the human colon carcinoma cell line Caco-2 (Fogh *et al.*, 1977), and Madine-Darby canine kidney cells (Rodriguez-Boulan and Sabatini, 1978).

The work of Emerman *et al.*, (1981) showed that the culture of mammary epithelial cells on floating rat tail collagen (type I) gels brought about morphological and biochemical differentiation. These cells were also induced to synthesize the entire pattern of milk-specific proteins, a situation which did not occur when the cells were grown on plastic (Parry *et al.*, 1982). Chambard *et al.*, (1981), had already shown that another epithelial cell type, thyroid cells, would differentiate to form follicle-like structures by embedding the cells inside collagen gels. Unlike the mammary cells these cells would not differentiate if seeded on the surface of the gel only. It is not only epithelial cells which are exposed to the effects of the ECM *in vivo* and as would be expected the ECM has been shown to effect non-epithelial cells. Kubota *et al.*, (1988) showed that a basement-membrane like matrix containing laminin could induce the differentiation of human endothelial cells into capillary-like structures. Anti-laminin antibodies prevented this induction of differentiation, thus illustrating the importance of the cell-laminin

interactions. Neuronal cells often require ECM components for their successful culture, for example, human fetal dorsal root ganglion cells depend upon laminin and fibronectin in particular for their culture (Yong *et al.*, 1988).

In view of the published data on the effects of rat tail collagen gel culture and the lack of success in causing the differentiation of HT-29_2 cells in a controlled manner by other methods, the hypothesis that collagen gel culture could induce a more functionally and morphologically differentiated phenotype was tested.

Each of the following sections will discuss the effects of collagen gel culture on the specific markers of enterocytic differentiation that were studied. Each marker will be discussed separately in its own section and each section will deal with three groups of cells. The first group were the control cells which were routinely grown on plastic substrata. The second group of cells studied were those which were derived from HT-29_2 cells grown on plastic and then seeded directly onto the collagen gels for the duration of the experiment only. These cells were further subdivided into those seeded onto gels which remained attached to the well bottom (non-released, NR) and those seeded onto gels which were allowed to float freely in the media (released, R). This second group of cells were therefore designated non-permanently passaged collagen (Non-PPC) NR and R gels. The third group of cells studied were produced by initially seeding HT-29_2 cells derived from plastic substrata onto collagen gels and then routinely passaging these cells continuously on the gels once a week. This third group of cells were designated permanently passaged collagen (PPC) NR and R gels and were used between passage 10 and 20. The rationale behind this prolonged exposure to collagen was that this may enhance the differentiation achieved, by increasing the length of exposure and thus the time for the cells to respond to this ECM component. Cells in the body are continuously exposed to the influences of the ECM and it was hoped that a prolonged exposure would enable the cells to reverse the loss of differentiation caused by years of culture on plastic and to achieve a greater degree of differentiation than those seeded onto the collagen for just the duration of the experiment (i.e. the second group of cells).

6.III.ii Results

Regular estimations of the growth rates of the three groups of cells cultured on plastic, non-passaged and passaged on collagen gels were performed in 24-well tissue culture plates. Cell numbers were determined at regular intervals during the growth of the cells as described in section 3.I. The results are presented in figures 40 and 41 for the HT-29_2 cells cultured on non-passaged collagen gels (Non-PPC) and passaged collagen gels (PPC) respectively.

Figure 40 shows that the growth rates were the same for the cells growing on all three substrata (plastic, non-released (Non-PPC NR) and released non-passaged collagen gels (Non-PPC R), with each population of cells having a doubling time of 27 hours and reaching a similar confluent cell density. However, figure 41 shows that the cells permanently passaged on collagen gels (PPC NR and R) had a prolonged lag phase of 48 hours which could not be explained. Lack of adherence may have been a possible explanation, but the cells were seen to adhere to the collagen gels without any problems. It may have been that a subpopulation of cells were dividing as normal, but that the growth rate was kept at a constant rate by an equal amount of cell death. However, cell death during the first few days of culture after passaging was not observed. The reason for their lack of an increase in cell number during the prolonged lag phase is at this point unexplained. Once the cells overcame the lag phase, both the PPC NR and PPC R cells grew with a doubling time of 39 hours, which was similar to that of the control cells grown on the plastic substrata (doubling time = 33 hours). They also reached a confluent cell density comparable to the control cells. Cells cultured on collagen gels required extra trypsinization as they remained clumped together following enzymic digestion of the collagen gel and it was very difficult to produce a single cell suspension of these cells. A possible explanation for this increased cell-cell adhesiveness will be presented in this section as part of the TEM data.

The morphological appearance of the cells growing on collagen gels was observed at LM, SEM, and TEM levels. Figure 42 shows the typical LM morphological appearance of HT-29_2 cells grown on various substrata. The cells grown on plastic (figure 42 (a)) form a tightly packed cell layer, with the outlines of individual cells being clearly visible.

However, some areas were hazy with the cell boundaries not clearly evident, suggesting multilayering of cells. The cells grown on Non-PPC and PPC gels looked similar to those growing on the plastic, but the cell boundaries were slightly more clearly delineated and the cells more cuboidal in shape. However, even these cells showed areas which were hazy with ill-defined cell borders. Unfortunately, the two micrographs (figure 42 (a) and (c)) were slightly out of focus which was due to transient problems with the microscope and the vibrations of the building in which it was housed. This problem was overcome by placing the microscope on top of layers of vibration absorptive material. The later micrographs showed an improvement in the focussing (figure 42 (a), (d) and (e)) and the cells appeared to be much clearer.

Scanning electron micrographs (figure 43) were produced from the HT-29_2 cells grown on plastic, non-released and released permanently passaged collagen gels (PPC NR and PPC R). The cells grown on the plastic were seen to have a much larger surface area and were more flat and elongated than the cells grown on collagen gels. Also, there was considerably more shrinkage caused by processing in the cells grown on plastic. The lack of obvious shrinkage in the cells on collagen was thought to result from the simultaneous contraction of the collagen, thus preventing the pulling apart of the cells. The cells grown on PPC NR and PPC R gels were smaller and more cuboidal with a higher density of microvilli on their plasma membranes. This may have been due to the cells being smaller and thus reducing their surface areas. This could have then resulted in an increase in the amount of membrane available for microvillus formation. The reduced density of microvilli in the cells grown on plastic was consequently due to the spreading out of the plasma membrane. This explanation for the increase in microvilli density on the collagen cultured cells was supported by the observation that the rounded-up, dividing plastic cultured cells, although still larger than the collagen cultured cells, did have a more dense microvillus arrangement than the flat, non-dividing cells cultured on plastic (figure 43 (a) and (b)).

TEM observations showed that the cells exhibited morphological characteristics of enterocytic differentiation which was comparable to that seen in the xenograft tumour (see section 4.I) when grown on collagen gels. This involved the production of microvilli-

lined intercellular lumen, with tight junctional complexes and numerous desmosomes between adjacent cells. The differentiation was significantly more advanced than that seen in cells grown on plastic substrata (figure 44 (a)), but was also heterogeneous like that of the xenograft tumour (see section 4, figures 8 and 9). This heterogeneity resulted in areas of undifferentiated cells which were more evident in the PPC NR and PPC R gels than the non-passaged gels, by virtue of the fact that they were sampled more often as passaging proceeded. The well developed desmosome formation seen in both Non-PPC and PPC gel cells may explain why the cells were so difficult to disperse into a single cell suspension after enzymic digestion of the collagen. The occurrence of intercellular lumen was not as frequent in the PPC gel cells compared to the Non-PPC cells, which was almost certainly due to the reduced culture time before samples were fixed and processed for TEM in the former cells. They were incubated for 7 days after passaging prior to fixation and although they were confluent by this stage, this incubation time was obviously not sufficient to allow them to develop more pronounced morphological differentiation in the form of intercellular lumen. Those that were seen tended to be underdeveloped with stunted microvilli (figure 45 (a) and (d)). Samples of all cells were later incubated for extended periods (7 to 28 days) for the immunocytochemical studies which will be discussed in sections 6.VI and 6.VII.

6.III.iii Discussion

It was apparent from the estimations of growth rates that the population doubling time was unaffected by the substrata upon which the cells were grown. However, the permanent passaging of HT-29_2 cells on NR and R collagen gels resulted in the cells acquiring a prolonged lag phase before commencing growth at a rate comparable to that of the control cells (figure 41). This increased lag phase was unexplained and was not the result of poor initial adherence of the cells to the collagen. LM studies revealed a more cuboidal appearance with more cells having distinct borders with their neighbours. The SEM data supported these findings with the cells on PPC NR and PPC R gels being smaller, more cuboidal with denser microvilli than the flat, elongated cells grown on plastic. The TEM data also showed that there were profound ultrastructural changes

occurring in response to culture on collagen gels with the appearance of microvilli-lined intercellular lumen. There was also improved junctional formation illustrated by the appearance of tight junctions and desmosomes between adjacent cells.

From the data presented in this section it can be concluded that collagen gel culture has enhanced the expression of morphological differentiation to a level similar to that already seen in the xenograft tumour. However, not all of the cells were differentiated and the heterogeneity of this differentiation, although comparable to that of the xenograft, has resulted in there being no change in the overall population doubling times. This was because the relatively small population of differentiated cells, whose possibly reduced growth rate was masked by that of the greater majority of cells. This masking effect, because differentiation was not universal, remained a problem throughout the studies on the effects of collagen gel culture on the differentiation of HT-29_2 cells. The reader must bear this in mind when considering the results presented in the following sections which will deal with the changes in functional markers of enterocytic differentiation.

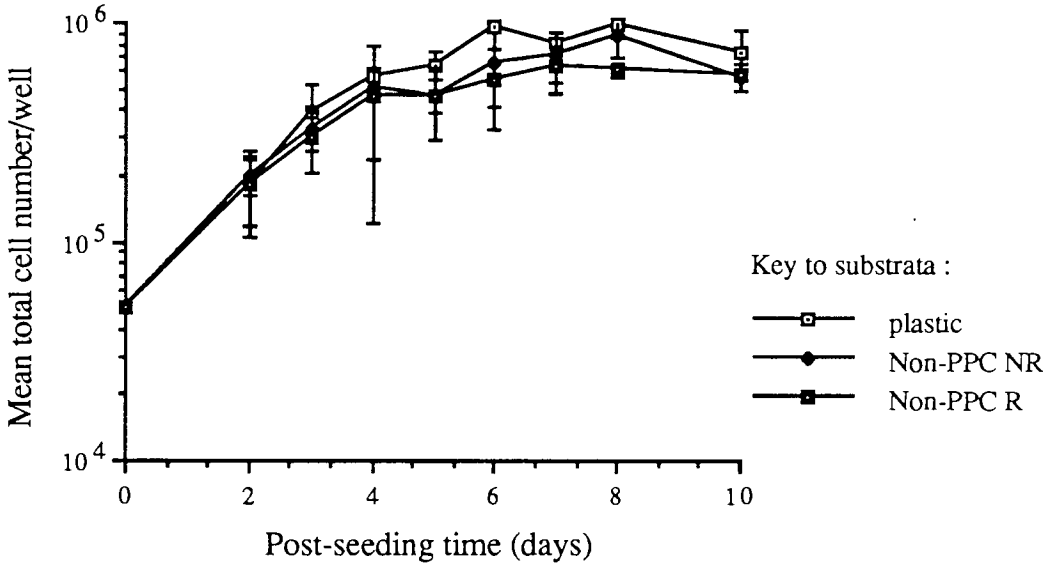


Figure 40 : Growth curves of HT-29_2 cells grown on plastic, Non-PPC NR and Non-PPC R gels.
Values are the means from several experiments (n = 3 ; ± sd).

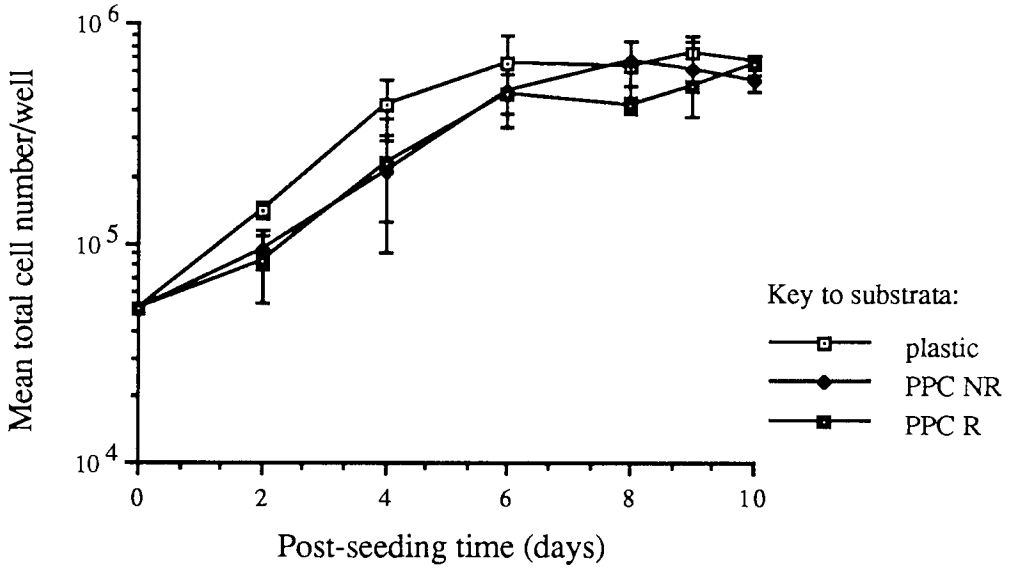


Figure 41 : Growth curves of HT-29_2 cells grown on plastic, PPC NR and PPC R gels. Values are the means of several experiments (n = 3-5 ; $\pm$ sd).

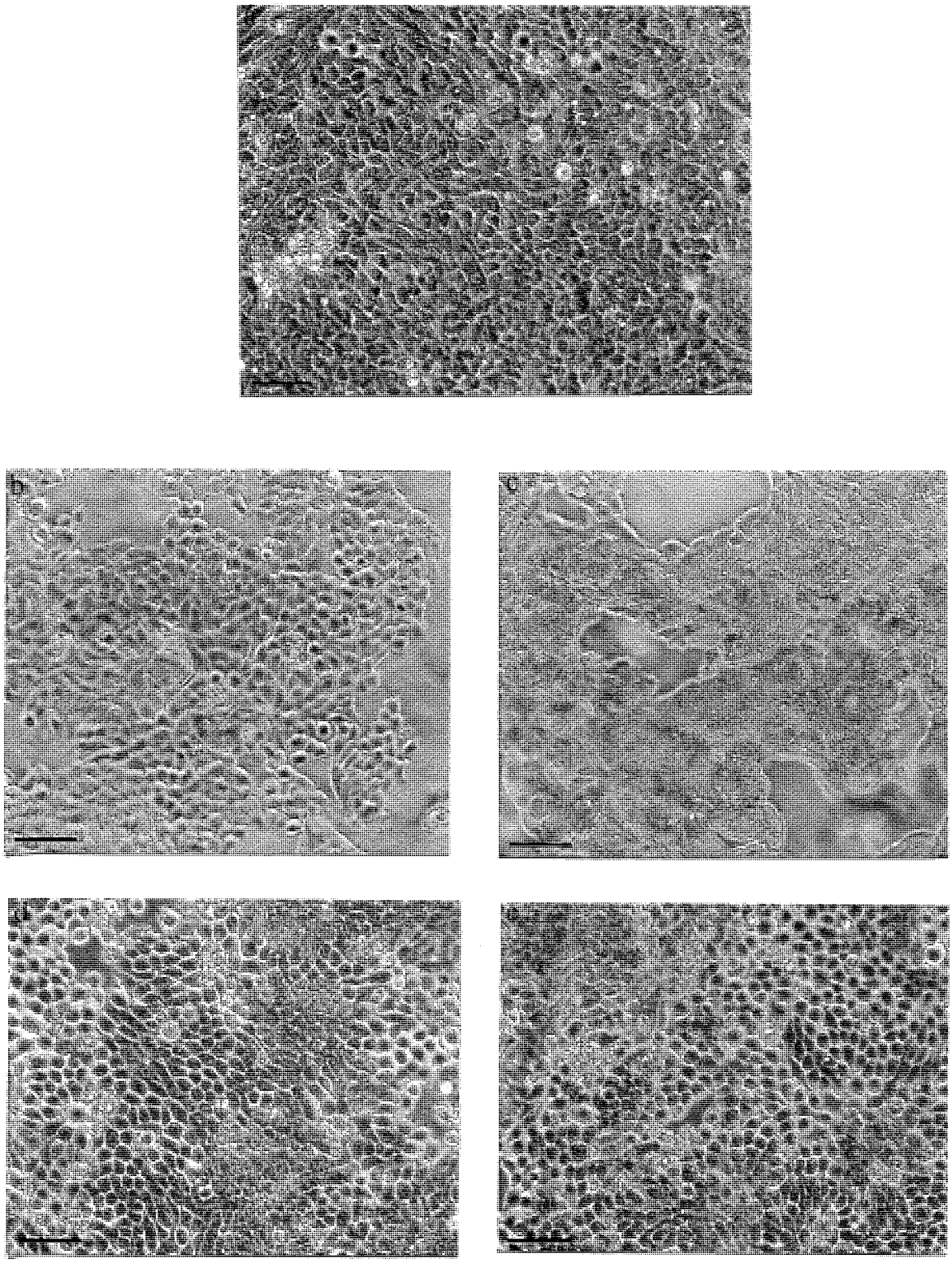


Figure 42 : Light micrographs of HT-29_2 cells grown on various substrata.
a) plastic, day 8 of culture, b) Non-PPC NR gel, day 7, c) Non-PPC R gel, day 7, d) PPC NR gel passage P+18,day 8, and e) PPC R gel passage P+18, day 8. *Scale bars*, 25µm.

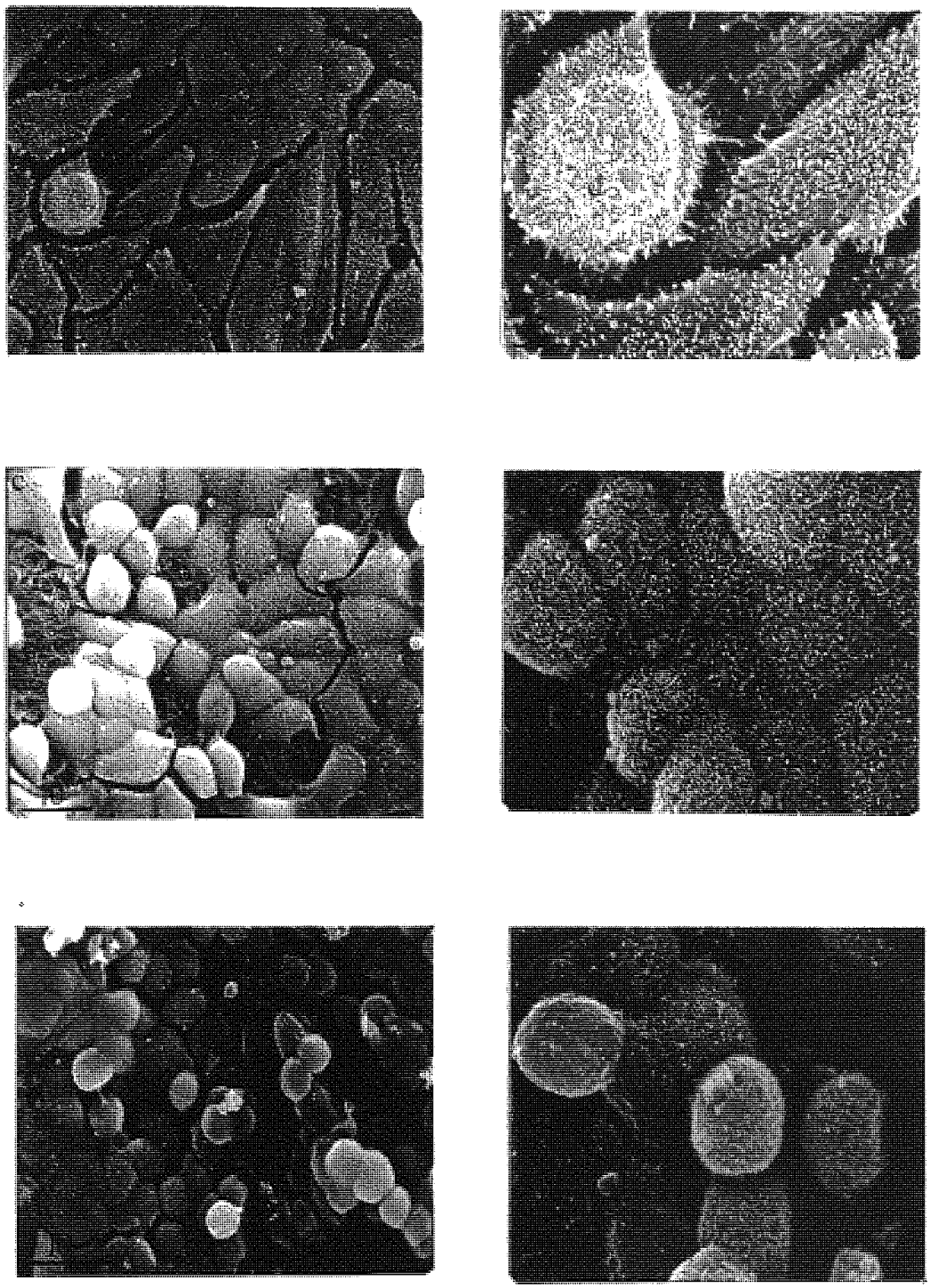


Figure 43 : Scanning electron micrographs of HT-29_2 cells.
a & b) cells grown on plastic substrata,
c & d) cells grown on PPC NR gel passage P +12,
e & f) cells grown on PPC R gel passage P + 12.
Collagen fibrils (cf), microvilli (mv) and dividing cells (d). *Scale bars*, as labeled in μm.

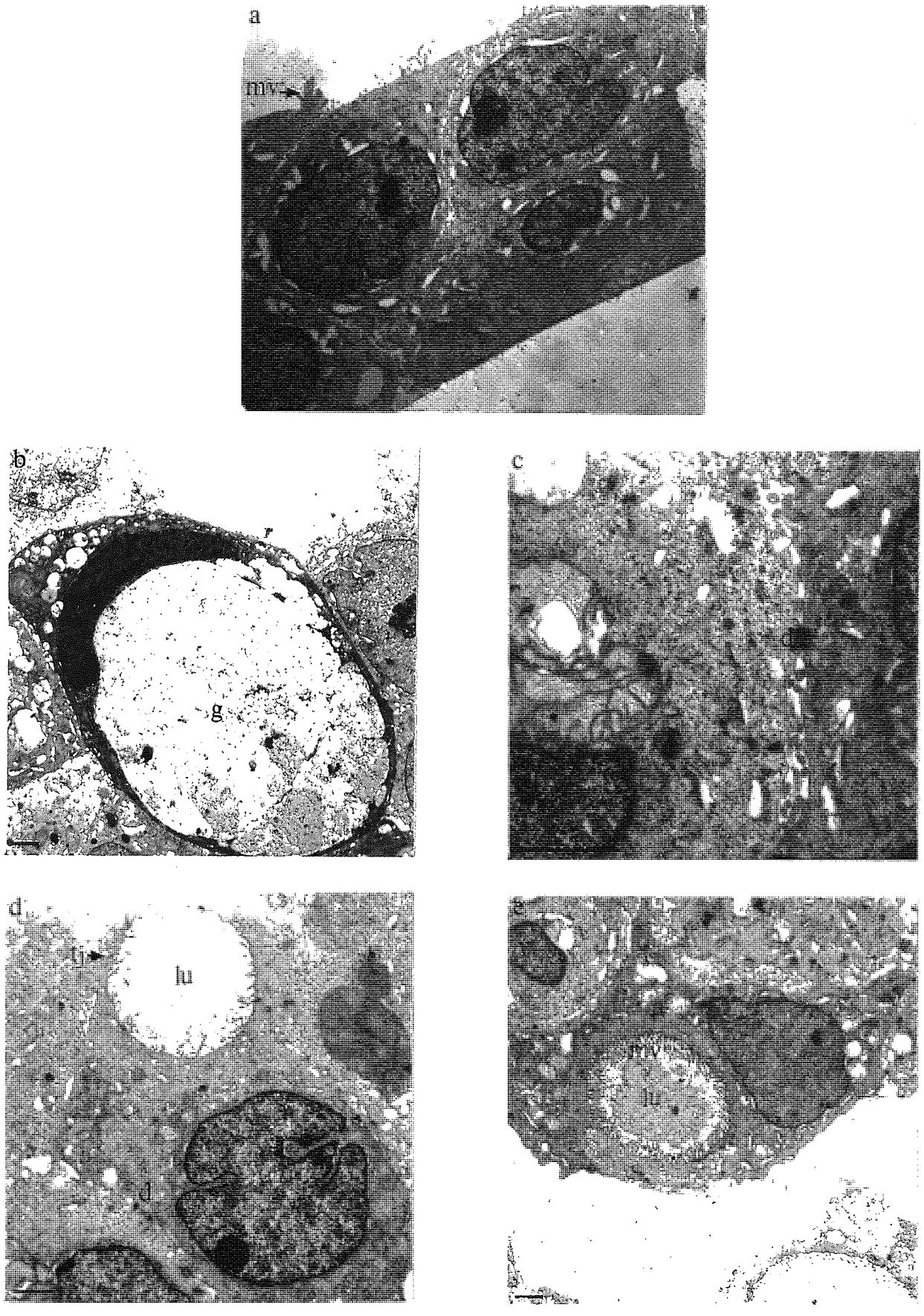


Figure 44 : Transmission electron micrographs of HT-29$_2$ cells grown on plastic and Non-PPC gels. Cells were grown on :
a) plastic day 7,
b) Non-PPC NR gel, day 9, c) Non-PPC NR gel, day 11,
d) Non-PPC R gel, day 9, e) Non-PPC R gel, day 11.
Intercellular lumen (lu), microvilli (mv), goblet-like cell (g), desmosomes (d), and tight junctions (tj). *Scale bars*, 1µm.

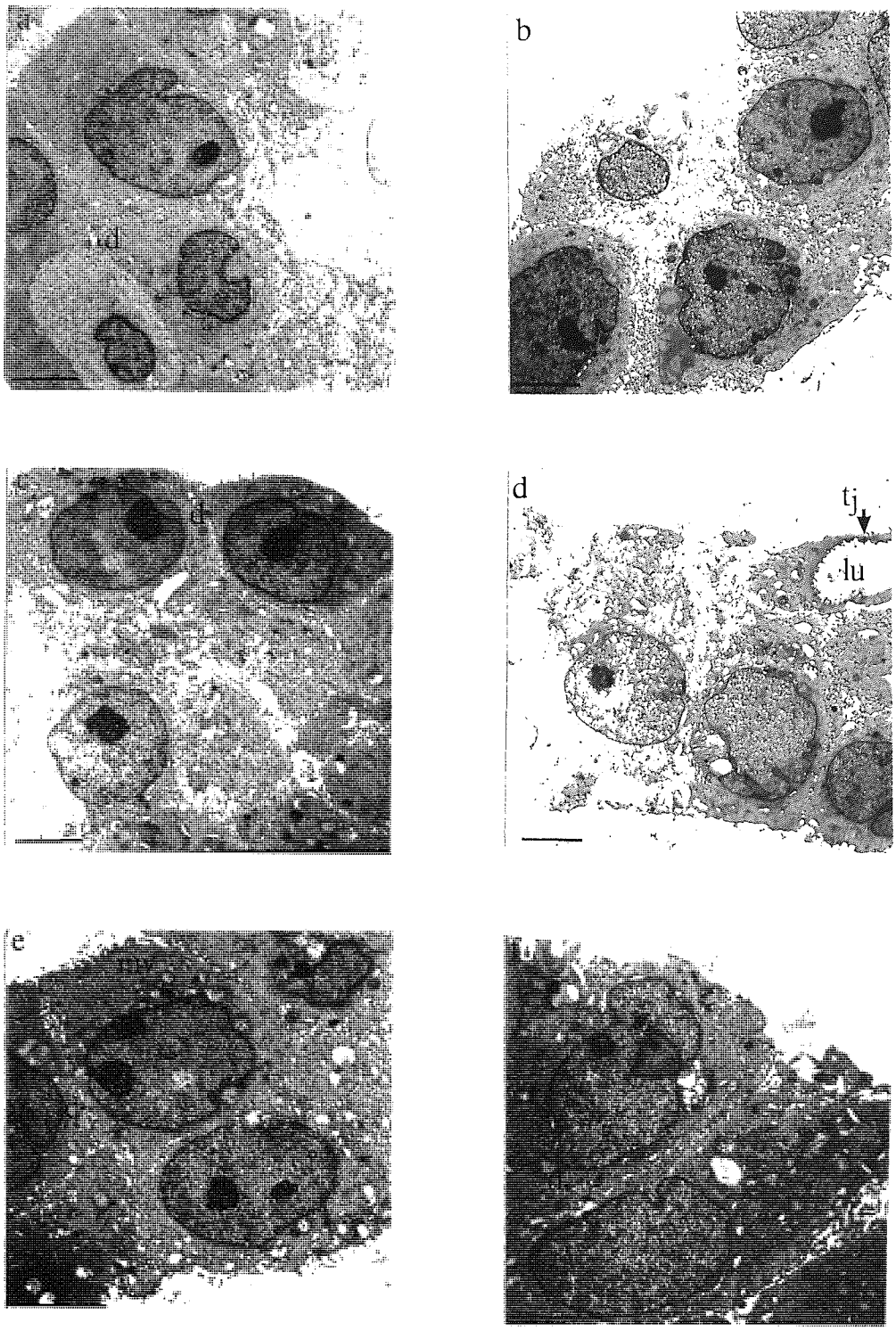


Figure 45 : Transmission electron micrographs of HT-29$_2$ cells grown on PPC gels.
a) PPC NR, passage P+1, b) PPC R, passage P+1,
c) PPC NR, passage P+5, d) PPC R, passage P+5 ,
e) PPC NR, passage P+15, f) PPC R, passage P+15.
All samples were fixed 7 days after seeding onto the substrata. Intercellular lumen (lu), microvilli (mv), desmosomes (d), and tight junctions (tj). *Scale bars*, 5µm.

6.IV The effects of collagen gel culture on the activity of alkaline phosphatase

6.IV.i Introduction

Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1, AlP), is a membrane-anchored glycoprotein, hydrolytic enzyme. It has several isoforms which are abundant in bone, liver, kidney, placenta, and the small intestine (McComb *et al.*, 1979). The enzyme acts on monoesters of orthophosphoric acid and has been implicated in the regulation and maintenance of intracellular phosphate levels. This is very important in tissues like bone and in those in which transepithelial transport is a major function e.g. the small intestine (Bitensky and Poulter, 1969). The presence of the enzyme in the lumenal margins of the brush border membrane of the small intestine is indicative of the high absorptive function of this tissue (Kenny and Maroux, 1982). It is only found in high levels in human fetal colon and after birth the levels decline to the low levels seen in the adult colon (Lacroix *et al.*, 1984 ; Lev and Griffiths, 1982). This differential distribution during development has lead this enzyme to be used as a biochemical marker for the *in vitro* enterocytic differentiation of cell lines of fetal or small intestinal origin. The levels of alkaline phosphatase activity have been previously shown to increase upon differentiation of HT-29 cells following glucose deprivation (Pinto *et al.*, 1982) and after treatment with the differentiating agent sodium butyrate (Herz *et al.*, 1981). It was therefore used as a marker of differentiation during these studies and the effects of collagen gel culture upon its activity were assessed.

6.IV.ii Results

The activity of alkaline phosphatase was determined by a fluorescent ultramicroassay (see section 3.III). A typical calibration curve for such an assay is shown in figure 46. The cells were prepared prior to the assay by trypsinization and/or collagenization from the test substratum. An experiment was performed to determine the effect that trypsinization may have on enzyme activity by possible perturbations to the plasma membrane. Cells were grown on tissue culture petri-dishes until confluency and then prepared for the fluorescent assay by either 1) trypsinization from the plastic, or 2) removal by gentle scraping with a rubber policemen. Figure 47 shows the results of this experiment which

was performed in triplicate, with each sample of cells being assayed 6 times during each experiment (n = 18). The activity of cells following trypsinization was significantly reduced (significance value, P = 0.02) when compared to the activity of those which were scraped from the plastic. The activity was 61 % of the activity of the control, scraped cells. However, it was not necessary to correct all subsequent results to allow for the detrimental effects of trypsinization because all cells were trypsinized at some point in their preparation, even those growing on collagen gels. It was not possible to perform such an experiment to determine the effects of collagenization because an alternative, less damaging method of removing the cells from the collagen gels was not available to serve as the control.

The experiments to determine the effects of collagen gel culture on alkaline phosphatase activity were performed on cells which had been removed from their substrata at two time points after initial seeding. Samples of cells from plastic substrata, Non-PPC and PPC gels were assayed on day 6 and 12. The assays on the Non-PPC and PPC gels were carried out separately each with its own corresponding sample of control cells which had been grown on plastic. These separate experiments were performed in triplicate and within each experiment each sample of cells was assayed in triplicate giving an overall n = 9. The levels of activity of Non-PPC and PPC gels were then compared to the control cells grown on plastic.

Figure 48 shows the mean activities of alkaline phosphatase of cells grown on Non-PPC gels and their corresponding control cells cultured on plastic. The activities of cells grown on both Non-PPC NR and R gels were significantly less on day 6 when compared to the control cell activity (t-test analysis: NR, P = 0.075 ; R, P = 0.016). Only the activity of the Non-PPC R gel cells was significantly lower than the control cells on day 12 of culture (P = 0.016). On day 6 there was no significant difference between the activities of cells from the NR and R gels (P = 0.710), but on day 12 the activity was significantly higher in the NR cells than in the cells from the R gels (P = 0.037). Activities of enzyme from cells cultured on both gel types were lower than the control cells on day 12. The results would therefore indicate that non-passaged collagen gel culture did not result in an elevation of alkaline phosphatase. Culture on the Non-PPC

NR gel resulted in higher activities than that from Non-PPC R gel culture on both assay days, but the difference was only significant on day 12.

Figure 49 shows the mean activities of alkaline phosphatase for cells grown on PPC gels for 6 and 12 days of culture. It was interesting to note that the day 6 basal activities and the elevation in activities seen on day 12 of the three substrata groups were higher than those seen previously during the Non-PPC experiments. This variation in activity of the control cells cultured on plastic for the two sets of experiments was unexplained. Despite this, the activities were higher in the cells grown on the PPC gels than the control cells cultured on plastic on both days of assay (6 and 12). The most significant increase in activity was seen with cells from the PPC R gels on day 12, for which a t-test analysis gave a significance value of 0.003 when compared to the activity of the control cells cultured on plastic for that day. However, there were no significant differences between the activities of cells from the PPC NR and PPC R gels on either day.

Activity of the enzyme in cells grown on permanently passaged on collagen gels (PPC) was also determined using an electron microscopical histochemical technique in order to give a visual, qualitative account of activity (figures 50 - 52) with increasing time on different substrata. The cells were washed well prior to fixation and staining to remove any exogenous alkaline phosphatase activity which may have been present in the sera of the tissue culture media. The activity was low on both days for the cells grown on tissue culture plastic being associated mainly with the cell membranes in contact with the media and with the microvilli of any intercellular lumen, an example of which is shown in figure 51 (a). Activity was higher in the cells grown on PPC NR and R gels and was found at the cell-cell borders where there were many microvilli-like interdigitations and again at the media surface. There was also some cytoplasmic staining evident in the PPC cells on day 6 which may have been due to enzyme on route to the membrane. By day 12 the activity had increased dramatically, with this being more evident in the cells cultured on the PPC R gels (figure 51 (c)). An electron micrograph taken at a higher magnification is shown in figure 52 to illustrate the localization of the staining at the microvilli-like interdigitations of the cell borders. Particulate staining can also be seen in some of the nuclei, a phenomenon already observed in section 6.II and thought to be due to the localization of

phosphatases which work in conjunction with the phosphokinases (Lord *et al.*, 1988). Controls for these experiments were performed in which the substrate sodium-ß-glycerophosphate was omitted from the incubation media and as a result there was no staining seen in any of the samples.

The histochemical and biochemical results for the cells grown on PPC gels would indicate that passaging continuously on collagen gels (NR and R) resulted in an elevation in alkaline phosphatase activity. There was no difference in the activity of the cells grown on the two different gels (NR or R) when biochemically assessed, however the histochemical data would suggest that the levels were much higher in the cells grown on the PPC R gels on day 12. The differences between the results of the two assays may have arisen from the slight pH differences of the reaction media used for each assay, which would then lead to the detection of different forms of the enzyme which had their own particular pH preferences for optimal activity. The fluorescent assay was performed at a pH of 10.4 and the histochemical assay at pH 9.6. However, the results still show an elevation above that of the control cells grown on plastic substrata following continual passaging on the two types of collagen gel (NR and R), although neither gel enhanced this elevation any more than the other.

6.IV.iii Discussion

The results would suggest that culture of cells on non-passaged collagen gels (Non-PPC) did not affect the activity of alkaline phosphatase. However, because there were no electron microscopical observations of these cells made it was not possible to determine if there had been any masking of the elevated activity which may have occurred in some of the cells in response to culture on the collagen. It should also be noted that masking can just as easily occur in the cells cultured on plastic where some cells may have had higher activities, but because there were relatively less of these high activity cells the biochemical assay did not detect the activity. Biochemical assays only produce an activity which in effect is the mean activity for the entire population and it will therefore be closer to the activity of the majority of the cells of that population than the minority. The histochemical assays performed on the cells permanently passaged on collagen gels (PPC) illustrates

these points nicely, where the heterogeneity of staining can be seen in all samples on both 6 and 12 days (figures 50 and 51). It was only when there was a significant increase in the individual cell enzyme activity coupled with an increase in the number of cells expressing that elevated activity, that the biochemical assay actually detected an overall increase. This was the case for the cells grown on the PPC gels in which the stimulation was sufficient enough not only to increase the individual cell enzyme activity, but also to enhance the number of cells expressing this elevated enzyme activity. It should also be noted that the histochemical observations showed that the activity of alkaline phosphatase was not only associated with polarized, differentiated cells, but was found around the entire plasma membrane of unpolarized cells. This would suggest that this enzyme is an early marker of the differentiation process and that it is not dependent upon morphological differentiation for expression of its activity.

In summary, it was necessary to passage the HT-29_2 cells on the collagen gels in order to induce an increase in alkaline phosphatase activity which was significantly greater than that of the control cells cultured on plastic substrata.

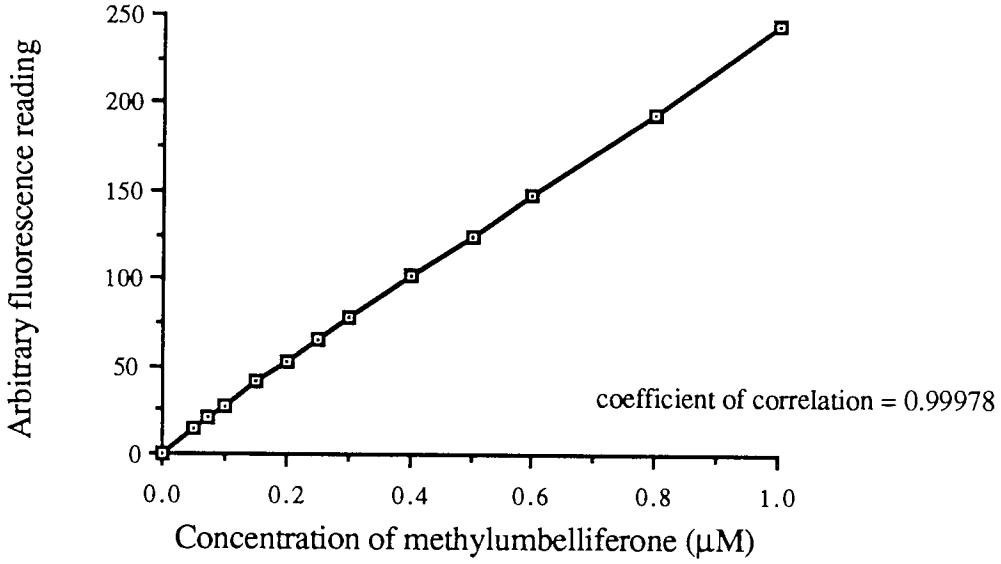


Figure 46 : Typical calibration curve for a fluorescent microassay for alkaline phosphatase activity.

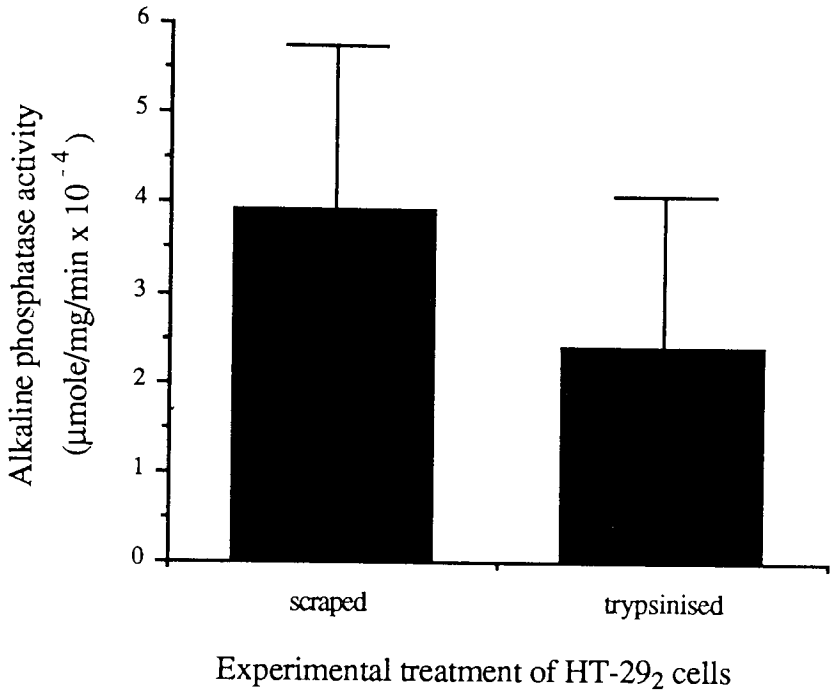


Figure 47 : Alkaline phosphatase activity of HT-29$_2$ cells removed from the plastic substrata by two different methods.
Values are the means of several experiments (n = 18 ; ± sd). t-test analysis, P = 0.02.

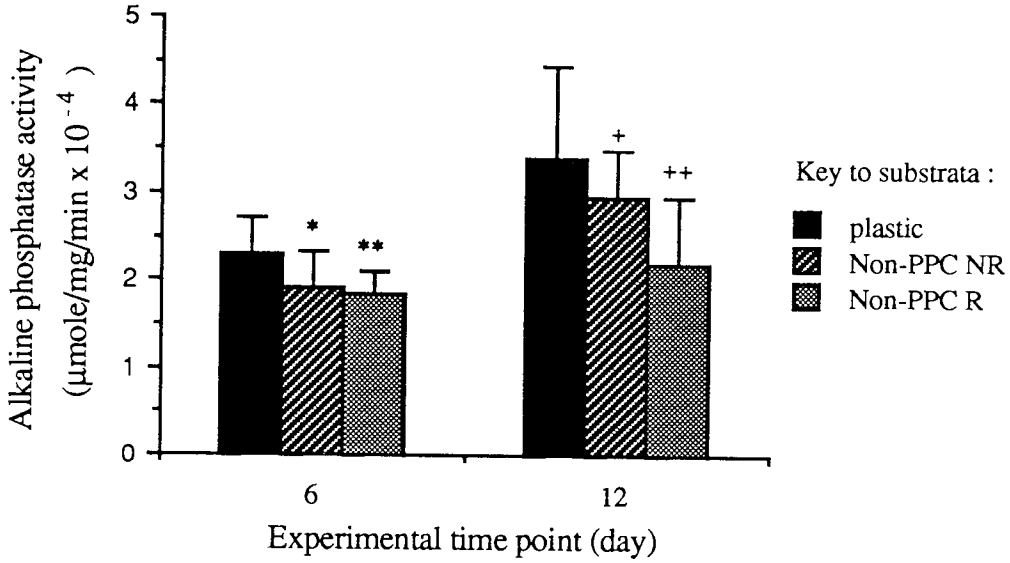


Figure 48 : Alkaline phosphatase activity of HT-29_2 cells grown on Non-PPC gels. Values are the means of several experiments (n = 9 ; ± sd).
Significance values from t-test analyses : * P = 0.075 ** P = 0.016 + P = 0.284 ++ P = 0.016.

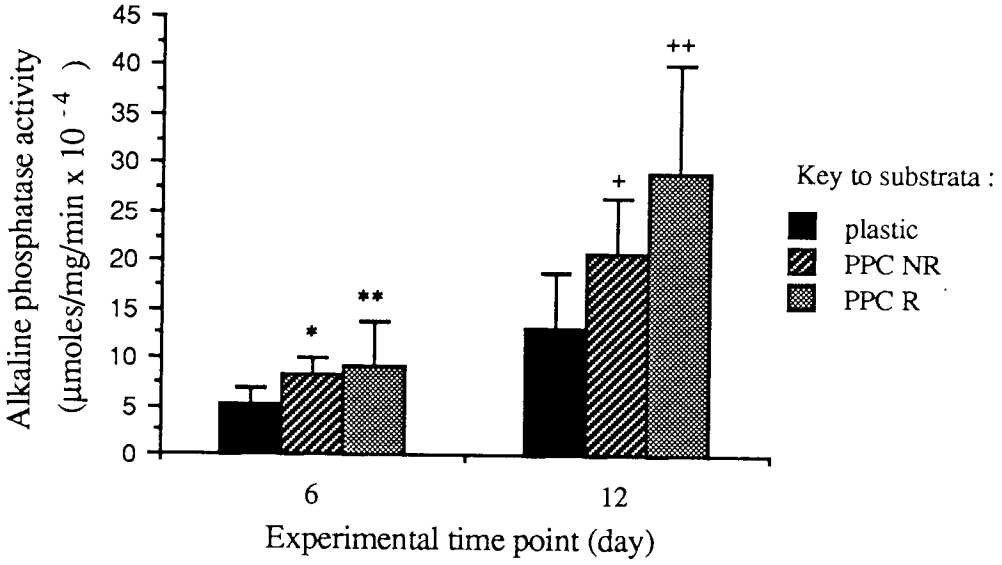


Figure 49 : Alkaline phosphatase activity of HT-29_2 cells grown on PPC gels.
Values are the means of several experiments (n = 9 ; ± sd).
Significance values from t-test analyses ; * P = 0.005 ** P = 0.041 + P = 0.021 ++ P = 0.003.

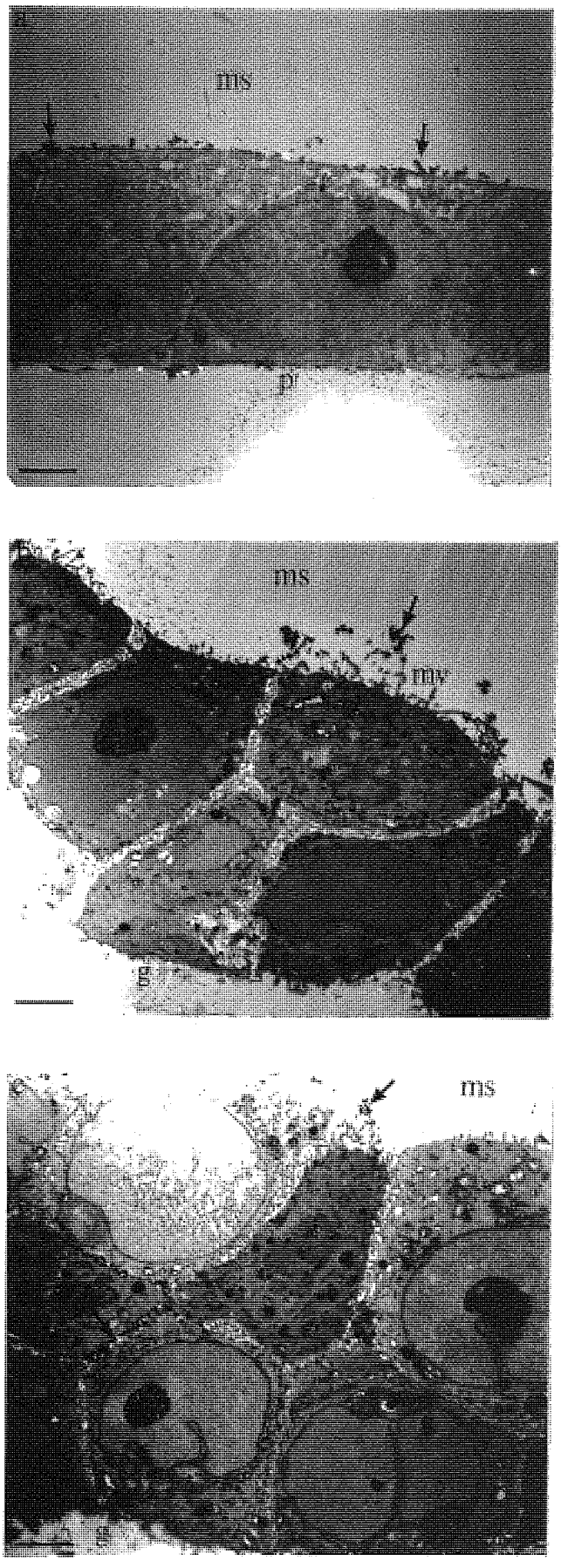


Figure 50 : Transmission electron micrographs of HT-29_2 cells stained histochemically for alkaline phosphatase activity on day 6.
a) cells grown on plastic, b) cells grown on PPC NR gel, and c) cells grown on PPC R gel. Alkaline phosphatase activity (arrows), microvilli (mv), media surface (ms), collagen gel (g), and plastic surface (p). *Scale bars*, 2μm.

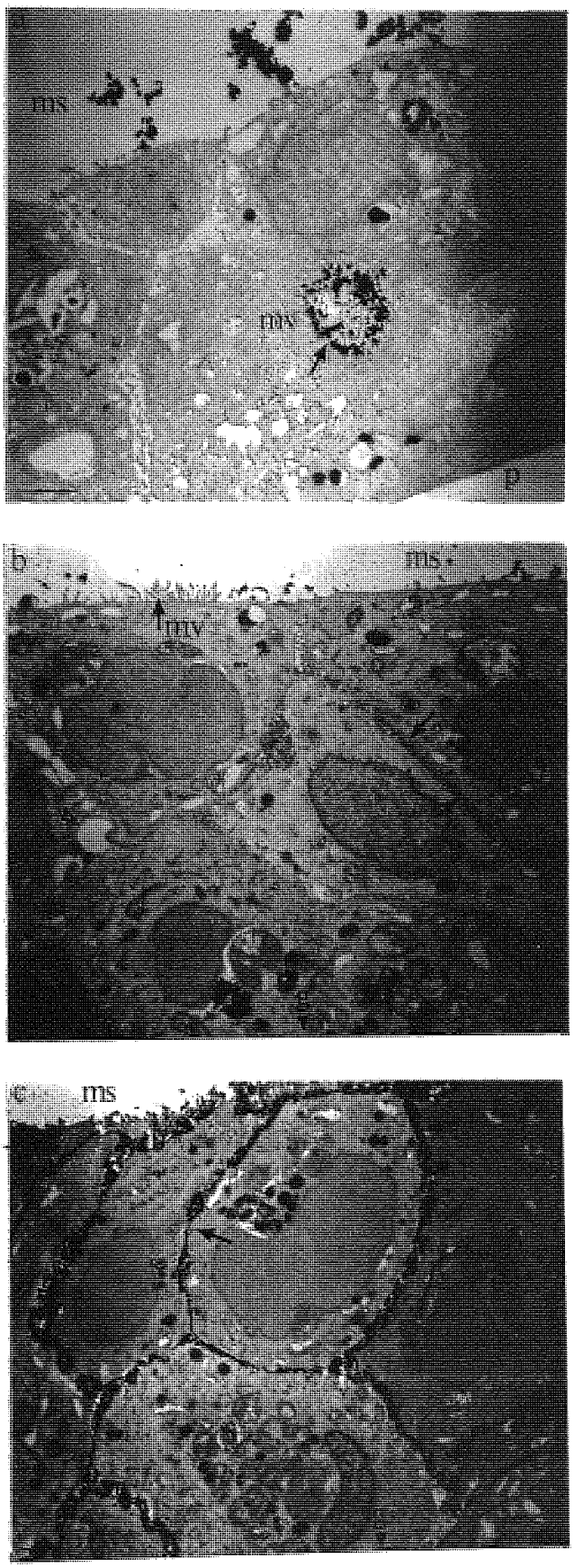


Figure 51 : Transmission electron micrographs of HT-29_2 cells stained histochemically for alkaline phosphatase activity on day 12.
a) cells grown on plastic, b) cells grown on PPC NR gel, and c) cells grown on PPC R gel. Alkaline phosphatase activity (arrows), microvilli (mv), media surface (ms), collagen gel (g), and plastic surface (p). *Scale bars*, 2μm.

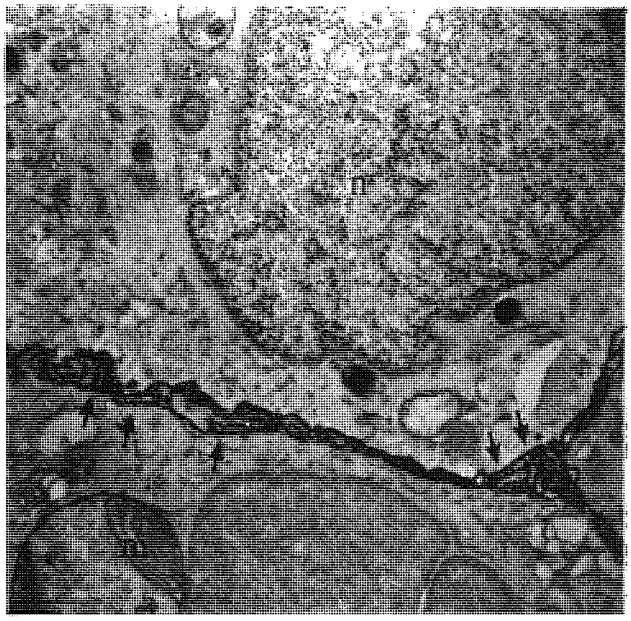


Figure 52 : High power transmission electron micrograph showing alkaline phosphatase activity. Sample of cells after 12 days in culture on PPC R gel.
Alkaline phosphatase activity (arrows), nucleus (n), and mitochondria (m). *Scale bar*, 1μm.

6.V. The effects of collagen gel culture on the secretion of CEA

6.V.i Introduction

Carcinoembryonic antigen (Gold and Freedman, 1965, CEA) is an oncofoetal glycoprotein whose function in normal development remains to be elucidated. However, its membership of the immunoglobulin superfamily which also includes the cellular adhesion molecules (CAMS) (Williams, 1987) and its localization to the plasma membrane has implicated it as having a role in cell-cell recognition and adhesion. It is overexpressed in almost all human colorectal carcinomas (Shuster *et al.*, 1980) and this overexpression has been shown to enhance colon carcinoma cell-cell aggregation. Its role as a CAM although aberrant has been implicated in tumorigenesis and the enhancement of metastasis (Benchimol *et al.*, 1989). The overexpression of CEA is also greater in well rather than moderately differentiated colorectal cancers (Davidson *et al.*, 1989) and the levels of this molecule in the blood of cancer patients is used as prognostic tool following surgery to indicate the extent of tumour removal and the recurrence of tumours, primary and metastatic. The cure of colorectal carcinoma is dependent on adequate primary surgery and recent work has shown that the expression of CEA by colorectal tumours may be able to help to improve peroperative radioimmunolocalization which could then enhance the surgeons assessment of the extent of tumour progression and thus influence the success rate of removal of the primary tumour (Blair *et al.*, 1990). CEA expression is also elevated in other types of cancer, including those of the pancreas and lung (Fahey and Lui, 1988).

Expression of CEA in the normal colon is associated with the undifferentiated basilar crypt cells and the microvillar surface of the mature absorptive cells (Ahnen *et al.*, 1987). Although the more differentiated colorectal carcinomas generally express more CEA some of the anaplastic tumours do not express any CEA at all, a situation which does not have a counterpart in the normal colonic epithelium. Even though the expression of CEA does not appear to follow a pattern which corresponds to the normal expression in intestinal tissue it is used as a marker of differentiation of malignant cell lines of intestinal origin *in vitro*.

Previous work has shown that the undifferentiated subclone HT-29-D4 both secreted and expressed at their plasma membranes very low levels of CEA (2.5×10^{-3} μg/10^6cells/24 hours). When these cells were induced to differentiate by glucose deprivation there was a considerable elevation in both surface expression of bound CEA and CEA secreted into the tissue culture media (max.= 1.3×10^{-1} μg/10^6 cells/24 hours). Also, CEA expression was localized to the apical membrane domain of these polarized cells as was the site of release (Fantini *et al.*, 1989). The levels of membrane-bound and secreted CEA were well correlated in both undifferentiated and differentiated HT-29-D4 cells and for this reason only the amount of secreted CEA was measured during these experiments.

In light of the published data on CEA expression, with particular reference to those results already discussed for HT-29 cells, the hypothesis that collagen gel culture could increase the production of CEA by HT-29_2 cells was tested.

6.V.ii Results

Cells were cultured on Non-PPC and PPC gels in separate experiments with their own corresponding control cells cultured on tissue culture plastic. Cells were cultured until confluency (day 7) then the media was replaced with serum-free media and the cells cultured for a further 48 hours. The conditioned media was collected, centrifuged to remove any cellular debris, concentrated and then stored at - 20 °C until being assayed with an ELISA for CEA. It was necessary to concentrate the samples 10 fold because preliminary assays of unconcentrated conditioned media had shown negligible activities. Concentrating the media enabled the detection of CEA. The collection of conditioned media was repeated for each group of cells in triplicate and the concentrated samples were assayed for CEA at least twice using an ELISA.

Controls were performed during the ELISA assays to ensure the validity of the assay. These controls involved incubating triplicate wells of the microtitre plate as for the test and calibration samples using the highest CEA calibration sample (75 μg/L) but with one of the following steps omitted :

1) coating of the plate with primary antibody overnight,
2) incubation with the second antibody,

3) incubation with substrate solution.

The corresponding control buffer was used in place of the above solutions in the control wells. These controls always gave lower levels of CEA than the control chicken sera of the calibration curve, therefore the assay was considered valid. A typical calibration curve is shown in figure 53.

The repeat ELISA assays revealed that the repeated thawing and re-freezing procedures and the storage in between assays lead to an overall decline in CEA detection. Figure 54 illustrates this point and shows the mean amount of CEA detected for each group of cells in three separate assays. For this reason only the results of the first ELISA assay for the Non-PPC and PPC gels will be presented and discussed in this section.

Figure 55 shows the mean amounts of CEA secreted into the media above cells grown on plastic, Non-PPC NR and R gels expressed as μg of CEA / mg of protein / 48 hours. There was no significant difference in the level of CEA secreted by cells on the three different substrata and the levels detected were of the same order of magnitude as the value obtained for the undifferentiated HT-29-D4 cells by Fantini *et al.*, (1989) when the results were adjusted to take into account the higher number of cells used and the longer collection time in our experiments. Growing the cells on collagen gels for the duration of the experiment without passaging therefore did not result in an increase in the secretion of CEA by the cells into the media.

Figure 56 shows the mean amounts of CEA secreted into the media above cells grown on plastic, PPC NR and R gels. There was no significant difference in the level of CEA secreted by the cells grown on plastic and PPC NR gels (t-test analysis: $P = 0.468$). However, there was a significant elevation in the secretion of CEA by the cells grown on PPC R gels when compared to those on plastic and PPC NR gels ($P = 0.001$ and $P = 0.000$ respectively). It should be noted that the values were all lower in these experiments than those observed during the Non-PPC experiments. It was thought that this may have been due to the effects of prolonged storage of the PPC samples of conditioned media prior to assaying. Even though the levels detected were lower the elevation of secretion seen in the cells grown on PPC R gels remains valid as prolonged storage would be expected to effect each sample equally.

6.V.iii Discussion

The culture of HT-29_2 cells on collagen gels (NR and R) for the duration of the experiment only did not enhance the production of CEA into the conditioned media above the cells when compared to those growing on plastic. However, by continuously passaging HT-29_2 cells on collagen gels (R only) it was possible to enhance the production of CEA, although the level reached was not as high as that seen in the differentiated HT-29-D4 cells (Fantini *et al.*, 1989). It must be borne in mind that the HT-29-D4 cells were induced to differentiate by different methods and they were not cultured on collagen gels. The results would suggest that by passaging HT-29_2 cells on floating collagen gels enhances the production of CEA which was being used as a marker of differentiation in these cells. This would imply that the cells cultured on the permanently passaged released collagen gels were more differentiated than those cells cultured on plastic substrata, passaged non-released gels (PPC NR) and non-passaged gels (Non-PPC).

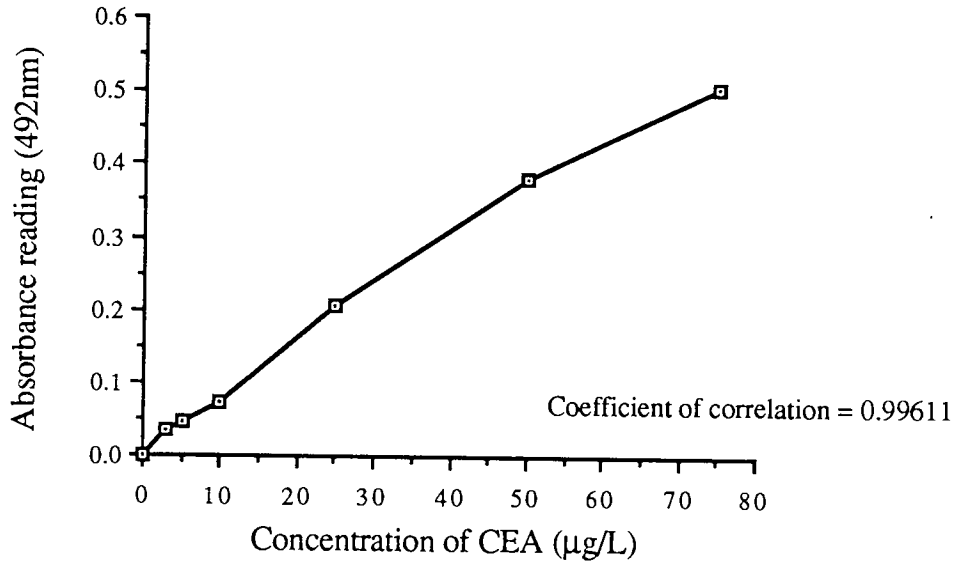


Figure 53 : Typical calibration curve for the CEA ELISA.

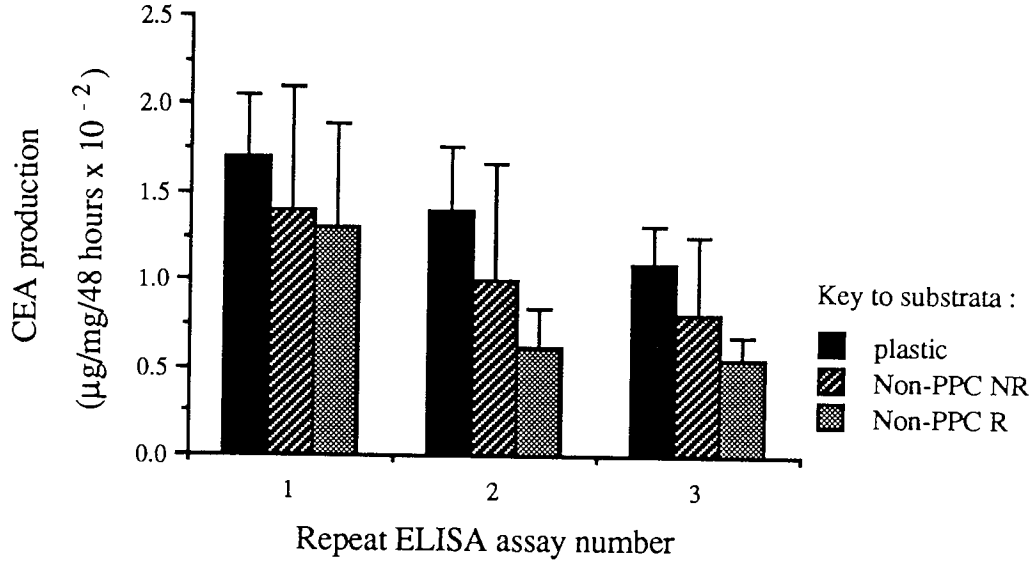


Figure 54 : The effect of storage upon detectable levels of CEA.

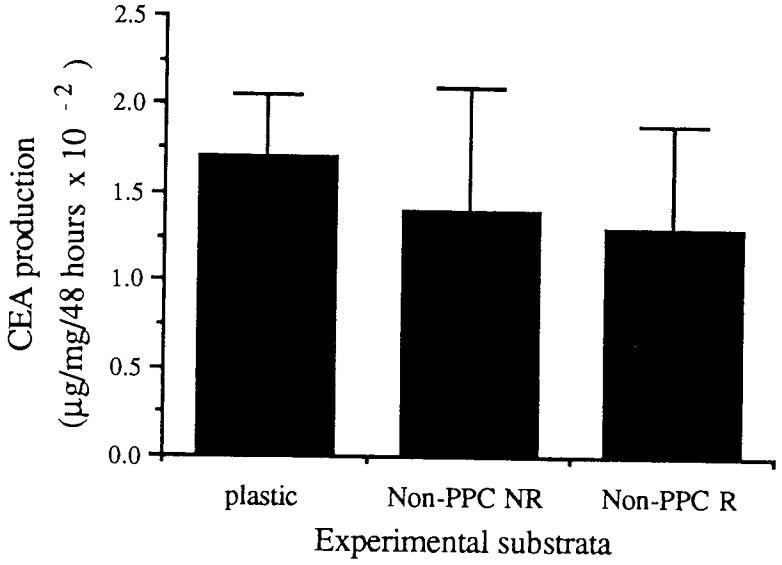


Figure 55 : Production of CEA by HT-29_2 cells grown on Non-PPC gels.
(n = 9 ; ± sd).

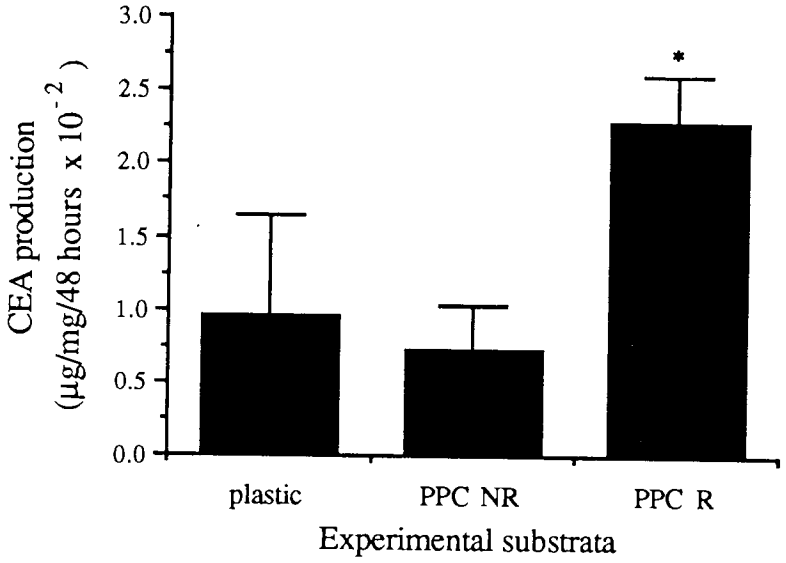


Figure 56 : Production of CEA by HT-29$_2$ cells grown on PPC gels.
* t-test analysis P = 0.001 (n = 9 ; ± sd).

6.VI. The effects of collagen gel culture on the activity of aminopeptidase

6.VI.i Introduction

Aminopeptidase N (EC 3.4.11.2, APN), is thought to play an important role in the digestion and absorption of small peptides in the gut (Kim *et al.*, 1974) and it is the most abundant peptidase in the small intestinal microvilli (Kenny and Maroux, 1982). High levels of activity are also associated with foetal and malignant colonic epithelium (Lacroix *et al.*, 1984 ; Zweibaum *et al.*, 1984), but activity is absent in normal adult colon. This differential expression of aminopeptidase activity has lead to the use of this enzyme as a functional marker of small intestinal differentiation.

The demonstration of aminopeptidase activity was shown by two methods, one biochemical (Roncari and Zuber, 1968), and the other, immunocytochemical using a mouse monoclonal antibody against human aminopeptidase N, kindly provided by Hauri *et al.*, (1985).

In light of the morphological changes previously observed (section 6.III) the hypothesis was tested that the culture of HT-29_2 cells on collagen gels may enhance the activity of aminopeptidase N.

6.VI.ii Results

P_2 membrane fragments were prepared from cells grown on the various different substrata for 6 and 12 days and these assayed biochemically for APN activity. K562, human chronic myelogenous leukaemia cells were first used as a negative control for each of the assays but it will be shown that these cells did have activity and therefore were not a suitable negative control.

A typical calibration curve is shown in figure 57, which was constructed using standard leucine aminopeptidase N enzyme (Sigma, England) diluted in phosphate buffer/TX-100. The calibration curves showed good coefficients of correlation and were considered to show suitable linearity over the range of activities used.

The detrimental effects of storage of the P_2 membrane fragments at +4 °C and -20 °C over night are illustrated by figures 58 and 59 (respectively). Storing the membrane fragments at -20 °C caused the greatest reduction in aminopeptidase activity, but storage

at +4 °C was also damaging. It was decided that the aminopeptidase activity of the membrane fragments would be determined on the same day of extraction to avoid the detrimental effects of storage. The fragments were stored at +4 °C until assayed.

Figure 60 shows the activity of aminopeptidase in membrane fragments extracted from cells grown on different substrata for 6 and 12 days. K562 cells were included in each assay as a negative control as it was believed that they would have negligible activity (personal communication with Mr. I. Hassan, Ciba-Geigy, Horsham). However, these leukaemic cells were shown to have activities similar to those of the HT-29_2 cells. They therefore did not serve as a good negative control and there was no other cell line available. It would appear from the figure that the activity of the K562 also significantly (t-test analysis: $P = 0.014$) increased form day 6 to 12. However, these cells were provided randomly from the tissue culture laboratory stocks and were therefore not necessarily incubated for 6 or 12 days. The trend seen was entirely due to cell variations observed for the activity of aminopeptidase of K562 cells.

On day 6 the activities of aminopeptidase of cells grown on all of the various collagen substrata were significantly lower than the activity of the cells on plastic substrata. There were also no significant differences in the individual activities of cells on the four collagen substrata (i.e. non passaged collagen gels (Non-PPC NR and R) and passaged collagen gels (PPC NR and R). However, by day 12 of culture there were significant elevations in activity of aminopeptidase of all cells cultured on collagen gels when compared with control cells cultured on plastic substrata (see figure 60 legend). There were no significant differences observed between cells on either Non-PPC and PPC gels, or between NR and R gels of each group. The collagen gel was therefore exerting a similar enhancing effect on all of the cells; that the culture on floating collagen gels (R) did not produce any additional inductive effects. The permanent passaging of cells (PPC) on collagen gel was unable to increase the activity of aminopeptidase above that of the cells cultured on non-passaged collagen gels (Non-PPC).

An alternative method was used to demonstrate aminopeptidase activity. This involved the use of a monoclonal antibody (HBB3/153/63) and the immunocytochemical localization of aminopeptidase in samples of cells embedded in wax and sectioned for

light microscopy. This was performed as described in section 3.II.ii. Controls were performed during the immunocytochemical assays to demonstrate the selectivity of staining and to isolate areas of non-specific staining. The controls were carried out on sections of formal-saline fixed normal human small intestine and for each control one of the following steps was omitted :

1) incubation with primary antibody (HBB3/153/63).
2) incubation with secondary antibody (Dako Z259).
3) incubation with tertiary antibody (Dako APAAP complex D651).
4) incubation with substrate solution 1 (see section 2.II.vi).

In each case the samples were incubated with the appropriate buffer, or in the case of control number 4 the substrate solution minus the substrate naphthol AS-MX phosphate. Also, a positive control of normal human small intestine was used in which all of the incubation steps were performed. Figure 61 shows the results of these control procedures for the localization of aminopeptidase, which was demonstrated by the red/pink stain against the blue counterstain of the haemotoxylin. It can be clearly seen that there was some non-specific staining in the control samples in which steps 1, 2 and 3 were omitted (figure 61 (a), (b), and (c)). Only by omitting the substrate (control number 4) was non-specific staining prevented completely. The activities seen in figure 61 (a) and (b) can be explained by the non-specific binding of the secondary and tertiary antibodies to the small intestinal epithelial cells. However, another explanation will be presented later in this section. The stromal cells showed no staining either specific or non-specific.

A particularly worrying observation was the activity seen in figure 61 (c) in which the tertiary antibody APAAP complex was omitted. It was this antibody which provided the conjugated intestinal alkaline phosphatase whose activity resulted in the production of the red staining which localized the antigen being studied. Therefore, the production of staining in the sample in which this antibody-enzyme complex had been omitted was unexpected. The cells of the small intestinal epithelium have high endogenous levels of the brush border associated enzyme alkaline phosphatase (intestinal). The substrate solution contained levamisole, a known inhibitor of alkaline phosphatase, to prevent any

endogenous non-specific activity interfering with the activity of the antibody-enzyme complex. However, levamisole is not a particularly effective inhibitor of intestinal alkaline phosphatase which explained its use in the substrate solution. For this reason the APAAP technique was not designed to be used for the immunolocalization of antigens in samples of intestine whose endogenous enzyme is resistant to levamisole inhibition. Fixation with formal saline (10%), tissue processing and embedding in paraffin wax should have resulted in the complete loss of endogenous alkaline phosphatase activity (Bitensky and Poulter, 1969). This information that lead me to believe that this technique would be valid for the detection of antigens in our tumour cell and control intestinal samples which were processed in this way. I would therefore suggest that the samples of small intestine were non-specifically stained as a result of endogenous alkaline phosphatase activity which remained following tissue processing, due to the extremely high original levels of activity. The effects of endogenous activity in the samples of HT-29_2 cells were considered to be negligible as it would be more likely that the general low level of activity seen in these cells (when compared to the small intestine) would have been completely destroyed by the tissue processing.

The control sample in which all of the steps were performed (figure 61 (e)) showed a greater degree of staining than the other negative controls and the activity was localized to the apical surfaces of the enterocytic cell lining the villi. Staining intensity was reduced in the crypt cell region. This was where the activity of aminopeptidase was expected and this confirmed its use as a marker of enterocytic differentiation. The was also more cytoplasmic staining seen in these cells when compared to the non-specifically stained negative control samples. The degree of non-specific staining must always be borne in mind when interpreting the results of the immunocytochemistry carried out on the actual HT-29_2 samples.

HT-29_2 cells were grown on the different substrata for 7, 14, 21, and 28 days and then samples fixed and prepared for immunocytochemistry as described in section 3.II. The immunolocalization of aminopeptidase was performed on all the samples on the same day, with the same antibody and substrate solutions in order to ensure minimal experimental variation, which is particularly critical with a procedure that provides

qualitative data of a visual nature. Figures 62 and 63 show the results of the immunocytochemical staining of HT-29_2 cells grown on various substrata for 7 and 28 days respectively. The results for days 14 and 21 showed the same trends (data not shown).

The samples prepared on day 7 of culture show higher cytoplasmic staining in the cells grown on plastic (figure 62 (e)) than the cells grown on the various collagen gels. This supports the biochemical data for day 6 (figure 60) of culture, where the activities of aminopeptidase were significantly lower in the cells grown on collagen gels. However, after 28 days of culture on the various substrata, although all of the levels of staining has increased from that observed on day 7, the levels were still lower in cells grown on collagen gels (figure 63). The staining was very intense in the cells grown on plastic (figure 63(e)) and considerably lower in the cytoplasms of cells grown on collagen gels (figure 63 (a),(b),(c), and (d)). Similar observations were obtained for the cells cultured for 14 and 21 days, with the cells on collagen displaying lower staining levels than the cells on plastic. Non-specific staining of the collagen gel was seen in all samples from each day of sampling.

These results therefore contradict the results obtained biochemically on day 12 (figure 60), which showed that the cells grown on collagen gels displayed significantly higher activities than the cells on plastic. One possible explanation that can be offered for this discrepancy in results is that the cells on collagen were producing a biochemically active enzyme which was detected by the biochemical assay, but which was not antigenically detected by antibody HBB3/153/63. Alternatively, the process of fixation may have damaged the antigen aminopeptidase resulting in minimal immunocytochemical detection. However, this would not explain the staining seen in the cells grown on plastic substrata, which would have been expected to have been similarly damaged by fixation.

The negative staining of the cells grown on collagen gels, which have been shown to have similar or higher levels of endogenous alkaline phosphatase activity (section 6.IV.) than the cells grown on plastic, supports the earlier suggestion that the staining seen in our cell samples was not due to endogenous alkaline phosphatase. If that had been the case the cells on Non-PPC gels would have been expected to display similar levels of

staining as those on the plastic and the cells on the PPC gels would have been expected to display a higher level of staining representing aminopeptidase activity.

6.VI.iii Discussion

The results of the biochemical assay would suggest that the culture of HT-29_2 cells on collagen gels for 6 days resulted in a significant reduction of aminopeptidase activity when compared with the cells cultured on the plastic substrata. However, by day 12 there was a significant enhancement in activity of all cells grown on collagen gels (i.e. both Non-PPC NR and R, and PPC NR and R). Culture on a released or passaged collagen gels did not enhance activity. The collagen gel alone was sufficient to enhance the aminopeptidase activity above that of the control cells cultured on plastic substrata.

The immunocytochemical detection of aminopeptidase in cells grown on the various substrata unfortunately provided contradictory data. On each of the four experimental time points (7,14,21, and 28 days) the cells cultured on collagen gels displayed consistently lower levels of staining for aminopeptidase than cells cultured on plastic. A possible explanation may be that the aminopeptidase expressed by the cells cultured on collagen was not antigenically detected but remained biochemically active in the aminopeptidase assay. Another, possible explanation was that the aminopeptidase was concentrated in the membrane fragments of the cells cultured on collagen giving a higher biochemical activity, but was not detected immunocytochemically because of poor resolution at the LM level. The control cells on plastic, on the other hand, showed higher levels of cytoplasmic staining by immunocytochemistry, which would have been lost during the preparation of the P_2 membrane fragments for the biochemical assay, resulting in lower levels of biochemical activity in these cells. The discrepancy in results from the two assays may therefore be due to the distribution of the enzyme i.e. cytoplasmic versus membrane-bound. In order to resolve this problem it would have been necessary to do immunolocalization at the EM level, and biochemical assays using whole cell preparations to show total cell activity, which was not possible to perform within the time constraints of this research.

Therefore, from the results obtained in this section by the two different methods, it was very likely that collagen gel culture enhanced the activity of membrane-bound aminopeptidase above that of the control cells grown on plastic. However, the results of the immunocytochemical analysis are difficult to interpret particularly in light of the biochemical data. It would appear that the levels of cytoplasmic aminopeptidase were lower in the cells cultured on collagen gel. The contradictory nature of the results make it difficult to state categorically what effect collagen culture had on the activity of aminopeptidase of HT-29_2 cells without further studies to define the exact distributional variations of aminopeptidase in the cells.

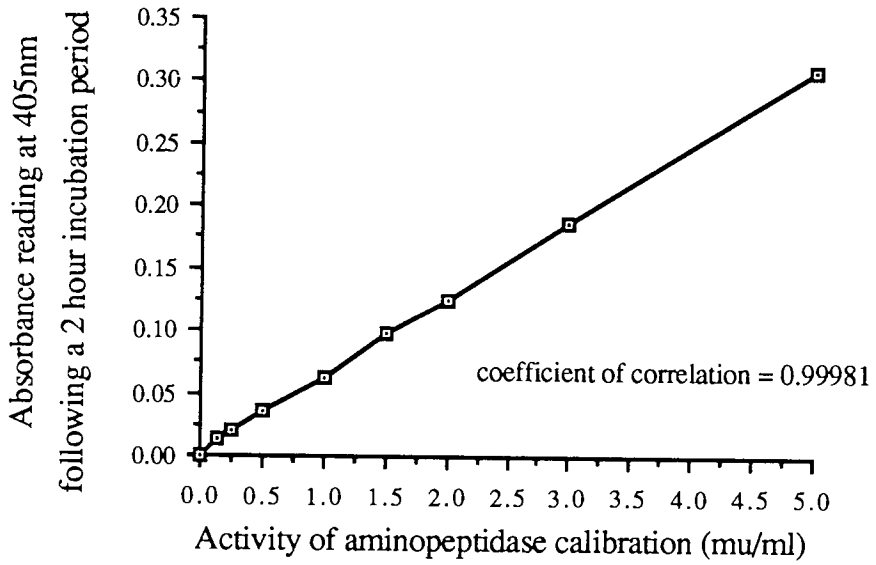


Figure 57 : A typical calibration curve for the biochemical determination of aminopeptidase activity.

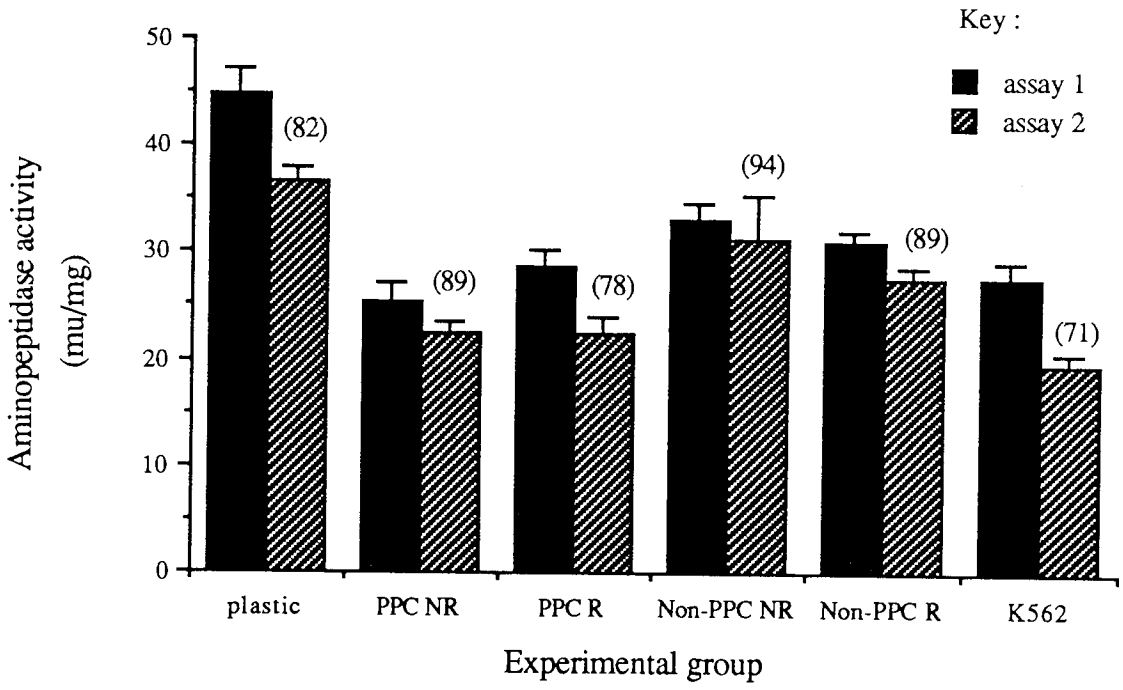


Figure 58 : Effect of storage of the P_2 membrane pellets at +4°C over night on aminopeptidase activity.
Numbers in parentheses are the values expressed as a percentage of first assay value. (n=3; ± sd).

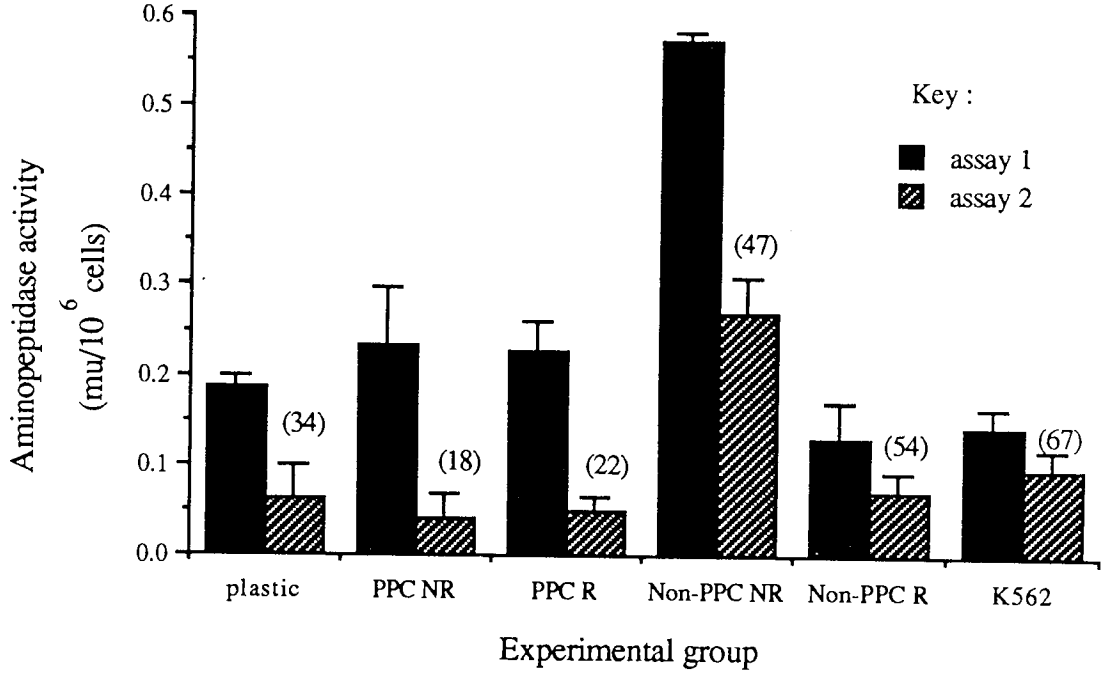


Figure 59 : Effect of storage of the P_2 membrane pellets at -20°C over night on aminopeptidase activity.
Numbers in parentheses are the values expressed as a percentage of first assay value. (n=3; ± sd).

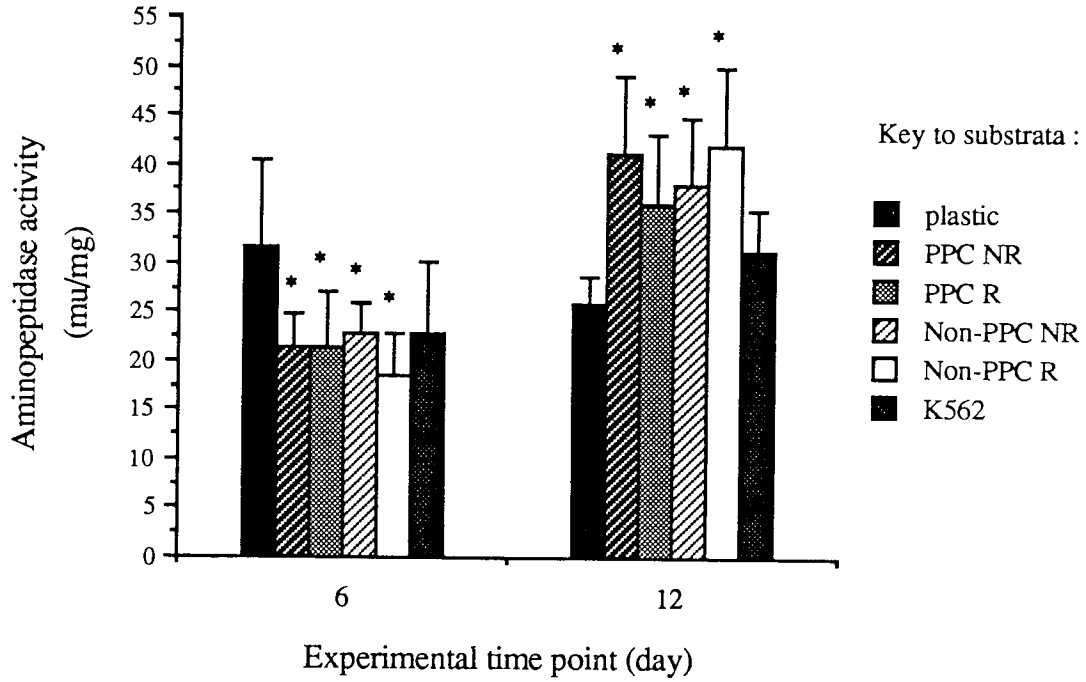


Figure 60 : Aminopeptidase activity of HT-29_2 cells grown on various substrata for 6 and 12 days.
Values are the means of several experiments (n = 9; ±sd).
* = results were significantly different from those of the cells grown on plastic. P values were from left to right : 0.009, 0.018, 0.022, 0.002, 0.000, 0.001, 0.000, 0.000.

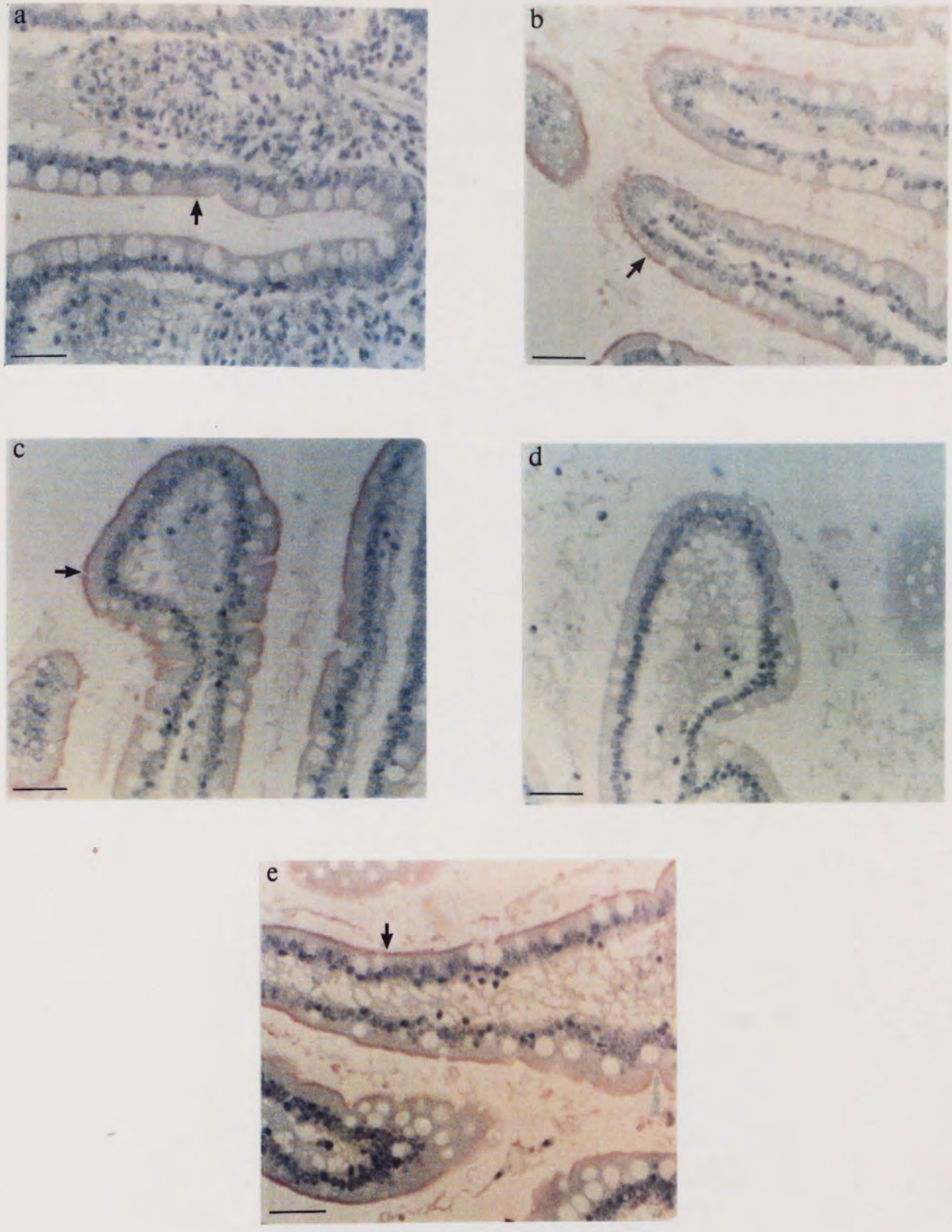


Figure 61 : Light micrographs of human small intestinal epithelium stained immunocytochemically for aminopeptidase.
Positive and negative controls performed as part of the immunocytochemical procedure : a) no primary antibody, b) no secondary antibody , c) no tertiary antibody , d) no substrate, and e) positive control with all steps performed. (arrows) pink staining localizing aminopeptidase. *Scale bars*, 20µm.

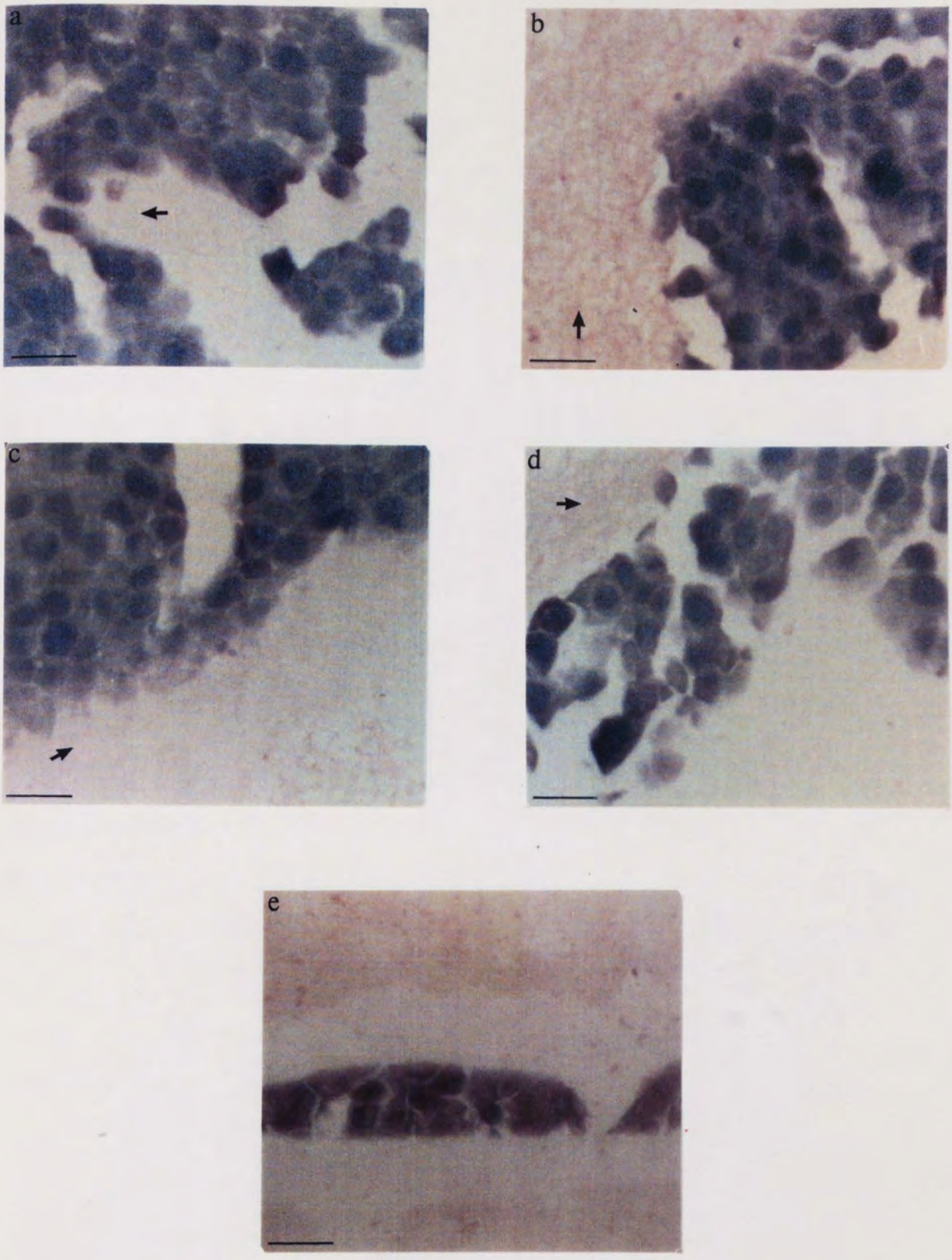


Figure 62 : Light micrographs of HT-29_2 cells grown on various substrata for 7 days, stained immunocytochemically for aminopeptidase.
Substrata :
a) Non-PPC NR, b) Non-PPC R, c) PPC NR , d) PPC R, and e) plastic.
(arrows) non-specific staining of collagen. *Scale bars*, 10μm.

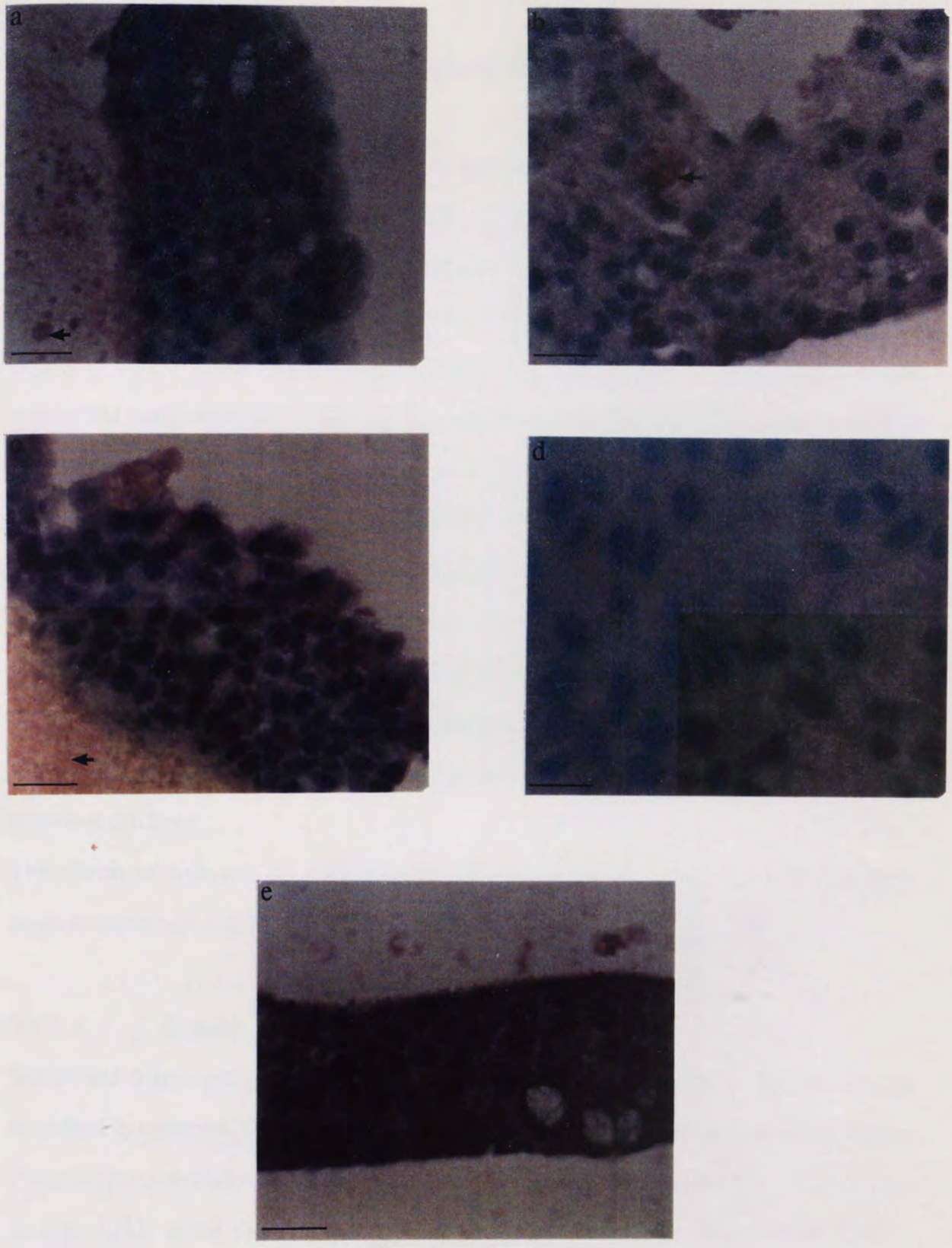


Figure 63 : Light micrographs of HT-29_2 cells grown in various substrata for 28 days, stained immunocytochemically for aminopeptidase.
a) Non-PPC NR, b) Non-PPC R, c) PPC NR , d) PPC R, and e) plastic.
(arrows) non-specific staining of collagen. *Scale bars*, 10μm.

6.VII The effects of collagen gel culture on activity of sucrase-isomaltase

6.VII.i Introduction

Sucrase-isomaltase (EC 3.2.1.48, SI) is a brush border associated hydrolytic enzyme which is responsible for up to 90 % of the microvillar maltase activity (Hauri *et al.*, 1985). It is one of the best characterized marker enzymes of the enterocytic villus cells of the adult small intestine (Kenny and Maroux, 1982). Activity has been observed to be higher in human malignant colonic epithelial cells than in normal adult colon, a result which was unexpected due to the lack of enzyme in adult colon (Zweibaum *et al.*, 1983). The expression of sucrase-isomaltase in malignant colonic cells, including HT-29 xenografts (Zweibaum *et al.*, 1983), suggested that these tumours were of foetal origins as this enzyme is expressed in developing colon (Grand *et al.*, 1976 ; Skovberg, 1982 ; Triadou and Zweibaum, 1985; and Mernard and Pothier, 1987). The activities of both maltase and sucrase have been previously shown to increase in HT-29 cells which has been induced to differentiate by glucose deprivation (Pinto *et al.*, 1982). It is often used as a marker of enterocytic differentiation when studying the *in vitro* differentiation of intestinal cell lines.

The effects of collagen gel culture on the levels of immunocytochemically detectable sucrase-isomaltase in HT-29_2 cells were therefore determined.

6.VII.ii Results

Additional 4 μm sections were cut from the samples embedded in paraffin wax as described in section 6.VI for the demonstration of aminopeptidase. These sections were immunocytochemically stained for the localization of sucrase-isomaltase as described in section 3.II.ii using the monoclonal antibody HBB 2/219/20 (Hauri *et al.*, 1985). Controls were performed during the immunocytochemical assays to demonstrate the selectivity of staining and to demonstrate any non-specific staining. The controls were carried out on sections of formol-saline fixed normal human small intestine and for each of the controls one of the following steps was omitted :

1) incubation with primary antibody (HBB 2/219/20).
2) incubation with secondary antibody (Dako Z259).*
3) incubation with tertiary antibody (Dako APAAP complex D651).
4) incubation with substrate solution 1 (see section 2.II.vii).

The controls were incubated with the appropriate buffer alone, and control number 4, with substrate solution minus the substrate, naphthol AS-MX phosphate. Also, as a positive control normal human small intestine sections were processed with all of the steps performed. Figure 64 shows the results from these control procedures for the localization of sucrase-isomaltase (* control number 2 was lost during processing). Control numbers 1 and 3 show low levels of non-specific staining (figure 64 (a) and (b)), localized to the apical surfaces of the villus enterocytes. Control number 4 shows no non-specific staining (figure 64 (c)) and the positive control sample displays considerably higher intensity staining of the villus enterocytes than all of the negative controls. This specific staining was localized in the cytoplasm as well as at the apical surfaces. The stromal cells show no staining in any of the samples, either specific or non-specific. The non-specific staining in the negative controls was much less intense than that observed during the immunolocalization of aminopeptidase (section 6.VI). Again, the non-specific staining in sample number 3 was thought to be due to low levels of endogenous activity which remained after tissue processing, which in theory should have destroyed all of the activity in the samples of small intestine. The nature of the staining was distinctly different in the positive control, with the intensity being much greater and being more widely distributed in the cytoplasm, as well as at the apical surfaces of the villus enterocytes.

The samples of HT-29_2 cells grown on various substrata used in section 6.VI were also used for the immunolocalization of sucrase-isomaltase, with fresh sections being cut from the wax blocks of tissue. The procedures followed were as described in section 6.VI, and only the results of the day 7 and 28 samples are presented (figures 65 and 66). The samples prepared on day 7 of culture showed very low levels of cytoplasmic staining in the cells grown on plastic and non-passaged (Non-PPC NR and R) collagen gels and

almost no staining in the cells grown on the passaged (PPC NR and R) gels. There was non-specific staining of the collagen gel as previously seen during the immunocytochemical detection of aminopeptidase (section 6.VI). Although, all of the levels of staining were very low the levels were significantly lower in the cells grown on the passaged (PPC NR and R) collagen gels. The levels of staining gradually increased with time and figure 66 shows the results of the staining of samples prepared on day 28 of culture. All samples have an increased intensity of cytoplasmic staining when compared to that of the day 7 samples. The staining was more uniform in the cells cultured on plastic (figure 66 (e)), with that of the cells cultured on collagen gel being more sporadic, showing areas of more intense staining than others. Some areas of intense staining were almost certainly areas of collagen trapped between cells non-specifically stained with the antibodies. However, staining can be clearly seen in cells whose cytoplasmic outlines are easily discernible. All of the samples showed similar levels of staining intensity and this would therefore suggest that culturing HT-29_2 cells on collagen gel does not enhance the expression of sucrase-isomaltase.

6.VII.iii Discussion

The results would suggest that the level of sucrase-isomaltase expressed by HT-29_2 cells increased with time and was independent of the substrata on which the cells were cultured. The collagen gel substrata (either non-passaged (Non-PPC NR and R) or passaged (PPC NR and R) did not have any enhancing properties with regards sucrase-isomaltase expression in these cells. However, it would be essential that this immunocytochemical data be confirmed by biochemical data, but due to practical problems it was not possible to perform these experiments. It is hoped that these experiments will be completed in the future.

These results may reflect the fact that in order to express sucrase-isomaltase the cells need to undergo a more profound morphological differentiation than that seen morphologically in these cells. Electron immunocytochemical localization of the sucrase-isomaltase would be an excellent way to determine whether or not those more morphologically differentiated cells were also expressing sucrase-isomaltase at their

apical surfaces. Unfortunately there was not time to perform such studies which would have enabled more definite conclusions to have been drawn.

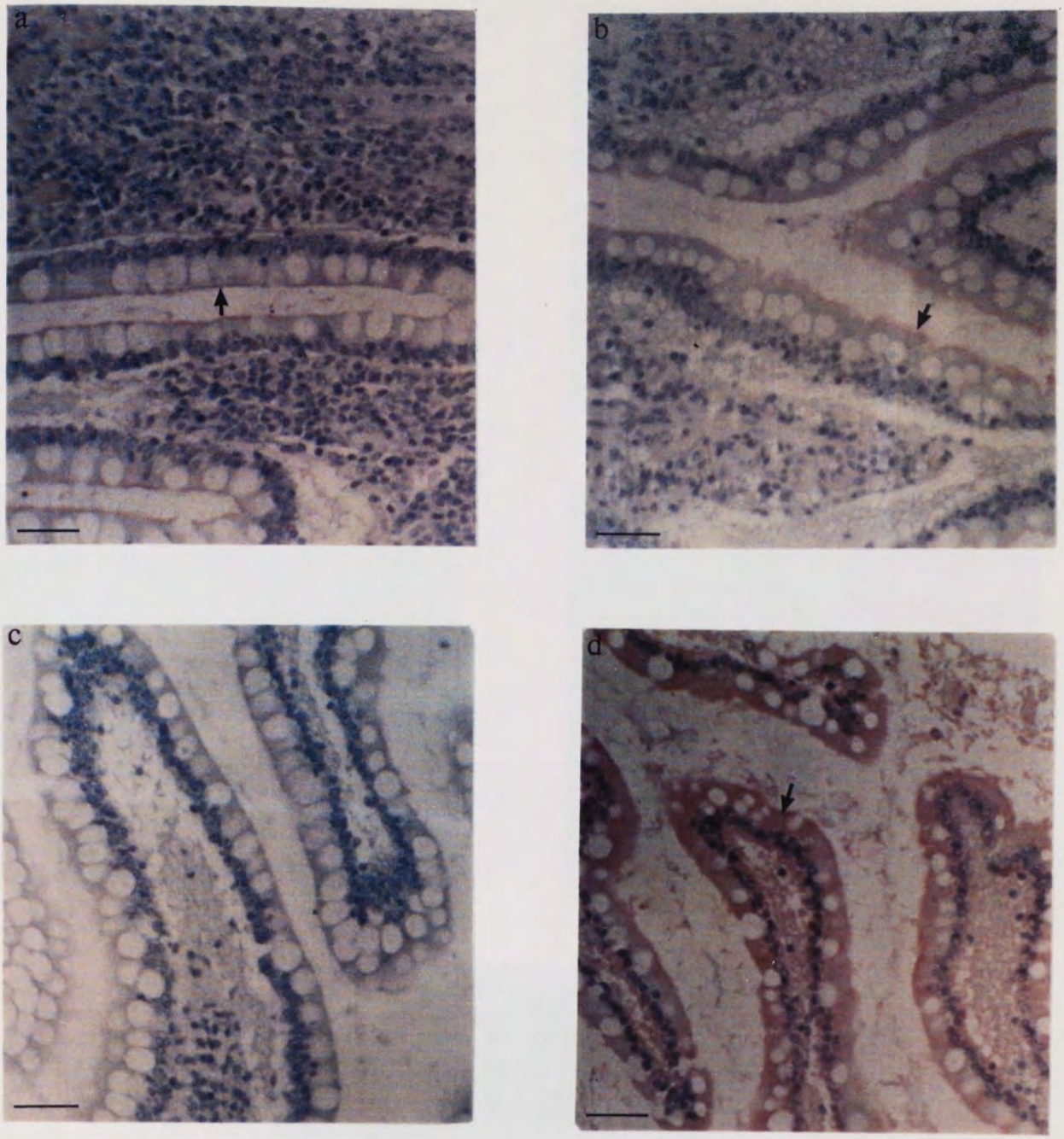


Figure 64 : Light micrographs of human small intestinal epithelium stained immunocytochemically for sucrase-isomaltase.
a) no primary antibody, b) no tertiary antibody, c) no substrate, and d) positive control with all steps performed. (arrows) non-specific staining. *Scale bars*, 20μm.

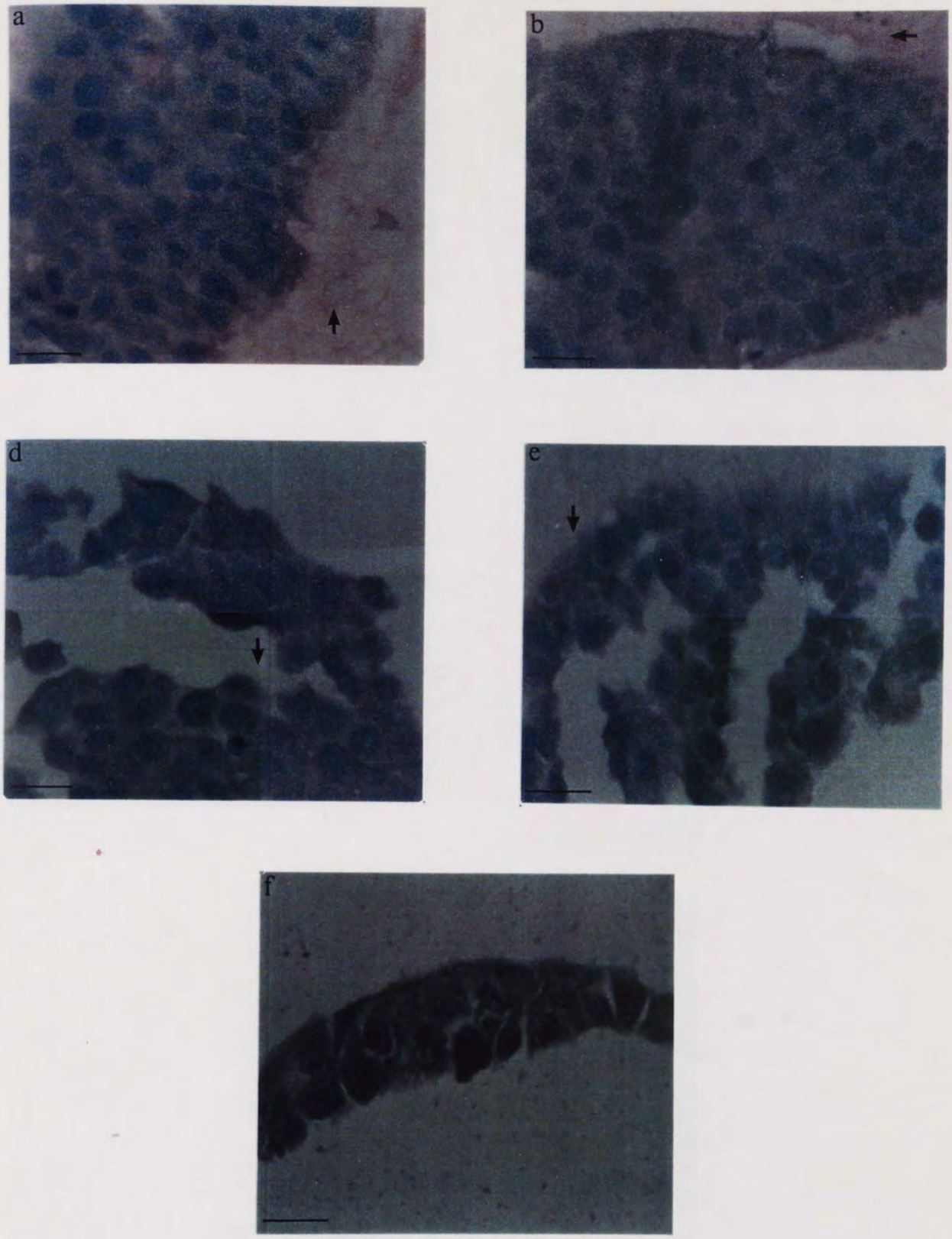


Figure 65 : Light micrographs of HT-29_2 cells grown on various substrata for 7 days, stained immunocytochemically for sucrase-isomaltase.
a) Non-PPC NR, b) Non-PPC R, c) PPC NR, d) PPC R, and e) plastic.
(arrows) non-specific staining of collagen. *Scale bars*, 10µm.

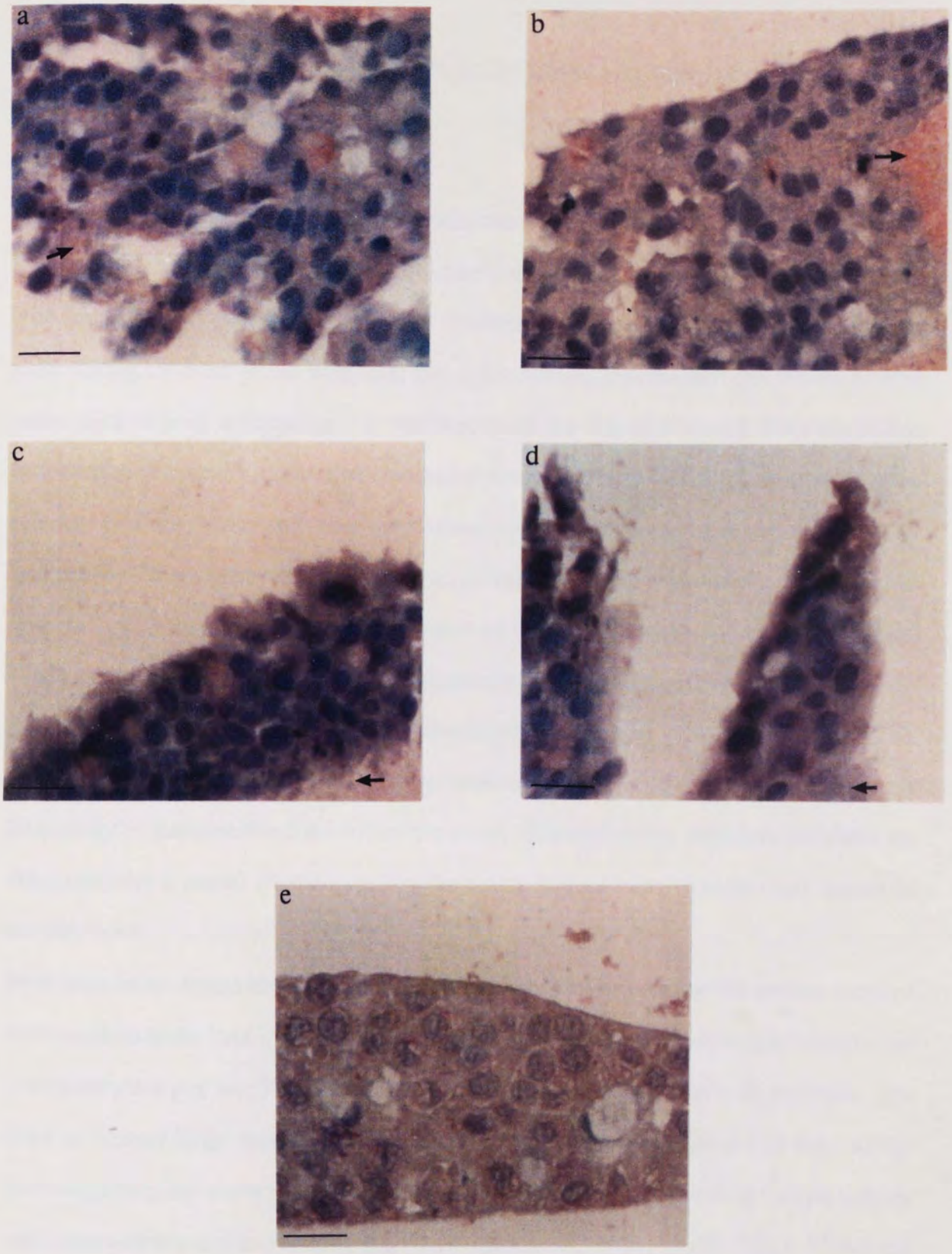


Figure 66 : Light micrographs of HT-29_2 cells grown on various substrata for 28 days, stained immunocytochemically for sucrase-isomaltase.
a) Non-PPC NR, b) Non-PPC R, c) PPC NR, d) PPC R, and e) plastic.
(arrows) non-specific staining of collagen. *Scale bars*, 10μm.

6.VIII The effects of collagen gel culture on the chemosensitivity of HT-29_2 cells to a panel of chemotherapeutic agents

6.VIII.i Introduction

Cancer is responsible for 22 % of all deaths in the most economically developed countries (Silverberg and Lubera, 1989) and therefore poses a serious problem for the clinician with regards to its successful treatment. The birth of cancer chemotherapy really took place during the First World War, with the chance finding that the nitrogen mustard nerve gases also caused leukopenia. It was not until the Second World War when this observation was made again, that research commenced that was to show that the side effects of these agents and their derivatives could be exploited for the treatment of leukaemia. Thus began the last 45-50 years of research into the anti-proliferative agents, with the use of some of these with mixed success in the clinic. Chemotherapy is essential in the treatment of cancer, because although surgery and radiotherapy can irradicate the primary tumour, these treatment regimes are limited at the sites of the distant metastases. Metastases often go undetected during their early stages of development and are frequently in inaccessible sites within the body. Chemotherapy therefore provides the clinician with a useful weapon against the metastatic tumours in their early stages of development.

New anticancer agents are synthesized with the hope of improving the present rates of therapeutic success and it is necessary that they are screened for their antitumour activity before they are put into Phase 1 clinical trials with human, terminally ill patients. The need to screen large numbers of potential chemotherapeutic agents has lead to the development of the use of *in vitro* screening assays using rapidly dividing human tumour cell lines, which are cost effective, quick and reproducible (Alley *et al.*, 1988). These cell lines are not necessarily representative of the tumours *in vivo* and this has lead to particular problems in selecting for drugs which are effective against the more slowly growing solid tumours. An abundance of antiproliferative agents exist which are highly effective against rapidly dividing tumours like the leukaemias and correspondingly these are the tumours with which the greatest success has been achieved. This can not be said for the solid tumours of which colorectal cancer is one. The most successful agent 5-FU,

can only produce response rates of 15-20 % (Mayer *et al.*, 1989). The problem may therefore lay in the choice of the screening system used to select for drugs useful in the treatment of the slower growing solid tumours. It has been known for sometime that when cells are removed from the tissue or tumour of origin to be cultured on tissue culture plastic or glass as adherent cell lines they loose almost all of their differentiated phenotype and specialized biochemical profile (Bissell, 1981). As Gregg (1972) stated : " The cell in culture is an adaptable organism; otherwise, it would not survive the environmental insults heaped on it by callous investigators". This is particularly relevant to the culture of polarized, epithelial cells such as those derived from intestinal tumours like the HT-29 cell line. When these cell lines adapt to their new environment they may through changes in gene expression loose their differentiated phenotype and develop a phenotypically changed pattern of drug sensitivity. Attempts have been made to improve the *in vitro* environment for cultured cells and this work, which has been extensively reviewed by Bissell (1982), has lead to the increased awareness of the role that the extracellular matrix plays in the control of induction of cellular differentiation and the maintenance of the differentiated state. However, the studies reviewed were primarily concerned with the effect that altering the culture environment would have on morphological and biochemical differentiation of any particular cell line studied. Altered cell response to exogenous agents has mainly dealt with responses to hormones e.g. lactogenic hormones and the culture of mammary epithelial cells on collagen and EHS (Emerman *et al.*, 1981; Lee *et al.*, 1984,1985; Schmidhauser *et al.*, 1990). There has been a study performed to assess the use of collagen gel culture of rat mammary tumour cells for determining the efficacy of anticancer agents (Sinha and White, 1988). The authors evaluated the efficacy of three commonly used anticancer agents for breast cancer on rat mammary epithelial tumour cells on collagen gels and compared the results to those of the *in vivo* efficacy. Their results suggested that collagen gel culture provided a useful system for determining drug efficacy. However, they did not determine the chemosensitivity of their model system when cultured on plastic substrata and thus could not compare the results with those obtained with the cells cultured on collagen gels. The use of cells cultured on plastic substrata for assays of toxicity *in vitro* is the chosen

system for screening new agents and to date this has proved to be unhelpful with regards to the discovery of new agents for the treatment of solid neoplasms.

The methods for determining the toxic effects of novel anticancer agents involve the culture of the human malignant cell lines *in vitro* and exposure to a range of concentrations of test agent over a fixed time. The number of cells which remain at the end of the test exposure time is then compared to that of the control, untreated cells. It is usual to construct a concentration-response curve for each agent tested, for each cell line used. It is then possible to determine the concentration of the agent which is required to kill 50% of the cells or the IC_{50} for that agent under those conditions. It is also standard practice to perform clonogenic assays on those remaining cells which have survived the treatment. Clonogenic assays are based on those first described by Hamburger and Salmon (1977a, 1977b). Clonogenic assays aim to determine the clonogenic or proliferative potential of those cells which remain after treatment. Clonogenic cells are thought to be representative of the population of stem cells which remains in the *in vivo* tumour, following chemotherapy, which can contribute to the repopulation of the tumour and thus cause a relapse (Mackillop *et al.*, 1983). However, clonogenic assays have several flaws which will be discussed fully in the results section (6.VIII.ii.a).

The aim of this section of work was to determine whether the culture of HT-29_2 cells on collagen gels could effect their chemosensitivity to a panel of known anticancer agents when compared to cells cultured on tissue culture plastic.

6.VIII.ii Results

Initial experiments were performed in order to determine the toxicity of each agent to HT-29_2 cells cultured on tissue culture plastic. These experiments aimed to determine the IC_{50} of the agent and a useful range of concentrations to use during the experiments to determine whether the substrata upon which the cells were cultured could alter the chemosensitivity. It was essential to keep the number of test groups to be used to three, plus the control, untreated group of cells on each substrata (i.e. plastic, non-passaged collagen gel (Non-PPC NR and R), and passaged collagen gel (PPC NR and R)). This was because of the considerable cost of collagen and the need to be economic with its

was because of the considerable cost of collagen and the need to be economic with its use. Three concentrations of each test agent were selected and the toxicity of each to HT-29_2 cells cultured on the different substrata were determined as previously described in section 3.V . The results were expressed as :

i) mean total cell number remaining at the end of the exposure period,

ii) cell number as expressed as a percentage of control,

iii) plating efficiency of the remaining cells following treatment,

iv) and the plating efficiency expressed as percentage of control.

The four sets of data for each agent were plotted against the concentration of the agent and each of the four graphs will be presented for each agent within the text or in Appendix 1. Experiments were performed initially to compare the chemosensitivity of cells cultured on plastic, with those cultured on non-passaged collagen gels (non-released,Non-PPC NR, and released, Non-PPC R). The experiments were then repeated for each agent using cells cultured on plastic and passaged on collagen gels (non-released, PPC NR, and released, PPC R) in order to assess the effects that continuous culture on collagen gel had on chemosensitivity. The results obtained for each agent will be discussed separately. A small introductory paragraph about the agent will be included at the beginning of the discussion of the results obtained for that agent. The four graphs each for cells cultured on non-passaged and passaged collagen gels will then be presented either in the text or in Appendix 1. This will be followed by a general summary and discussion. First, there will be a discussion of the problems that were encountered using the clonogenic assay as a measure of proliferative potential following cytotoxic treatment.

6.VIII.ii.a Clonogenic analysis of proliferative potential

The clonogenic assay used in this series of experiments was an adaption of the original human tumour colony assay (HTCA), which was developed by Hamburger and Salmon (1977a and 1977b). Their assay was an *in vitro* culture system which used semisolid medium support for the culture of colonies of cells and was used originally to investigate the growth and chemosensitivity of clonogenic tumour cells derived from human myelomas. There have been extensive studies which have assessed whether there is a correlation between *in vitro* chemosensitivity of tumour biopsies and the response of patients with metastatic tumours to chemotherapy. These studies have shown that HTCA has had a 71% true-positive rate and a 91% true-negative rate for prediction of the drug sensitivity and resistance, respectively, of cancer patients to specific antineoplastic agents (Salmon, 1984). The predictive values were lower when the assay was performed in a murine adenocarcinoma of the colon (MAC) system, with these experiments giving a 44% true-positive rate and a 85% true-negative rate for drug sensitivity and resistance, respectively (Phillips *et al.*, 1990). However, the predictive value of this clonogenic assay using the MAC system was still consistent with those performed with material derived from actual cancer patients, in that the drug resistance was predicted with greater accuracy than the drug sensitivity. There are several problems associated with the use and interpretation of clonogenic assays which strictly follow the original Hamburger and Salmon protocol and with those which are adaptions of that original method (Selby *et al.*, 1983). Only those that are pertinent to the cloning of HT-29_2 cells will be discussed here.

The most important problem which was encountered with the use of the adapted clonogenic assay with all of the HT-29 cells which were used was the inconsistent number of colonies produced by the control cells. Often the numbers of control colonies were too low to be able to detect any detrimental effects due to the exposure to the anticancer agent. This problem occurred in the first batch of HT-29 cells used (HT-29_1) as previously described in Part Two (sections 5.II and 5.III), but was attributed to the infection which they were later discovered to have been carrying. It was not possible to explain why the control plating efficiency was so inconsistent at the time of the

experiments. However, later work on the second batch of HT-29 cells, HT-29_2, which also showed control colony inconsistencies pointed to a more likely explanation for the problem with both cell lines. The sudden drop in control cell plating efficiency observed during the experiments to assess the effects of substrata on the chemosensitivity of HT-29_2 cells coincided with a change in the batch of foetal calf serum being used in the laboratory. Although all prospective sera was batch tested on all cell lines currently being used in the laboratory prior to selection of the most suitable one for all cell lines, these screening procedures of new sera only used growth kinetics as the test parameter. Colony formation was not assessed in the test batches of sera, as it was at that time thought that if the cells grew equally well in the test sera as in the existing batch (Imperial Laboratories, IMP 180414) as determined by doing parallel growth estimation studies, then their respective colonies would also grow optimally in that serum. This assumption proved to be wrong and although the HT-29_2 cells grew very well in the new batch of Imperial sera (Imperial Laboratories, IMP 5810714) the control colony formation was very low (figure 67). This resulted in the loss of a considerable number of experiments which had already been set up using this new batch of general laboratory serum. In order to repeat these experiments and to produce valid clonogenic data, a range of sera was tested from different companies in order to establish which one permitted optimal control colony formation as well as adequate cell growth. This batch was then to be used exclusively with the HT-29_2 cells for the remainder of the studies. Figure 67 illustrates the mean plating efficiencies of control, untreated HT-29_2 cells cultured in test sera from different companies (see the figure legend for the meanings of the abbreviations). 7 out of the 8 test sera produced plating efficiency values of less than 15%. Advanced Protein Products sera (APP AF1167, column 5) gave a better plating efficiency than the existing laboratory sera (IMP 180414, columns 6 and 7) which was used prior to the sudden drop in plating efficiency described here.

A second problem encountered when using the clonogenic assay was the inter-experimental variation in cloning efficiency. This is highlighted with the control cells on figure 67, columns 6 and 7, which show the mean control plating efficiency for HT-29_2 cells cultured in the same sera (Imperial Laboratories, IMP 180414) on two separate

occasions using cells from the same resurrection and within one passage of each other. The colonies were incubated in the same incubator and were incubated for the same length of time (14 days). However, the mean plating efficiencies were significantly different (t-test analysis, P = 0.000). The only way to minimize this biological variation in cloning efficiency was to produce as much raw data as possible by repeating the chemosensitivity assays a minimum of 3 times for each agent and to incubate 6 wells of colonies for each treatment group during each chemosensitivity assay. However, the data for the plating efficiencies produced during the chemosensitivity experiments which will be discussed in this section still showed large inter-experimental variations and consequently large standard deviations. Therefore, the results will be presented as the mean plating efficiencies versus concentration of chemotherapeutic agent, with the corresponding error bars (= standard deviation) and also as the plating efficiency expressed as percentage of control (= 100%) versus concentration of chemotherapeutic agent.

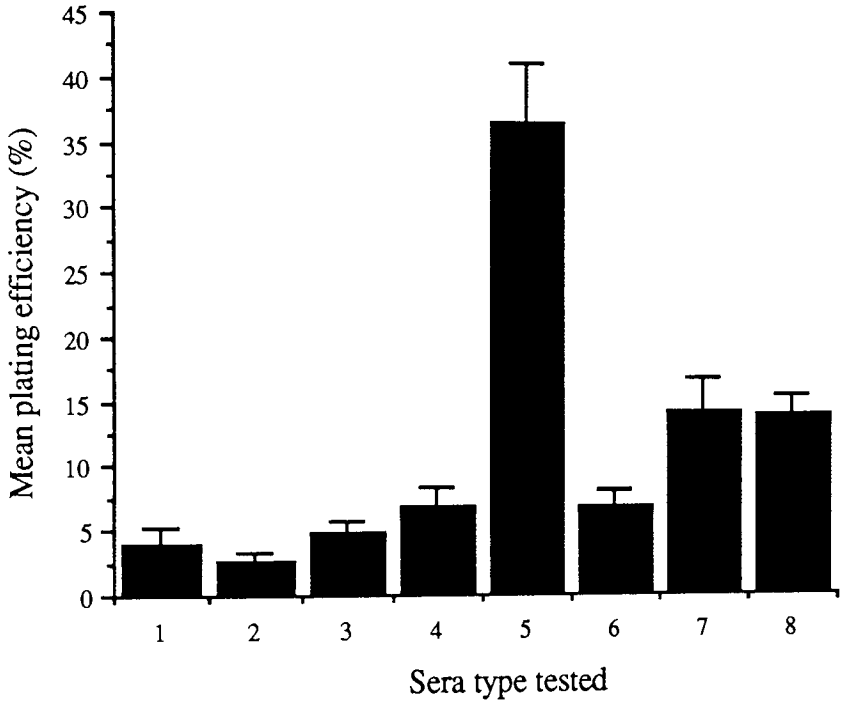


Figure 67 : Mean plating efficiencies of HT-29_2 cells cultured in 10% FCS from different sources. (n = 6 ; ± sd). Key to the source of sera :

1) Northern Biologicals (35923); 2) Northern Biologicals (30926);
3) Imperial Laboratories (5810714); 4)Imperial Laboratories (5810715);
5) Advanced Protein Products (AF1167); 6)Imperial Laboratories (180414)*;
7) Imperial Laboratories(180414)*; 8) Gibco (40G6394A).

* Same serum tested on two different passages of HT-29_2 cells on consecutive weeks.

6.VIII.ii.b Chemosensitivity of HT-29_2 cells to 5-Fluorouracil

The antimetabolite 5-Fluorouracil (Heidelberger, 1957, 5-FU) remains one of the most effective agents used in the treatment of adenocarcinoma of the colon even though it was first discovered over 25 years ago. However, it produces only partial response rates of 15-20% (Mayer *et al.*, 1989). It is a fluorinated pyrimidine, with a fluorine atom substituted for the hydrogen in the position 5 of the pyrimidine ring of uracil. The fluorine-carbon bond is extremely stable and prevents the addition of a methyl group which is necessary for the conversion of uracil to its thymine analogue, an important part of the DNA molecule. Thus, 5-FU prevents the incorporation of thymidine into DNA and consequently results in the inhibition of cell proliferation.

5-FU has been reported to have a half-life of 14 days in medium at 37°C (Hildebrand-Zanki and Kern, 1984) and was therefore administered only at the beginning of the 6 day treatment period. The IC_{50} for 5-FU against HT-29_2 cells was calculated to be 0.7 μM from initial experiments. Three concentrations of 5-FU, 0.77 μM, 3.85 μM, and 77 μM, were selected from these experiments which were performed on HT-29_2 cells cultured on plastic. These concentrations were shown to produce 39%, 12% and 4% cell survival following an exposure of 6 days, respectively. Only the results of the control, 0.77 μM and 3.85 μM treatment groups will be presented. This is because by including the results of the 77 μM treatment group, the x-axis scale is too elongated and the changes occurring in the lower dose groups are not clearly evident.

The HT-29_2 cells were seeded at a density of 5 x 10^4 cells/well on day 0, onto either plastic or collagen-coated plastic 24-welled plates. On day 1, the control media was removed, and replaced by media containing the appropriate concentration of 5-FU. The gels designated as "released" (R), were released from the plastic and floated in the medium. The cells seeded onto the plastic only were derived from stocks of HT-29_2 cells being routinely passaged in culture. The cells seeded onto the collagen gels were either i) seeded directly from cells obtained from cultures of HT-29_2 cells growing in plastic flasks (as for those on plastic) and were designated non-passaged collagen gel cells (Non-PPC NR and Non-PPC R), or ii) seeded from cells permanently passaged on either non-

released (PPC NR) or released (PPC R) collagen gels. The cells were exposed for 6 days, recovered from the substrata and the number of cells determined.

The chemosensitivity experiments performed to determine whether or not the substrata on which the cells were cultured could alter those cells responses to the chemotherapeutic agents tested, were carried out on the Non-PPC and PPC gel cells at different times. The initial experiments were performed on cells cultured on non-passaged collagen gels (Non-PPC) and following the lack of any overt substrata-dependent differences, passaging of cells on collagen gels commenced. After a minimum of 10 passages the cells were used in a second series of identical experiments performed to determine what effect extensive exposure to collagen had on the chemosensitivity of the HT-29_2 cells to the panel of anticancer agents. Each set of experiments had their own control cells cultured on plastic and because of the general variation in control cell response to the test agents during the two phases of this experiment, the data will be presented separately for the two groups of cells cultured on collagen (i.e. Non-PPC and PPC) for each agent discussed.

Figure 68 illustrates the effect that 5-FU had on HT-29_2 cells cultured on plastic and Non-PPC collagen gels (NR and R), expressed as a) mean cell number/well, and b) cell number expressed as percentage of control versus concentration of 5-FU. Figure 68(a), shows that the number of cells that remained following treatment were higher on the plastic substrata. This may suggest that the cells were more sensitive to 5-FU when cultured on either of the collagen gels (non-released, NR or released, R). However, when the results are expressed as percentage of control, untreated cell number (figure 68(b)) it can be clearly seen that this was not the case, with there being no substrata dependent differences in sensitivity to 5-FU. There was a slight elevation in percentage of remaining cells in the Non-PPC NR cells when compared to the plastic and Non-PPC R cells, but this was only at the lower concentration of 5-FU (0.77μM) (figure 68 (a)). The reason for the consistently lower numbers of cells derived from the collagen gels (NR and R) when compared with those cultured on plastic was possibly due to the difficulty of obtaining a 100 % retrieval of cells following removal of the collagen by

enzymic digestion. This phenomena was observed in both the non-passaged (Non-PPC) and passaged (PPC) collagen gel cultured cells during all of the chemosensitivity experiments and the regular estimations of growth rate.

Figure 69 illustrates the effects that 5-FU had on HT-29_2 cells during the second phase of experiments, cultured on plastic, and PPC collagen gels (NR and R), expressed as a) mean cell number/well, and b) cell number expressed as percentage of control, versus concentration of 5-FU. As with the initial experiments with the cells cultured on Non-PPC collagen gel (figure 68) there was no substrata dependent difference in the sensitivity of HT-29_2 cells cultured on plastic and PPC NR and R gels to 5-FU. There was slightly more variation in the cell number remaining after 5-FU treatment than was seen with the Non-PPC experiments (figure 68(a)) and this resulted in larger error bars, particularly for the 0.77μM treatment group. The results, when expressed as percentage of control suggested that the cells cultured on PPC NR gels (53% of control) may have been more sensitive to the 5-FU (figure 69(b)) than those cultured on plastic and PPC R gels (68% of control), after 0.77μM 5-FU. However, the inter-experimental variation observed resulting in the large errors at this concentration make it difficult to state that the cells cultured on PPC NR collagen gels were definitely more sensitive to 5-FU than the cells cultured on plastic or PPC NR collagen gels.

Figures 70 and 71 show the results of the clonogenic assays performed during the experiments to determine the chemosensitivity of HT-29_2 cells to 5-FU. The results are expressed as a) mean plating efficiency (%), and b) plating efficiency as expressed as percentage of control (%) for the cells cultured on plastic and non-passaged collagen gels (Non-PPC) (figure 70); and for the cells cultured on plastic and passaged collagen gels (PPC) (figure 71). It can be seen from both figures that the amount of inter-experimental variation was considerable. The results would therefore suggest that there was no substrata dependent differences in the effects of 5-FU on the clonogenic potential of the HT-29_2 cells, as assessed during the two phases of the experiments. However, a very interesting observation seen in both sets of experiments was the enhancement of clonogenic potential with the mildest concentration of 5-FU (0.77 μM) in all treatment groups (figures 70 and 71). This concentration resulted in a 35-50% reduction in cell

number (figures 68 and 69), but those remaining cells were able to clone better than the untreated control cells from all substrata groups. There was no definite trend with regards which substrata produced the greatest enhancement of clonogenic potential above that of the control, untreated cells, but the enhancement was clearly seen in all cases, ranging from 112 % to 166% of the control plating efficiency. The second concentration of 5-FU (3.85 μM) produced a reduction in clonogenic potential to approximately 40% of the control, as would be expected following a concentration which resulted in a cell kill of 80-85%. The enhancement of clonogenic potential of cells treated with a subtoxic concentration of 5-FU was unexpected and difficult to explain initially. However, it will be shown that this phenomena was seen repeatedly with the other anticancer agents studied except SRI 62-834 (see section 6.VIII.ii.f). The consistency of the phenomena suggested that it was in fact a true result and not just an artifact of the assay. This idea was also supported by the earlier results of clonogenic assays performed on the HT-29_2 cells following treatment with sodium butyrate and DMF (section 6.I). Although in these experiments the plating efficiencies were not enhanced above that of the control cells, they did remain remarkably high even after extremely toxic concentrations of agent. For example, a concentration of 150 mM DMF which caused a 92% reduction in cell survival resulted in a plating efficiency of 98% of the control value (figure 31). The idea that anti-neoplastic drug treatment may in fact increase the malignant behaviour of *in vivo* tumours and *in vitro* tumour cells, which could manifest itself in such a way that the clonogenic potential would be enhanced above that of the untreated cells, was introduced in section 6.I. It is hoped that it will be possible to suggest a plausible explanation for this apparent "mitogenic" effect of mildly toxic concentrations of anticancer agents on malignant cells incorporating these ideas. This explanation will be discussed in full in the discussion (6.VIII.iii) for this section.

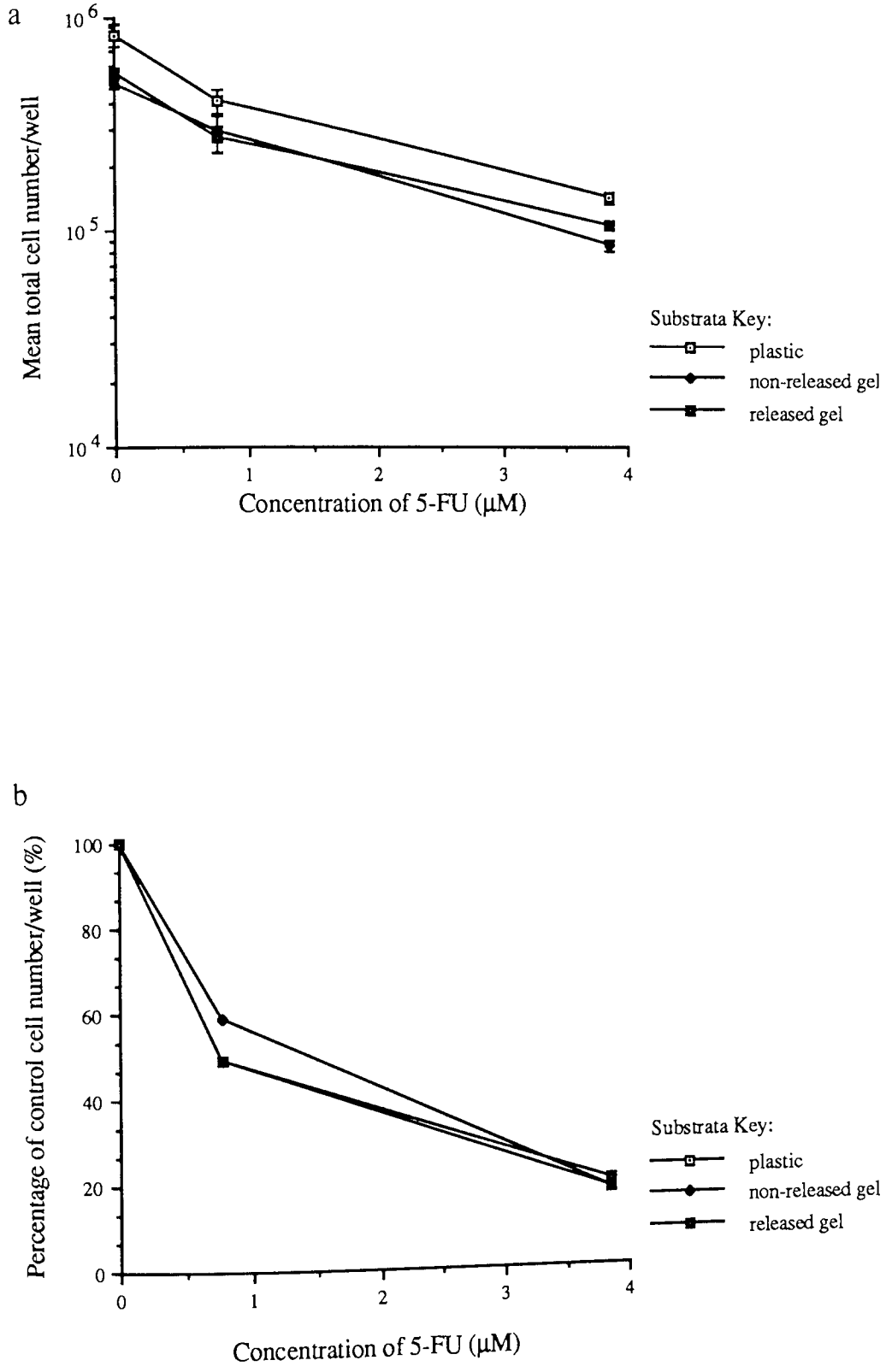
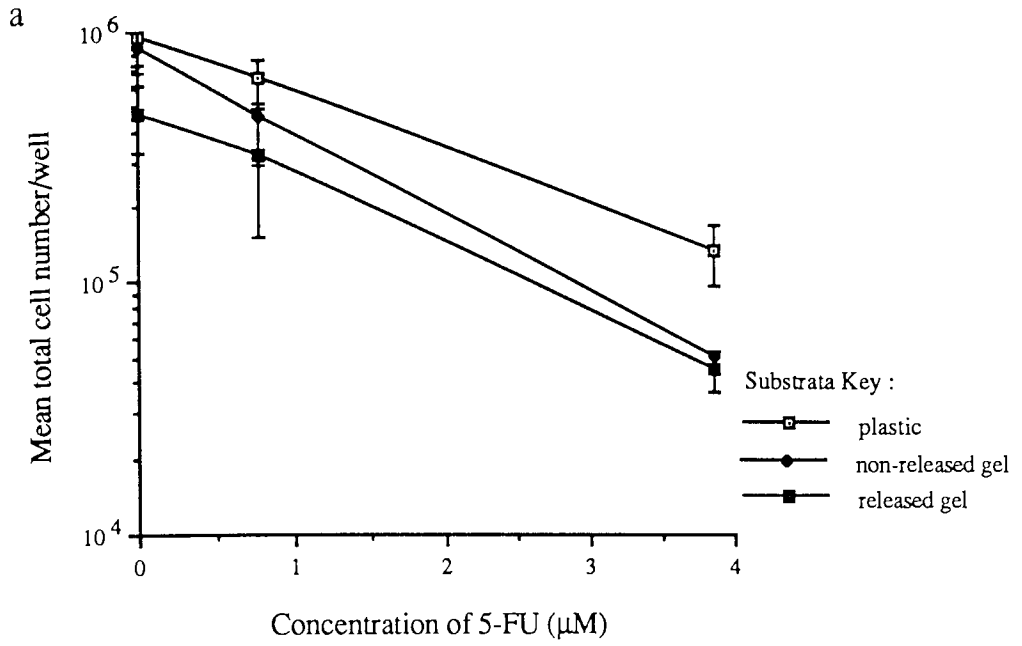


Figure 68 : Concentration-response curves for 5-FU toxicity to HT-29_2 cells cultured on plastic substrata and non-released (NR), and released (R) non-passaged collagen gels (Non-PPC).
Results expressed as a) mean total cell number/well following treatment for 6 days, and b) cell number expressed as a percentage of the control cell number/well (%) following treatment for 6 days. (n = 3 ; ± sd).



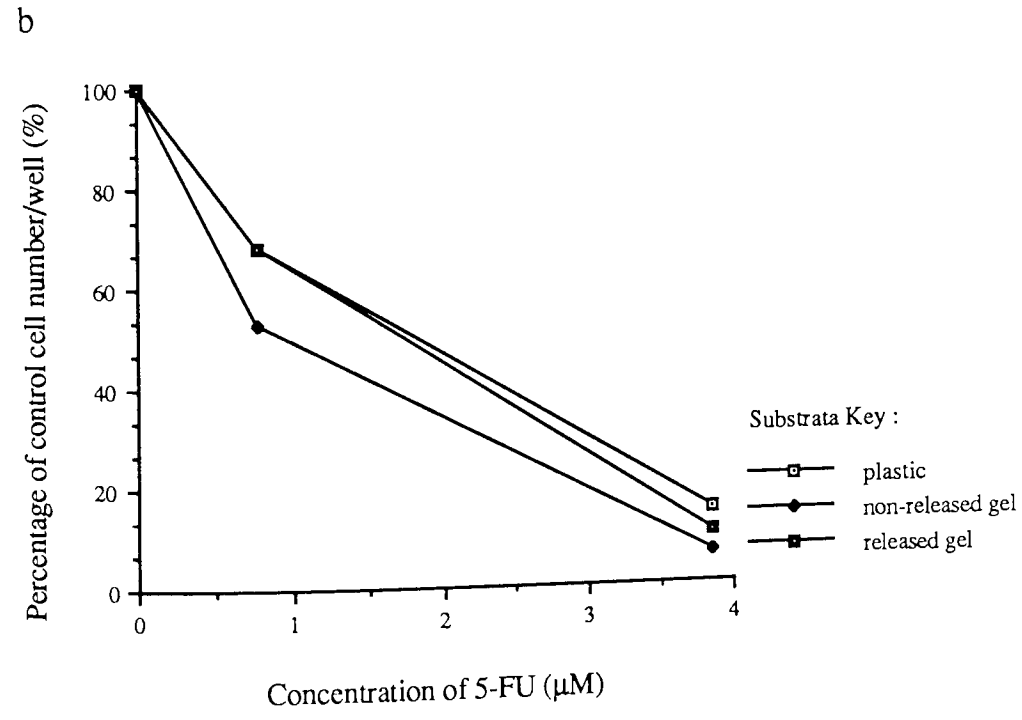
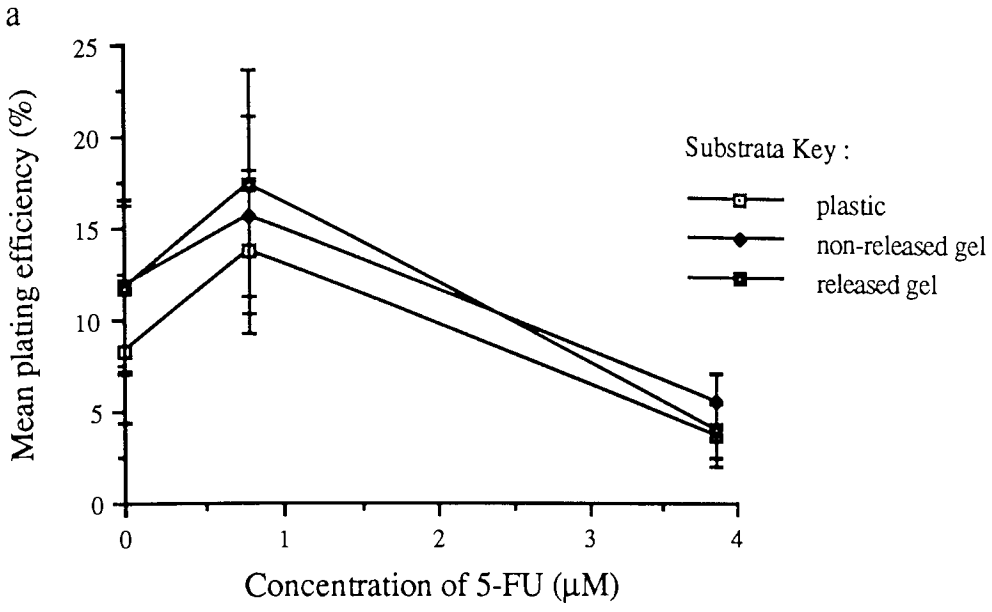


Figure 69 : Concentration-response curves for 5-FU toxicity to HT-29_2 cells cultured on plastic substrata and non-released (NR), and released (R) passaged collagen gels (PPC). Results expressed as a) mean total cell number/well following treatment for 6 days, and b) cell number expressed as a percentage of the control cell number/well (%) following treatment for 6 days. (n = 3 ; ± sd).



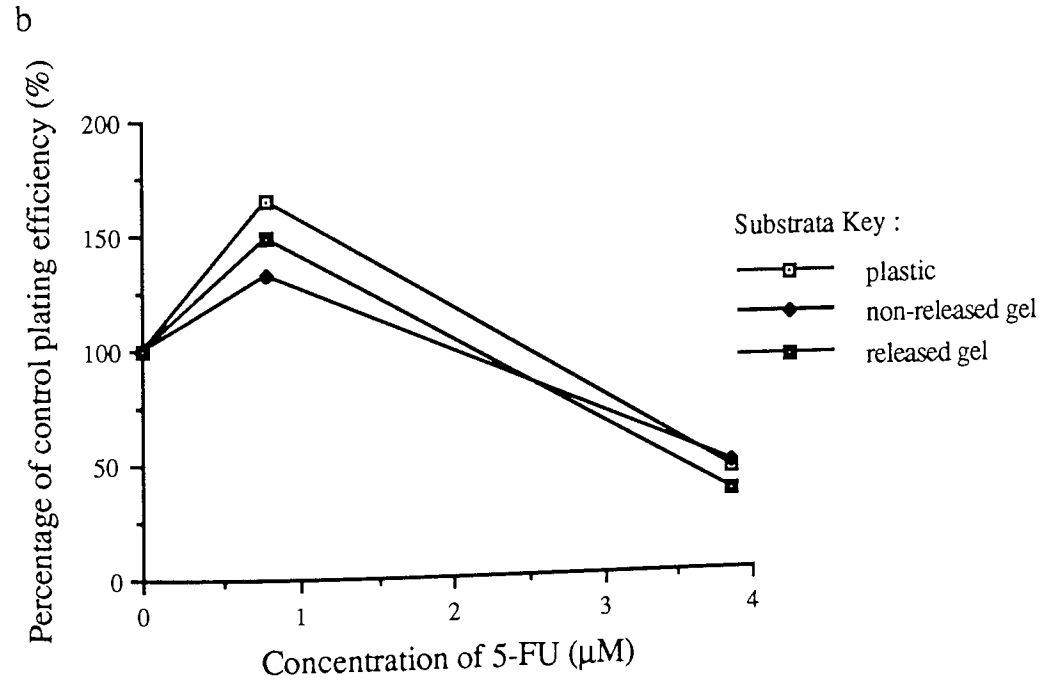
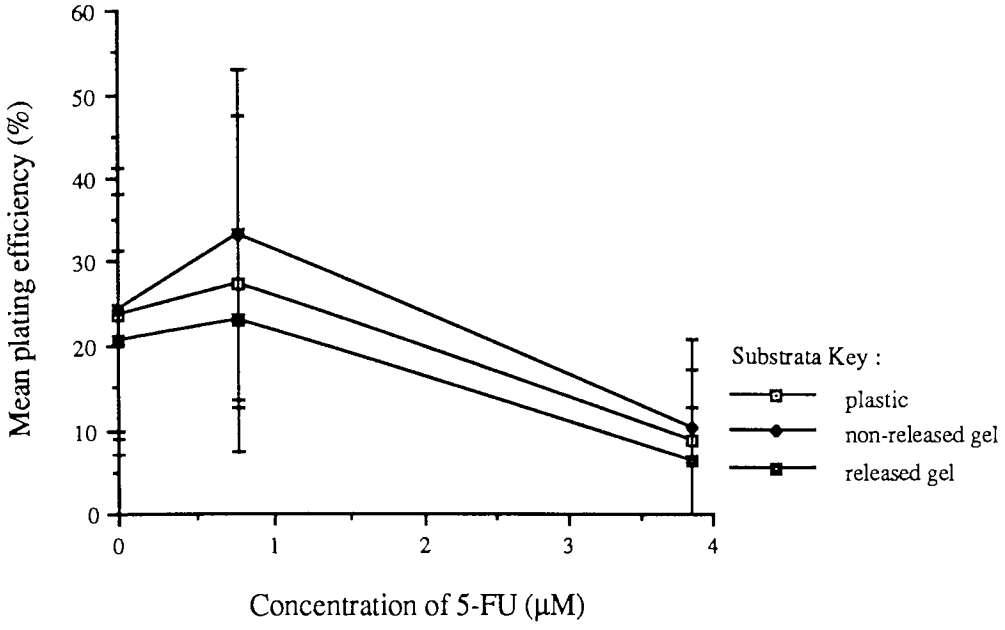


Figure 70 : The effect of 5-FU on clonogenic potential of HT-29$_2$ cells cultured on plastic substrata and non-released (NR), and released (R) non-passaged collagen gels (Non-PPC).
Results expressed as a) mean plating efficiency (%) of remaining cells following treatment for 6 days, and b) mean plating efficiency as expressed as percentage of control (%) following treatment for 6 days. (n = 18 ; ± sd).

a



b

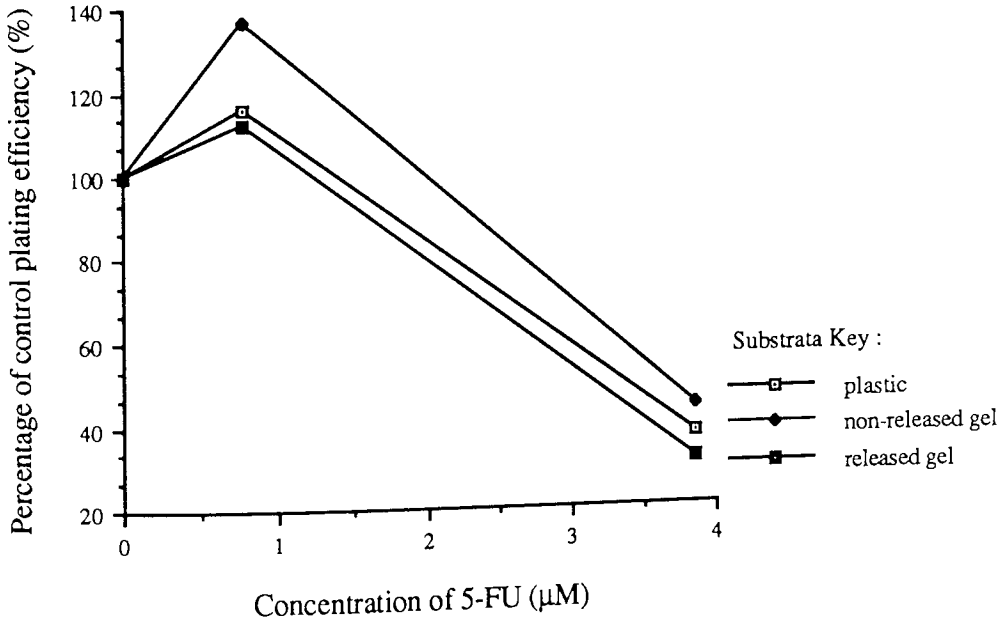


Figure 71 : The effect of 5-FU on clonogenic potential of HT-29$_2$ cells cultured on plastic substrata and non-released (NR), and released (R) passaged collagen gels (PPC). Results expressed as a) mean plating efficiency (%) of remaining cells following treatment for 6 days, and b) mean plating efficiency as expressed as percentage of control (%) following treatment for 6 days. (n = 18 ; ± sd).

6.VIII.ii.c Chemosensitivity of HT-29_2 cells to 1-(2-chloroethyl)-3-[2-(dimethyl-aminosulfonyl)ethyl]-1-nitrosurea (TCNU)

The alkylating agent, 1-(2-chloroethyl)-3-[2-(dimethylaminosulfonyl)ethyl]-1-nitrosurea (TCNU, taumustine) is related to the nitrosureas and its structure is based on the ß-amino acid taurine. Initially, it was reported to have little anticancer effect, but more recent studies have shown marked activity in several murine tumours systems (Pierson *et al.*, 1985). The nitrosurea family of drugs have found a limited role in the chemotherapy of malignant disease particularly for tumours of the brain (Weiss and Issel, 1982). Chloroethylnitrosureas decompose to produce alkylating fragments which alkylate the O^6 position of guanine residues in DNA. The relatively slow reaction of the chloroethyl group attached to the O^6 position of guanine with the cytosine on the opposite strand brings about the cross-linking of DNA (Kohn, 1977). The initial damage can be reversed by the enzyme DNA O^6-alkylguanine alkyltransferase (Harris *et al.*, 1983) which removes the chloroethyl group from the guanine. Human cell lines with very low levels of the transferase (Mer-) are more sensitive to chloroethylnitrosureas than those with high levels of activity (Mer+) which have the ability to repair the damage. Unfortunately, the levels of the enzyme are often higher in tumours than in the normal tissues, leading to toxicity to the host and little chemotherapeutic activity (Scudiero *et al.*, 1984). TCNU also shows carbamoylating activity resulting in the inhibition of glutathione reductase activity (Tew *et al.*, 1985, 1987).

HT-29 cells have been shown to contain high levels of O^6-alkylguanine alkyltransferase, and have been designated Mer+ (Day *et al.*, 1980). TCNU was chosen as a member of the panel of chemotherapeutic agents in order to test whether the substrata on which the cells were cultured could effect their Mer phenotype, and subsequently their chemosensitivity to TCNU.

Initial experiments carried out on HT-29_2 cells cultured on tissue culture plastic produced an IC_{50} value for TCNU of 96 μM. Three concentrations of TCNU were selected as a result of these experiments in order that chemosensitivity experiments could be performed on HT-29_2 cells cultured on the different substrata as previously described for 5-FU in section 6.VIII.ii.b. The concentrations used during these studies were 70 μM,

and 349 μM which resulted in a cell survival of 67%, 23%, and 3% respectively, with cells cultured on tissue culture plastic. As for the 5-FU studies, the first experiments with TCNU were performed on cells cultured on plastic, non-passaged non-released and non-passaged released (Non-PPC NR and R) collagen gels. The second series of experiments were performed on cells cultured on plastic, passaged non-released and passaged released (PPC NR and R) collagen gels. Again, as for the 5-FU experiments the results will be presented in the form of concentration-response curves consisting of either cell number data or plating efficiency data, for each set of experiments. These graphs can be found in Appendix 1.

Figures 72 and 73 (see Appendix 1) illustrate the effects that TCNU had on the cell number following an exposure of 6 days. The data is expressed as a) mean total cell number/well, and b) mean total cell number expressed as a percentage of the control (%). The results of both sets of data, i.e. for the cells cultured on Non-PPC and PPC collagen gels, which displayed larger inter-experimental variation, would suggest that there were no substrata dependent differences in the chemosensitivity of the HT-29_2 cells. Figure 73(b) shows a moderate trend towards the cells cultured on both PPC collagen gels being more sensitive to the agent than the control cells cultured on plastic, but the large standard deviations shown in figure 73(a) prevent any definite conclusions being drawn. Therefore, even the more permanent exposure to collagen gels by passaging (PPC NR and R gels) did not result in any conclusive change in the chemosensitivity of the cells.

Figures 74 and 75 (see Appendix 1) show the results of the clonogenic analysis of the cells in response to exposure to TCNU for 6 days. The data is expressed as a) mean plating efficiency (%) and b) mean plating efficiency expressed as a percentage of control (%) for the cells cultured on plastic and non-passaged collagen gels (Non-PPC) (figure 74); and for the cells cultured on plastic and passaged collagen gels (PPC) (figure 75). The large inter-experimental variations do not allow any clear conclusions to be drawn from these clonogenic assays. However, it was interesting to note that the mildest concentration of TCNU (70 μM) resulted in an enhancement in the plating efficiency above that of the controls, for all treatment groups except the two groups of cells cultured on the passaged collagen gels (PPC NR and R) (figure 75(b)). These two groups of cells

also showed a more marked reduction in their clonogenic potential following treatment with 140 μM TCNU than the cells in all other treatment groups. These results may suggest that the passaging of HT-29_2 cells on collagen gels resulted in an increase in their chemosensitivity to TCNU, with these cells displaying reduced clonogenic potentials at both concentrations (70μM and 140μM) (figure 75(b)) when compared to cells cultured on plastic substrata and non-passaged collagen gels. It was not possible to determine the reason for this possible increase in sensitivity or whether the Mer phenotype had been altered, with a possible reduction in the activity of the repair enzyme O^6-alkylguanine alkyltransferase as a result of culturing the cells continuously on collagen gels. Molecular biological studies could be used to elucidate the exact mechanism of increased sensitivity if that is what is occurring and the results are not due to an artifact of the clonogenic assay. Larger numbers of clonogenic assays including more treatment groups would need to be performed in order to reduce the inter-experimental variation observed during these experiments and to make definite conclusions easier to draw.

A possible reason for the enhancement in clonogenic potential has been discussed in section 6.VIII.ii.b., as this phenomena was also observed during the treatment with 5-FU. This will also be discussed during the discussion (section 6.VIII.iii).

6.VIII.ii.d Chemosensitivity of HT-29_2 cells to sodium butyrate

The four carbon fatty acid sodium butyrate (NaB) has been shown to be a potent inducer of differentiation of both haemopoietic cell lines (Friend *et al.*, 1971; Leder and Leder, 1975) and anchorage-dependent cells such as hamster fibroblasts, murine embryonal carcinoma cells (Leavitt *et al.*, 1978; McCue *et al.*, 1984) and the human breast carcinoma cell line MCF-7 (Prasad and Sinha, 1976). It is a natural fatty acid normally present in lipids and generally exerts its biological properties at the concentration of 5mM (Kruh, 1982). It is also interesting to note that short chain fatty acids are normally produced in the colon by the naturally occurring colonic bacteria and that the levels of these compounds in the stools reach on average a concentration of approximately 190mM (Prizont *et al.*, 1975; Cummings, 1981). Several laboratories have demonstrated the induction of enterocytic differentiation of some lines of HT-29 cells by sodium butyrate (Augeron and Laboisse, 1984; Wice *et al.*, 1985) although this was not observed during the extensive and long term studies carried out for this thesis (section 5.I).

Initial experiments performed on HT-29_2 cells cultured on tissue culture plastic produced an IC_{50} value of 1.9 mM (section 6.I) and provided data in order that the appropriate concentrations could be selected for the experiments to determine chemosensitivity. Three concentrations of NaB were selected for further investigations to determine the effect of collagen gel culture on the chemosensitivity of HT-29_2 cells to this agent. These concentrations were 2 mM, 5 mM and 15 mM. Each resulted in a reduction in cell number expressed as a percentage of the control, of 45%, 11%, and <1% respectively (section 6.I).

As for the previous studies, the first experiments with NaB were performed on cells cultured on plastic, non-passaged non-released and non-passaged released (Non-PPC NR and R) collagen gels. The second series of experiments were performed on cells cultured on plastic, passaged non-released and passaged released (PPC NR and R) collagen gels. The results will be presented in the form of concentration-response curves consisting of either cell number data or plating efficiency data, for each set of experiments. The graphs are in Appendix 1.

Figures 76 and 77 (see Appendix 1) show the effects that the selected concentrations of NaB had on the cell number following an exposure of 6 days on cells cultured on the different substrata. These experiments showed less inter-experimental variation than previous studies using 5-FU and TCNU. However, the results would suggest that there was no difference in the chemosensitivity of HT-29_2 cells cultured on the plastic substrata, non-passaged collagen gels (figure 76(a)) and passaged collagen gels (figure 77(a)).

Figures 78 and 79 (see Appendix 1) show the results of the clonogenic assays performed during the chemosensitivity experiments. Again, there were large inter-experimental variations making definite conclusions difficult to draw and one is forced to say that there would appear to be no differences in the effect that the agent had on the clonogenic potential of the cells on different substrata. However, it is interesting to note that an enhancement in the plating efficiency was again observed with the two mildest concentrations of NaB (2 mM and 5mM). This was most pronounced in the cells cultured on plastic substrata (figures 78(b) and 79(b)), but was also observed in the cells cultured on the non-passaged collagen gels (Non-PPC). An enhancement of plating efficiency was not observed in the cells cultured on the passaged collagen gels (PPC) (figure 79(b)). Further experiments would need to be performed in order to determine if this was a true result of the passaging on the collagen gels because of the extent of the present inter-experimental variation.

Another interesting observation is the variability in the clonogenic potential of the parental HT-29_2 cells cultured on the plastic substrata as controls for the two phases of these experiments. In the first experiments carried out with cells cultured on plastic and non-passaged collagen gels the enhancement of plating efficiency seen in those on the plastic was very profound, reaching a maximum enhancement of 257% of control with a concentration of 5mM. Whereas the plating efficiency of the corresponding control cells cultured on plastic for the second phase of the experiments was only enhanced to a maximum value of 105% of control in response to a concentration of 2mM NaB. One possible explanation that can be offered for these huge variations in the response of the control HT-29_2 cells to the clonogenic enhancing properties of NaB is that the cells had

undergone phenotypic drift during culture. However, the cells were regularly resurrected from the cell bank and used for only 10 passages to reduce the chance of such phenotypic drift. The stock supply of cells in the cell bank were all banked within 1 or 2 passages of each other upon receipt from the donor institution. Another possible explanation would be the use of different batches of sera for the two phases of the experiment. As already discussed earlier the sera can have profound effects on the clonogenic potential of cells in culture (section 6.VIII.ii.a). Unfortunately, there was not sufficient time to investigate the role that sera may play in the cellular response to exogenous agents, particularly chemotherapeutic agents like NaB.

The enhancement of plating efficiency observed during these studies will be discussed fully in the discussion (section 6.VIII.iii) when the effects on HT-29_2 cell clonogenicity of all of the agents will be compared.

6.VIII.ii.e Chemosensitivity of HT-29_2 cells to N,N,-dimethylformamide

The polar solvent N,N,-dimethylformamide (DMF) has been shown to modulate differentiation in a number of cell lines such as murine rhabdomyosarcoma cells (Dexter, 1977) and human colon carcinoma cell lines (Hager *et al.*, 1980; Dexter *et al.*, 1981). It has also been shown to have anti-tumour and differentiation-inducing activity in xenografts of head-and-neck cancer (Van Dongen *et al.*, 1989). However, it was not shown to induce differentiation of the HT-29_1 cells (section 5.II).

Initial experiments performed on HT-29_2 cells cultured on plastic demonstrated an IC_{50} value of 81 mM and allowed the selection of three concentrations of DMF to be used in the experiments to determine if the culture on collagen gels altered the cells chemosensitivity. These concentrations were 100mM, 150mM and 250mM, which reduced the cell number to 32%, 8% and <1% of control cell number respectively (section 6.I).

As for the previous chemosensitivity assessment experiments, the first experiments were performed on cells cultured on plastic substrata and non-passaged collagen gels (Non-PPC NR and R). These experiments were then repeated using cells cultured on plastic substrata and passaged collagen gels (PPC NR and R). The results for each set of experiments will be presented as a) mean total cell number/well, b) percentage of control cell number (%), c) mean plating efficiency (%), and d) mean plating efficiency expressed as percentage of control (%) (see Appendix 1).

Figures 80 and 81 (see Appendix 1) show the results of the effects of DMF on cell number following an exposure of 6 days to cells cultured on the different substrata. These experiments show very low levels of inter-experimental variation and the results clearly show that there were no substrata-dependent differences in the chemosensitivity of HT-29_2 cells to DMF and that the passaging of cells on collagen did not alter the chemosensitivity either (figure 81).

Figures 82 and 83 (see Appendix 1) show the results of the clonogenic analyses and again they show large inter-experimental variations. The results would suggest that there were no substrata-dependent changes in the clonogenicity of HT-29_2 cells in response to DMF. However, an enhancement of clonogenic potential was observed in all substrata

groups with the two lowest concentrations of DMF (100mM and 150mM). Both of these concentrations produced a substantial loss of cells (figures 80 and 81), but resulted in a considerable enhancement of plating efficiency above that of the control, untreated cells on all substrata (figures 82 and 83). There was no definite trend observed with regards one particular substrata producing the greatest enhancement, with the cells cultured on the non-passaged collagen gels showing the greatest elevation in the first experiments compared to the control cells on plastic (figure 82). However, the cells displaying the greatest enhancement during the second experiments were those cultured on plastic (figure 83). It would be easy to suggest that the cells had become less susceptible to enhancement of clonogenicity by the passaging on collagen gels, but there were considerable variations in the effects of DMF on the clonogenicity of the control cells cultured on plastic. An indicator of this variability was that the cells cultured on plastic during the first experiments (figure 82, max.= 153%) had a lower enhancement of plating efficiency than their equivalent cells used during the second series of experiments (figure 83, max.= 210%). This may explain the apparent reduction in enhancement of the cells cultured on passaged collagen gels (figure 83) and suggest that it was not due to the passaging on collagen. The results would therefore suggest that there were no substrata-dependent or passaging-dependent differences in the chemosensitivity of HT-29_2 cells to DMF.

6.VIII.ii.f. Chemosensitivity of HT-29_2 cells to the ether lipid SRI 62-834

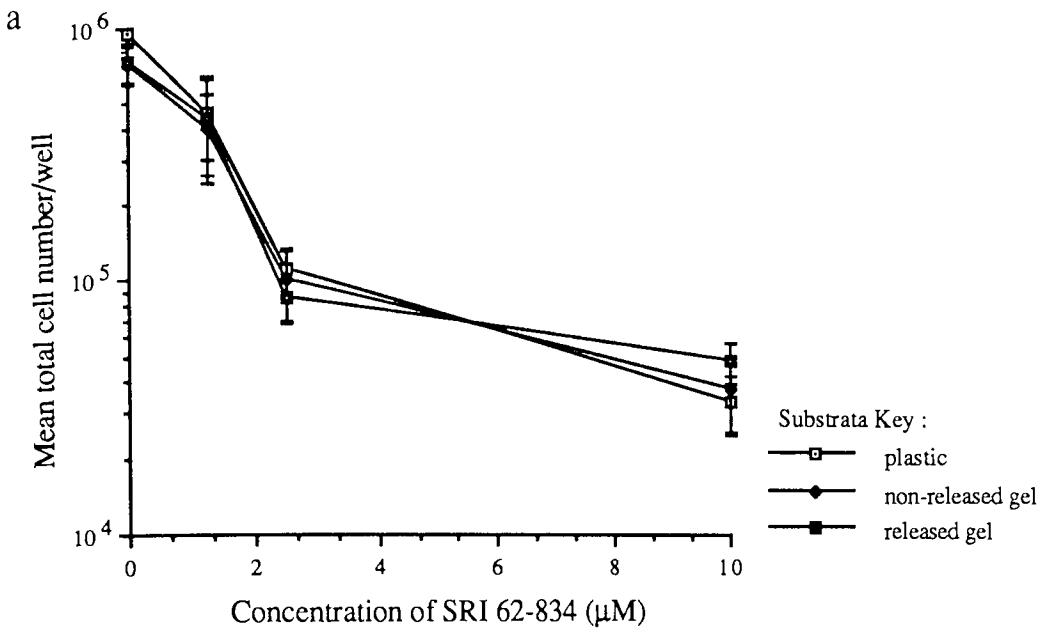
The ether lipid SRI 62-834 is a novel anticancer agent presently in Phase I clinical trial, which has both *in vitro* and *in vivo* activity (Houlihan *et al.*, 1987), but whose mechanism of action is ill defined. This compound has generated a lot of interest because it has highly selective toxicity towards leukaemic cells compared to normal bone marrow cells (Bazill and Dexter, 1989). This molecule is capable of interacting with the plasma membrane in a detergent like manner resulting in physical disruption (Noseda *et al.*, 1989) and this has been suggested as its mechanism of cytotoxicity. However, Lazenby *et al.* (1990) have shown that at moderately cytotoxic concentrations of SRI 62-834 this was not the primary event. Their data showed that there was an elevation in intracellular calcium concentration in leukaemic cells following treatment with SRI 62-834, which was most probably modulated by protein kinase C activation. However, the exact mechanism by which SRI 62-834 raises the intracellular calcium concentration and the cause of the selectivity observed between normal and malignant cells are still to be elucidated. The possibility that this agent may be acting via the membrane and not by direct or indirect effects on DNA synthesis like so many anticancer agent lead me to investigate the toxicity of this agent to HT-29_2 cells which show the usual resistance common to solid tumour cell lines.

The initial experiments which were performed on HT-29_2 cells cultured on plastic in order to determine their chemosensitivity to SRI 62-834 gave an IC_{50} value of 0.9 μM. This value can be compared with those determined for the human leukaemic cell lines HL-60 and K562 of 8 μM and 65 μM respectively, although these values were determined by a different method (Lazenby *et al.*, 1990). The HT-29_2 cells were considerably more sensitive to SRI 62-834 producing a steeper concentration-response curve than any of the four agents previously discussed in this section. The IC_{50} values of the other agents being 0.7 μM for 5-FU, 96 μM for TCNU, 1.9 mM for NaB and 81 mM for DMF. Only 5-FU had an IC_{50} comparable to that of SRI 62-834, with the cells being as sensitive to both agents, but as with the other agents tested the concentration-response curve for 5-FU was not as steep as that for SRI 62-834. SRI 62-834 was therefore the most toxic agent used as part of this panel of chemotherapeutic agents.

Three concentrations of SRI 62-834 were selected from the initial experiments for further investigation using cells cultured on the various substrata, as for the other four agents already discussed in this section. These concentrations were 1.25 μM, 2.5 μM and 10 μM, which reduced the cell number to 26 %, 3 % and 1 % of control cell number respectively. The experiments were performed on cells cultured as described in previous sections.

The toxic effects of SRI 62-834 following a 6 day exposure period can be seen for HT-29_2 cells cultured on both non-passaged (figure 84) and passaged collagen gels (figure 85). Both sets of data show the steepness of the concentration-response curve for HT-29_2 cells in response to SRI 62-834 and also that there were no substrata-dependent differences in the chemosensitivity to this agent. A very interesting finding from these experiments was the effect that SRI 62-834 had on the clonogenic potential of HT-29_2 cells which was unlike those observed from all of the previous experiments : it can be seen that this agent did not enhance the clonogenic potential of the cells even at the lowest concentration (1.25 μM, figures 86 and 87) as seen with the milder concentrations of the other four agents studied. This may be because this concentration resulted in a reduction in cell number to 26% of the control which was lower than that seen for the lowest concentration of each of the other agents. However, concentrations of 5 mM NaB or 150 mM DMF which resulted in reductions in cell number to 11% and 8% respectively still enhanced the clonogenic potential of HT-29_2 cells above that of the control, untreated cells. It would therefore seem that the proportion of cells surviving treatment was unrelated to the ability of the agent to enhance clonogenicity.

Alternatively, the results may suggest that those agents which have shallow concentration-response curves and are relatively unsuccessful against inherently resistant cell lines such as HT-29 do not actually have direct mitogenic capabilities, but do allow the cells to respond to the stress of the initial treatment by becoming more anaplastic. This increased anaplasticity manifests itself as an increased clonogenic potential in *in vitro* assays as a result of the treated cells being able to withstand the secondary stress of the cloning procedure which the control cells can not. The possible reasons for these results and those for the other agents used will be discussed later (section 6.VIII.iii).



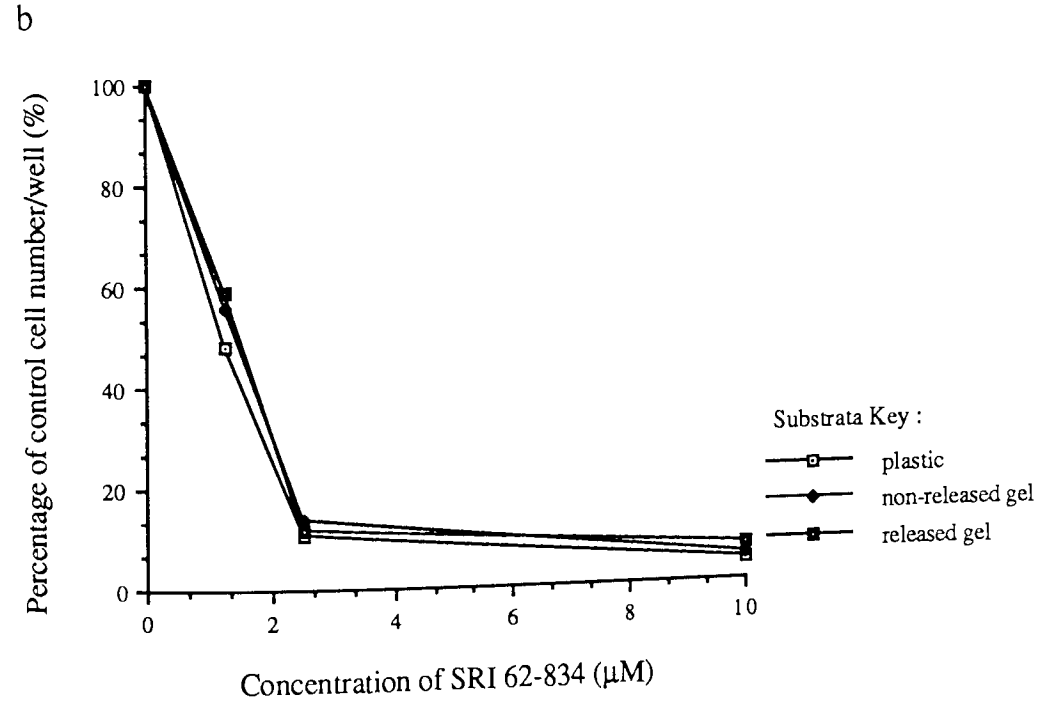
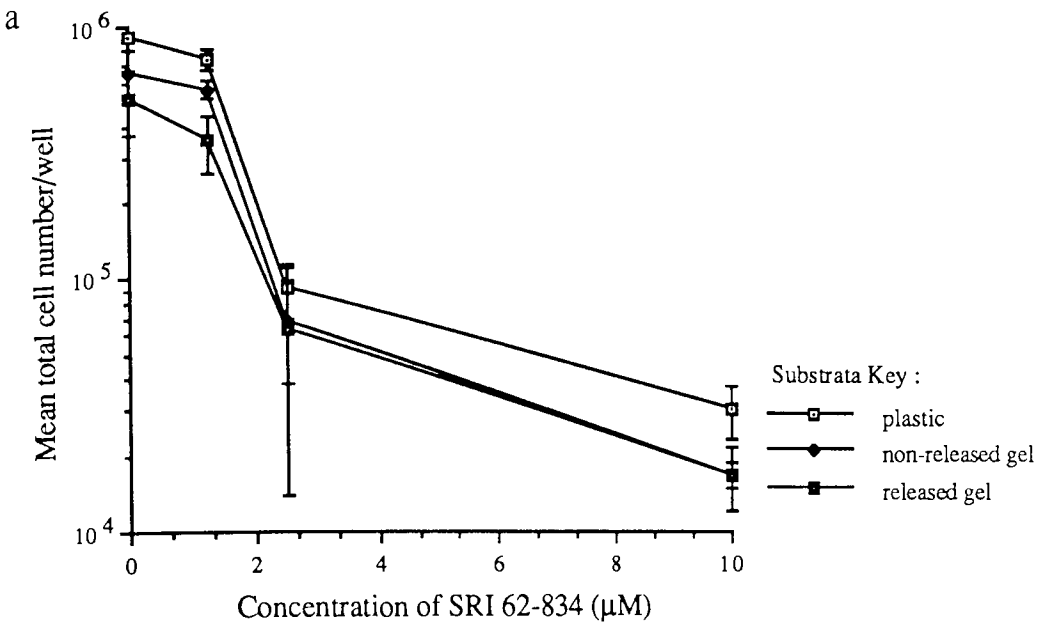


Figure 84 : Concentration-response curves for SRI 62-834 toxicity to HT-29_2 cells cultured on plastic substrata and non-released (NR), and released (R) non-passaged collagen gels (Non-PPC).
Results expressed as a) mean total cell number/well following treatment for 6 days, and b) cell number expressed as a percentage of the control cell number/well (%) following treatment for 6 days. (n = 3 ; ± sd).



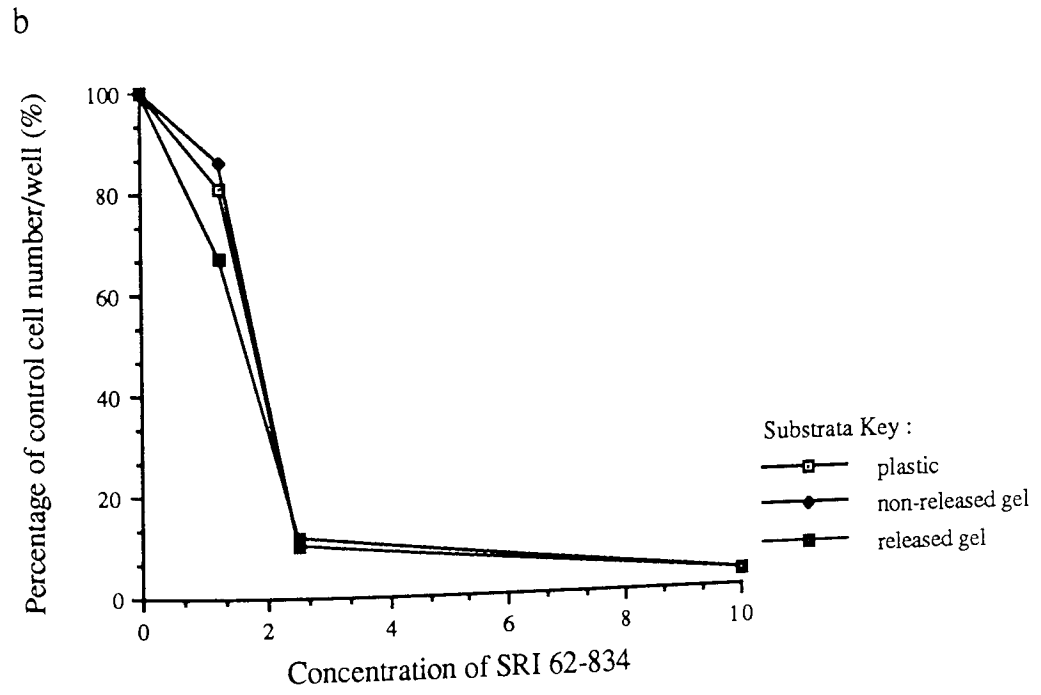
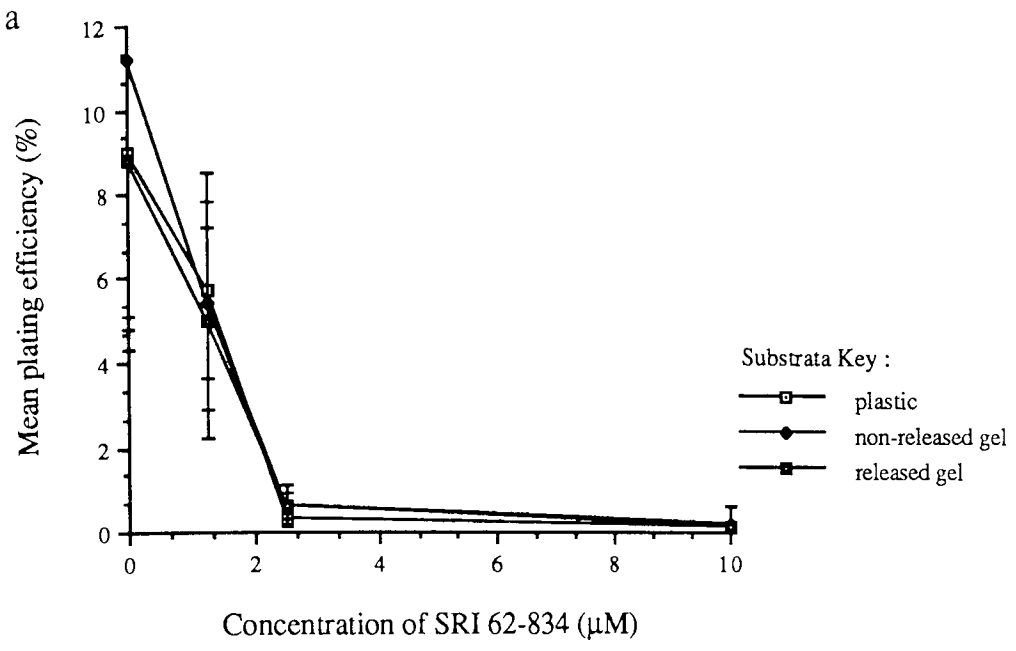


Figure 85 : Concentration-response curves for SRI 62-834 toxicity to HT-29_2 cells cultured on plastic substrata and non-released (NR), and released (R) passaged collagen gels (PPC).
Results expressed as a) mean total cell number/well following treatment for 6 days, and b) cell number expressed as a percentage of the control cell number/well (%) following treatment for 6 days. (n = 3 ; ± sd).



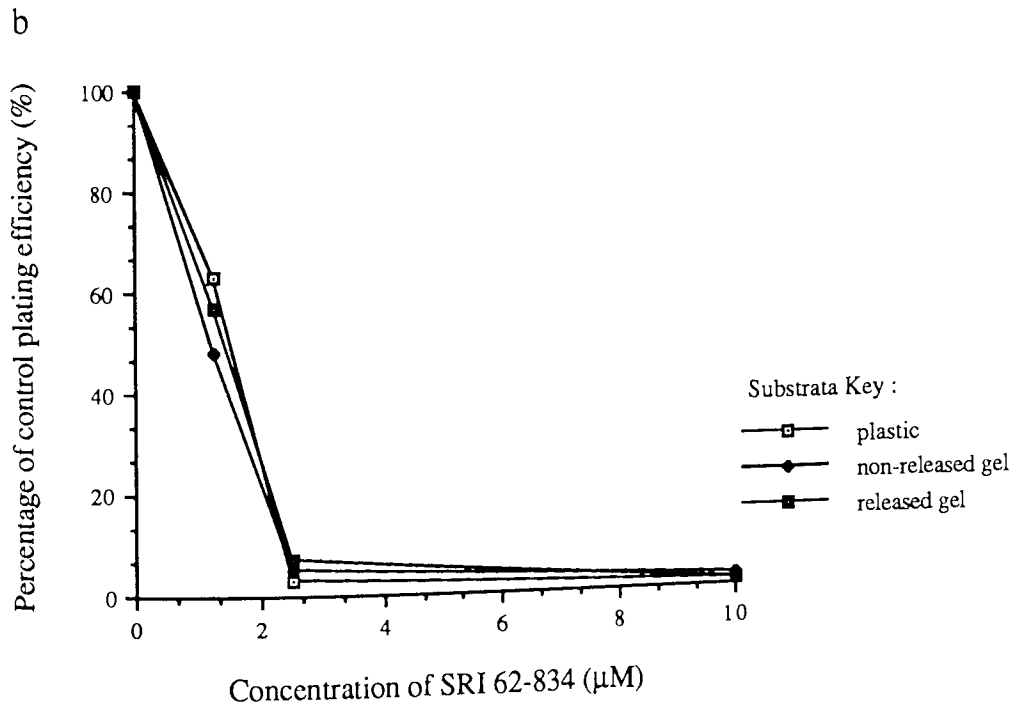
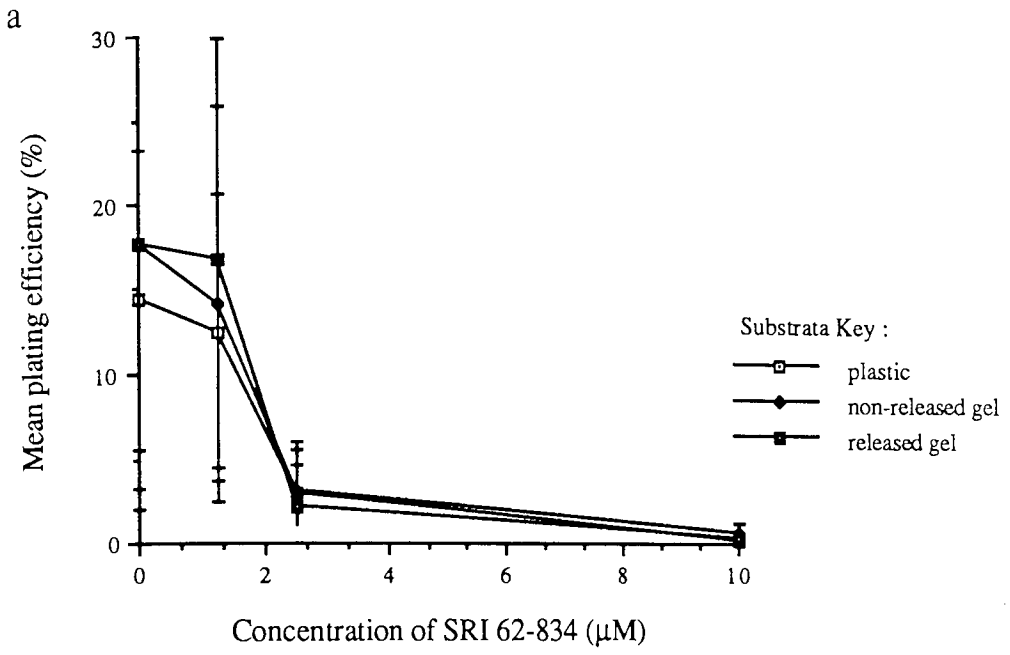


Figure 86 : The effect of SRI 62-834 on clonogenic potential of HT-29_2 cells cultured on plastic substrata and non-released (NR), and released (R) non-passaged collagen gels (Non-PPC).
Results expressed as a) mean plating efficiency (%) of remaining cells following treatment for 6 days, and b) mean plating efficiency as expressed as percentage of control (%) following treatment for 6 days. (n = 18 ; ±sd).



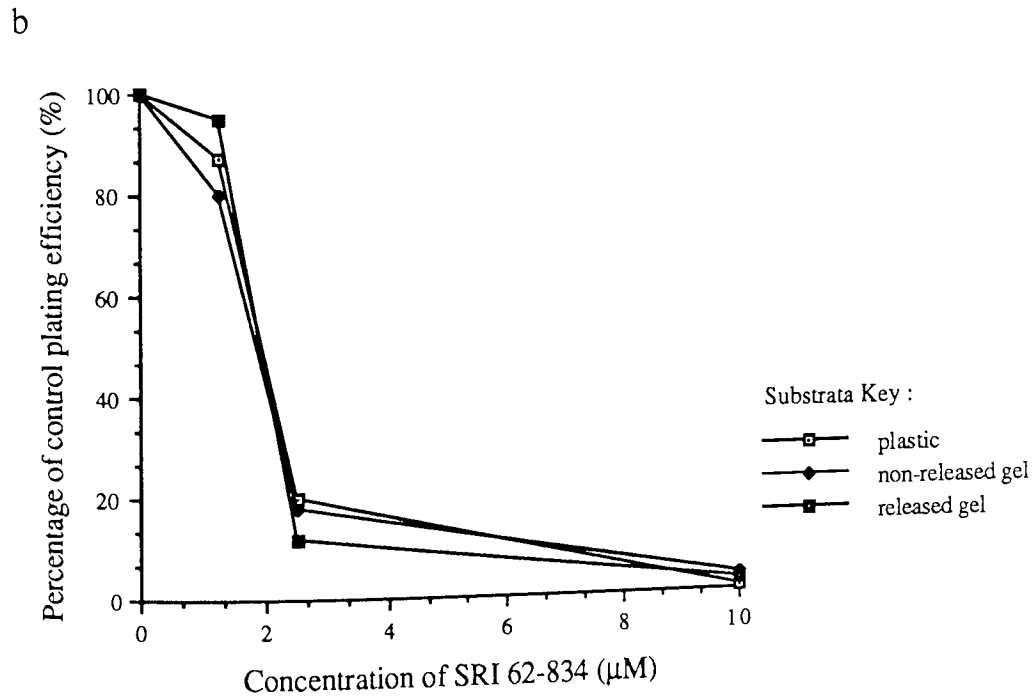


Figure 87: The effect of SRI 62-834 on clonogenic potential of HT-29$_2$ cells cultured on plastic substrata and non-released (NR), and released (R) passaged collagen gels (PPC). Results expressed as a) mean plating efficiency (%) of remaining cells following treatment for 6 days, and b) mean plating efficiency as expressed as percentage of control (%) following treatment for 6 days. (n = 18 ; ±sd).

6.VIII.iii. Discussion

The experiments discussed in this section aimed to determine whether the substrata on which HT-29_2 cells were cultured could affect their chemosensitivity to a panel of anti-neoplastic agents with different mechanisms of action. During the course of carrying out the experiments a number of problems were encountered. These included cell clumping which occurred after culture on collagen gels and the poor reproducibility of the clonogenic assays used to determine the drug effect on cell proliferative capacity. The results for each agent tested showed that there were little or no substrata-dependent differences in the chemosensitivity of HT-29_2 cells as measured by cell numbers and plating efficiencies following a 6 day exposure. The increased exposure of cells to the collagen gels as achieved by continuously passaging on both non-released (PPC NR) and released (PPC R) gels also had no effect on the chemosensitivity of the cells.

However, a very interesting observation was made during the course of these experiments which was both unexpected and highly reproducible. The results of the clonogenic assays suggested that four of the five agents tested, those which also had shallow concentration-response curves and were relatively ineffective against the HT-29_2 cells, enhanced the clonogenic potential of cells above that of the control, untreated cells on all substrata. In contrast to these agents the ether lipid SRI 62-834 which was highly toxic to the HT-29_2 cells and possessed a sharp concentration-response curve did not enhance the clonogenicity of the cells. *In vitro* clonogenicity has been described as one measure of proliferative potential (Hamburger and Salmon, 1977a; 1977b), and the results suggested that 4 out of 5 of the anticancer agents tested (5-FU, TCNU, NaB, and DMF) were capable of enhancing the proliferative potential of HT-29_2 cells. This idea has already been put forward as a possible explanation for the poor advances in the treatment of cancer despite the improvements that have been made with regards early detection and medical care (Kerbel and Davies, 1982; McMillan and Hart, 1987). There is experimental evidence which supports the idea that cancer chemotherapy can enhance the malignancy of the remaining population of cells following treatment. Initially it was thought that this resulted entirely from the detrimental effects that many of the anticancer agents have on the host immune system reducing immunosurveillance and on vascular

endothelium leading to damage which can enhance tumour cell arrest and growth (McMillan and Hart, 1987). Research has advanced with the use of *in vitro* models of metastasis and data from such experiments has shown for example that treatment of malignant B16 cells with the anti-metastatic agent ICRF-159 prior to i.v. injection into recipient mice increased the number of pulmonary tumour nodules (Lazo *et al.*, 1978). Other experiments have shown similar metastasis enhancing capabilities of a number of anticancer agents including hydroxyurea, methotrexate, cytosine arabinoside and 5-azacytidine, although melphalan and 5-fluorouracil were not enhancers of metastasis in the cell model used (McMillan *et al.*, 1986; McMillan and Hart, 1987). How these agents may bring about such a devastating change in the malignant properties of the residual tumour cells remains to be elucidated and may well prove to be a response of the individual cells to the stress of treatment and not due directly to any particular property of the drug. This may explain how anticancer agents with different mechanisms of action can bring about similar changes in the clonogenic potential of HT-29_2 cells. Those remaining cells which survive the initial treatment with the anticancer agent (which are often also mutagenic agents) may undergo further mutational changes resulting in the increased generation of more malignant variants (Kerbel and Davies, 1982). In the *in vitro* clonogenic assays this may be represented by an initial response of the treated cells to the agent which prepares them for the secondary stress of the cloning procedure which they are capable of surviving and proliferating after, which the control untreated cells are not. Alternatively, the selective pressures resulting from treatment may lead to the amplification of a gene responsible for increasing malignant or metastatic activity, although there is little evidence for this (McMillan and Hart, 1987). It may be that anticancer agents are altering gene expression and some agents such as sodium butyrate and the polar solvents have been shown to do this and at the same time altering the malignancy of Lewis lung carcinoma cells (Takenaga,1986). 5-Azacytidine, which is also known to cause hypomethylation of DNA and increased gene expression (Jones and Taylor, 1980) has been shown to increase *in vitro* clonogenicity and growth rates, which are often used as the markers of malignancy of human cell lines (Olsson *et al.*, 1985). This situation is analogous to that observed with four of the agents tested during these

studies. The observations made during these experiments therefore support previous published reports of cancer chemotherapy enhancing the malignant properties of cancer cells and further experimentation would be required in order to elucidate the mechanism by which the disparate agents are having this effect. A common cellular response to the stress of treatment may pin-point a new target for cancer chemotherapy not yet discovered or exploited for therapeutic purposes.

6.IX The effects of simultaneous collagen gel culture and glucose deprivation on the morphological differentiation of HT-29_2 cells

6.IX.i Introduction

The use of glucose deprivation as a tool for inducing the enterocytic differentiation of HT-29 cells (Pinto *et al*, 1982) has been discussed fully earlier (sections I and 5.III). Although metabolic manipulation did not prove to be successful at inducing the differentiation of HT-29_1 cells (section 5.III) the procedure was repeated for the HT-29_2 cells as these were a new batch of cells from a different source (section 6.II). Even though the cells were successfully subcultured in the glucose deficient media, the cells did not achieve a polarized morphology indicative of enterocytic differentiation (section 6.II). However, at the same time that those experiments were performed on HT-29_2 cells cultured on plastic substrata parallel studies were carried out on cells cultured on collagen gels, both non-released and released. These experiments aimed to determine whether the exposure of HT-29_2 cells to two putative inducers of enterocytic differentiation would be more successful at inducing differentiation than each of these inducers on their own.

6.IX.ii Results

The experimental measurements in this section were limited to recording morphological changes by electron microscopy. The combined effects of the reduced growth rate when HT-29_2 cells were cultured in glucose-deficient medium and the reduced surface area of the collagen gels compared to a plastic tissue culture flask would have resulted in too few cells for biochemical assays to be performed.

HT-29_2 cells were cultured on non-released and released type I collagen gels in DMEM medium containing glucose (25mM) until the cells reached confluency (approximately 7 days after seeding). The cells were then switched immediately in one step into glucose-deficient medium (2.5mM inosine) and cultured for a number of weeks. Observations were made using a light microscope and these revealed that the cells remained attached to the collagen with cell loss similar to that seen during the deprivation of cells cultured on plastic substrata (section 6.II). Samples of cells cultured on non-released and released collagen gels were fixed and processed for EM visualization after 16 and 43 days of

culture. Electron microscopical examination of these samples revealed that although the HT-29_2 cells still grew as a multilayer similar to that observed for cells cultured on plastic, there were microvilli-lined intra- and intercellular lumen seen amongst these typically anaplastic cells. The degree of polarization around the intercellular lumen was highly variable, as was the extent of microvilli and tight junction formation. Some cells which were arranged around an intercellular lumen exhibited morphological features indicative of enterocytic differentiation, with the cells being polarized and possessing distinct microvilli-lined apical membranes separated from the basolateral membranes by well formed tight junctions (figure 88 (a) and (b)). Microvilli-lined intracellular lumen contained within the cytoplasm of individual cells were also evident and the intercellular lumen were often very large (figure 88 (c)). The morphological appearance of the well differentiated cells arranged around an intercellular lumen resembled very closely those observed *in vivo* when HT-29_2 cells were grown as xenografts, as was the degree of heterogeneity of the response (section 4.I).

6.IX.iii Discussion

This increased differentiation displayed by cells cultured on collagen gels which were being simultaneously glucose-deprived compared to cells cultured on collagen gels in normal medium may indicate the combined inductive effects of the two inducers under investigation. However, it may also be a result of the increased length of culture under these conditions which were not repeated for those on collagen only at the EM level. Light microscopical observations of 4 μM sections of these latter cells, cultured for 7, 14, 21 and 28 days did not reveal morphological changes indicative of enterocytic differentiation as advanced as that seen in these experiments (section 6.III). These results may therefore suggest that by combining the two inducers of differentiation, which alone were not successful at bringing about profound morphological differentiation of the HT-29_2 cells, it was possible to induce a higher degree of differentiation. Unfortunately, the limited nature of these experiments prevent further discussion as to the role of both glucose-deprivation and collagen type I gel in regulating the enterocytic differentiation of HT-29_2 cells. Future experiments using HT-29 cells

cultured on plastic which are capable of undergoing enterocytic differentiation when glucose-deprived may be more fruitful for the elucidation of the roles of these inducers when combined. The HT-29 cells used for these experiments were unresponsive to metabolic manipulation (section 6.II) and this may have reduced the responses seen.

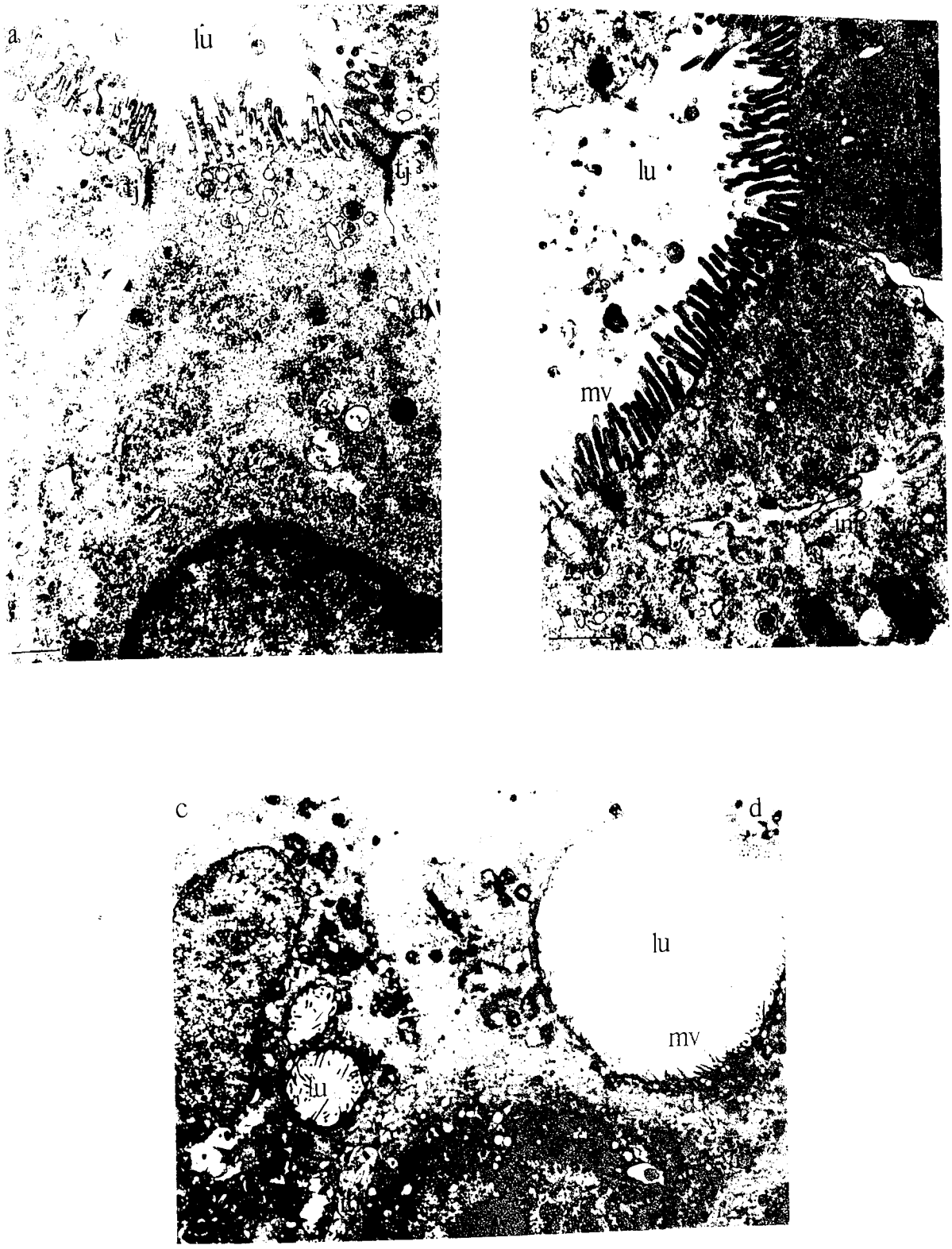


Figure 88 : Transmission electron micrographs of HT-29$_2$ cells cultured in the absence of glucose on : a and b) a released collagen gel for 16 days, and c) a non-released collagen gel for 43 days. Nucleus, (n); microvilli, (mv); tight junctions, (tj); lumen, (lu); interdigitations, (int); desmosomes, (d). *Scale bar*, 1 µm.

6.X The effects of culture on permeable filter inserts on the morphological and biochemical differentiation of HT-29_2 cells

6.X.i Introduction

A very important feature of epithelial cells which determines their function is their ability to polarize producing two distinct membrane domains. The apical and basolateral plasma membranes are separated by the formation of tight junctions between adjacent cells of the epithelial cell layer. Each membrane has a specific set of functions which govern the overall function of the differentiated cell. The intestine is lined with typical epithelial cells or enterocytes which are polarized and lay upon a specialized extracellular matrix called the basement membrane. The majority of cells of epithelial origin when cultured *in vitro* on plastic substrata do not readily polarize as they would *in vivo*. Some cell lines can polarize under these restrictive conditions by forming domes which are indicative of transepithelial transport (Vega-Salas *et al.*, 1987; Salas *et al.*, 1988). Other cells such as Caco-2 cells and glucose-deprived HT-29 cells can polarize whilst remaining attached to the plastic substrata (Blais *et al.*, 1987; McCormack and Johnson, 1989; Pinto *et al.*, 1982). During the investigations carried out to determine the inductive effects of culture on collagen type I gels as described in section 6.VIII the gels which were released from the tissue culture well bottoms were used to provide an environment more like the *in vivo* one which may allow polarization. An alternative method of providing such an environment is to culture the cells on inert permeable filter inserts which fit into the wells of a plastic plate. These filters provide the cells with the possibility of polarization by separating the medium into two distinct compartments. The cells may then arrange themselves in order that they may derive the majority of their nutrients via the basolateral membrane as is the *in vivo* situation. These filter inserts are used extensively to study both epithelial differentiation (Koh, 1989) and transepithelial transport across polarized cell sheets (Fantini *et al.*, 1989). These filter inserts can be used successfully without any special coating and can thus be used to determine the role that being able to polarize alone may play in regulating differentiation.

The experiments described in this section aimed to determine the degree of morphological and biochemical differentiation which could be achieved by HT-29_2 cells when cultured on inert porous inserts without any inductive effects of an added ECM.

6.X.ii Results

HT-29_2 cells were cultured on porous inserts in standard DMEM medium for up to 35 days with regular re-feeding. Samples of cells were fixed and processed for electron microscopy after 9, 14, 21, 28, and 35 days. The results of these experiments revealed an increased degree of differentiation with increasing time in culture. Cells cultured for 9 days revealed a poor degree of enterocytic differentiation (figure 89 (a)), but they did exhibit a greater expression of desmosome formation than HT-29_2 cells cultured on plastic substrata (figure 7, section 4.I). Profound enterocytic differentiation was not observed until the cells had been cultured for 28 days (figure 89 (b)), with cells being arranged around intercellular lumen similar to those observed in the xenograft samples (figure 9 section 4.I). These well differentiated cells displayed features typical of enterocytic cells of the intestine and goblet-like cells containing characteristic mucus granules were also observed (figure 89 (d)). Another similarity between the cells cultured on the inserts and those grown as a xenograft was the degree of heterogeneity of the response to their environment. Only a small proportion of the total cell population displayed differentiated features in both situations.

These results suggested that the HT-29_2 cells were capable of achieving a state of differentiation *in vitro* similar to that achieved *in vivo* when cultured on permeable filter inserts. The extent of enterocytic differentiation in this situation would appear to be more advanced than that observed for these cells cultured on collagen type I gels (section 6.III). A possible reason for these observations may be that the cells differentiated better when allowed to produce their own ECM when cultured on the filter insert, than when exposed to one component of the ECM only. In order to determine whether the cells were producing any ECM of their own and whether this production correlated with the onset of morphological differentiation, the experiment was repeated incorporating ruthenium red in the fixation solution (sections 2.II.xiii and 3.II.iv). Ruthenium red labels extracellular

materials which are thought to be acidic mucopolysaccharides (Luft, 1971) and can be used to show the extent of deposition of extracellular matrix using the electron microscope (Dr. T. Allen, personal communication). Figure 89 (e) and (f) illustrate the time-dependent increase in the deposition of the extracellular matrix components located external to the plasma membrane of HT-29_2 cells cultured for 7 and 21 days respectively. Even though the micrograph shown in (e) was under-exposed compared to that in (f) the increased staining intensity was significant in the latter. Practical problems with the samples prevented any of the later time point samples from being processed. It is hoped that these problems will be resolved in the future so that these results may be verified.

In sections 6.VI and 6.VII the immunolocalization of aminopeptidase and sucrase-isomaltase was described for HT-29_2 cells cultured on plastic and collagen gels. As part of those experiments HT-29_2 cells were also cultured on permeable filter inserts for up to 28 days, with samples being fixed and processed for immunocytochemistry every 7 days (section 3.II). Figure 90 shows representative results of these experiments. The level of staining for aminopeptidase was seen to be greater in the cytoplasm of cells cultured on filter inserts than in those of cells cultured on collagen gels and similar to that observed for cells cultured on plastic (section 6.VI, figure 62). The intensity of staining decreased with time as shown in figure 90 (a) and (b) which show 4 μM sections of HT-29_2 cells cultured on permeable filters for 7 and 28 days respectively stained for aminopeptidase. The intensity of staining observed for the samples stained for sucrase-isomaltase was greater on day 7 (figure 90 (c)) than that observed for the cells cultured on plastic and collagen gels (section 6.VII, figure 65) and the intensity increased with time (figure 90 (d)).

Non-specific staining of the mixed esters of cellulose filter was observed and was thought to be due to the gradual absorption of sera proteins onto the filter during culture. Attempts were made to block this staining by adding rabbit serum to the secondary antibody (Z 259 rabbit anti-mouse immunoglobulins, Dakopatts) as suggested by a Dako representative. This method was unsuccessful at preventing non-specific staining at the two concentrations (10% and 50%) of rabbit serum used (figure 90 (e) and (f) respectively).

6.X.iii Discussion

The results obtained during these experiments would suggest that HT-29_2 cells were capable of achieving differentiation *in vitro* that was comparable to that observed when these cells were grown as xenografts in nude mice. The degree of morphological differentiation was better than that obtained when cells were cultured on plastic substrata and collagen type I gels. Preliminary data obtained from the use of ruthenium red as a EM stain for extracellular mucopolysaccharides would suggest that the HT-29_2 cells were producing their own ECM when cultured on the inert filter inserts. The deposition of these extracellular molecules increased with time and this may explain the delay in onset of the morphological changes indicative of differentiation seen from day 28 onwards. Further studies are needed in order to elucidate which components of the ECM the cells may be producing and when the induction of this production begins. HT-29 cells have been shown to deposit their own ECM onto plastic substrata and this ECM material was demonstrated to promote neurite outgrowth of PC-12 cells (Bellot *et al.*, 1985). Fluorescently labeled antibodies to individual components of the ECM which are commercially available could be used to characterize the ECM produced and also molecular biology studies could be used to produce a detailed picture of the molecular events taking place within the cell in response to culture in this environment.

The immunocytochemical data revealed an increased expression of both aminopeptidase and sucrase-isomaltase in cells which had been cultured on permeable inserts when compared to those cultured on plastic or collagen gels. However, the expression of aminopeptidase decreased with time for the former cells, which was in direct contrast to the increased expression observed with time for the cells cultured on plastic and collagen gels. An explanation for the reduction in expression of a marker of enterocytic differentiation in cells which have been shown to exhibit the highest level of morphological differentiation aside from that of the xenograft can not be offered. The expression of sucrase-isomaltase was seen to increase with time in all cells observed independent of substrata, which would support the idea that with increasing time in culture the cells can acquire a greater degree of differentiation. The expression was greatest in the cells cultured on permeable filters and this would support the

morphological observations which showed that these cells were capable of achieving a degree of differentiation *in vitro* similar to that seen in the samples of xenograft.

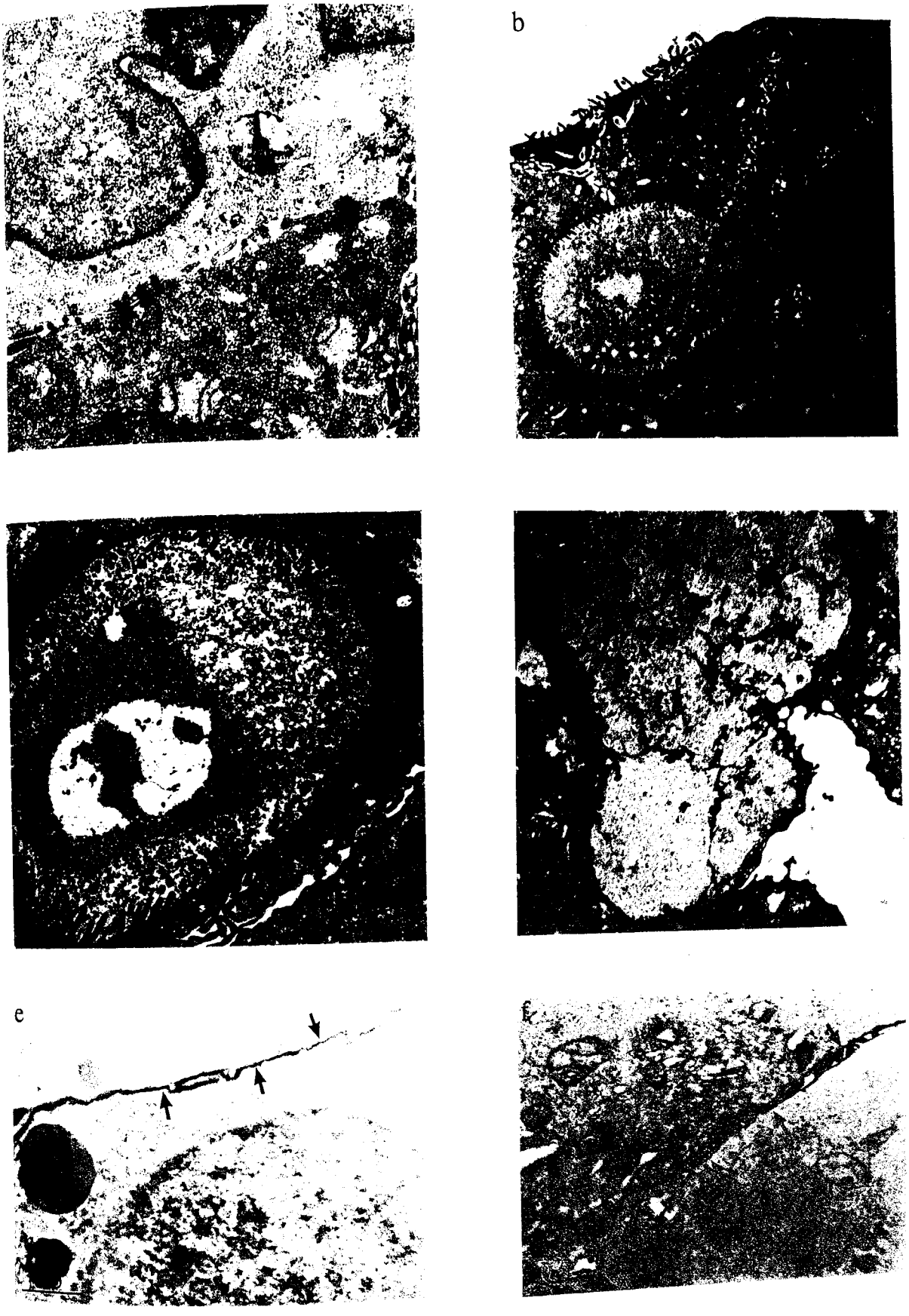


Figure 89 : Transmission electron micrographs of HT-29_2 cells cultured on porous filter inserts for :
a) 9 days, b) 28 days, c and d) 35 days, e and f) 7* and 21* days.
(*these latter samples were stained with ruthenium red (arrows) to show extracellular mucopolysaccharides). *Scale bars*, as labeled in µm.

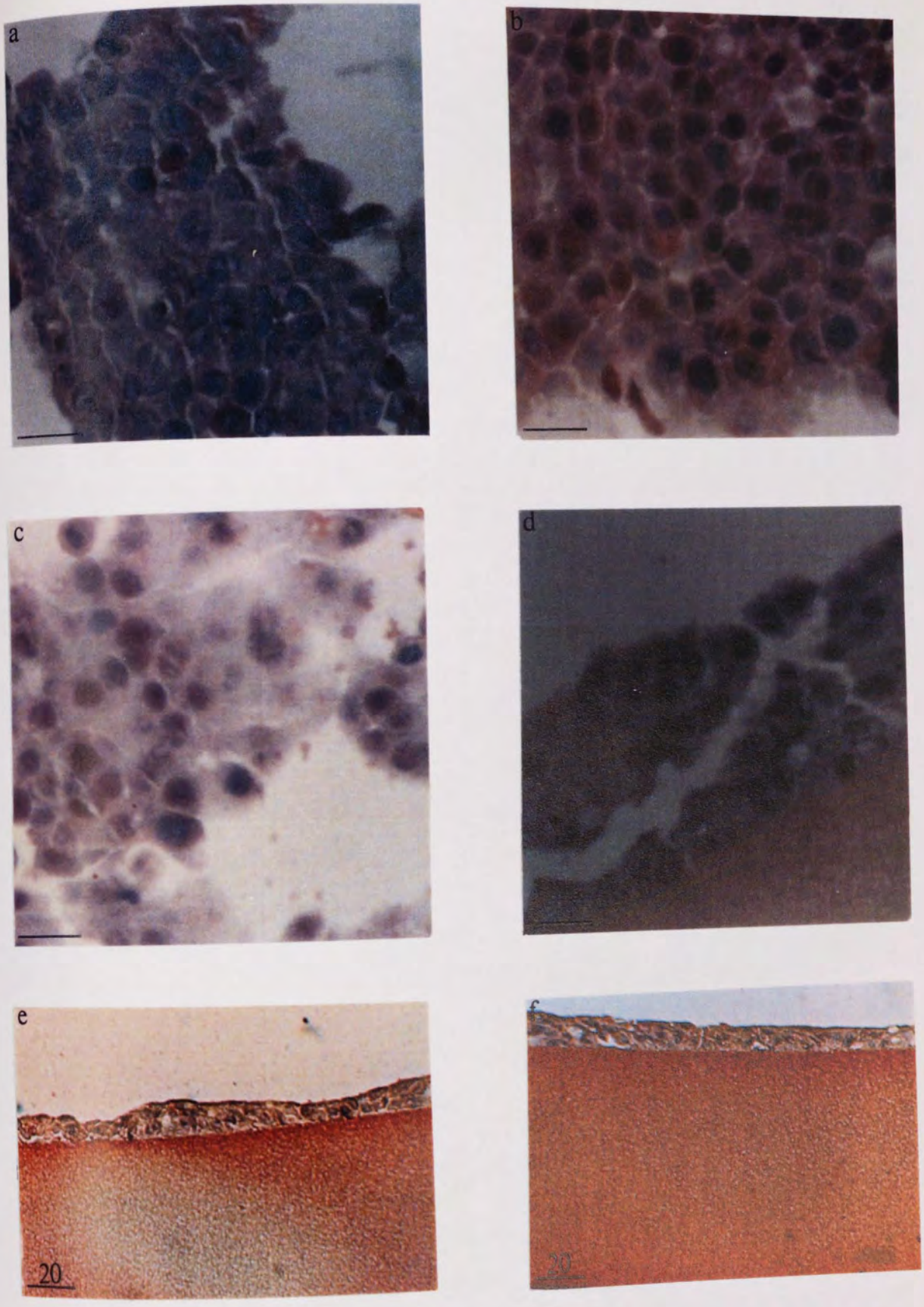


Figure 90 : Light micrographs of HT-29_2 cells cultured on porous filter inserts for: a and b) 7 and 28 days respectively and stained immunocytochemically for aminopeptidase; c and d) 7 and 14 days respectively and stained immunocytochemically for sucrase-isomaltase; e and f) 28 days stained immunocytochemically for sucrase-isomaltase with rabbit serum as a blocking agent against non-specific staining (10% and 50% respectively). *Scale bars*,10µm.

SECTION 7 : GENERAL DISCUSSION

SECTION 7 : GENERAL DISCUSSION

Many laboratories interested in the synthesis and development of novel anticancer agents incorporate into their early evaluation of efficacy, the use of *in vitro* screening models consisting of malignant cell lines. Even large institutions like the National Cancer Institute in the United States uses *in vitro* screening models to select possible anticancer agents (Alley *et al.*, 1988). However, it is generally accepted that although this method of selection has generated agents which are useful against the more rapidly proliferating malignancies i.e. the leukaemias, it has not produced agents efficacious against the more slowly proliferating solid tumours such as that of the colon. It has been known for some time that the *in vitro* environment used for such screening models i.e the culture on plastic or glass surfaces does not usually facilitate the maintenance of the polarized morphological and functional differentiation of epithelial cells (Bissell, 1981), which are responsible for 90% of human malignancies (Wright and Alison, 1984c). Consequently, the malignant cell lines used as part of the screening panels being used to select for clinically effective antineoplastic agents may not be representative of the tumours from which they were derived, with respect to their morphology, functional differentiation and more importantly their chemosensitivity.

Although there have been many studies to investigate the generation of more physiological environments *in vitro* by for example, the use of coatings of individual components of the ECM; the use of coatings of complete basement membrane; the use of fibroblast feeder layers; and the use of inert porous filter inserts, these studies have concentrated solely on the morphological and biochemical differentiation of the particular cells being studied. Each one of these more representative *in vitro* environments has been shown to facilitate the maintenance of a more differentiated state in both normal and malignant cells (see sections 1.II.iii and 1.III.). There have been very few investigations into the effect of the culture conditions upon the chemosensitivity of malignant cells *in vitro*. One such study which was performed unfortunately did not compare the chemosensitivities of rat mammary cells cultured on collagen type I gels to those of the same cells cultured on plastic or glass, but to the *in vivo* mammary tumour from which the cells were derived (Sinha and White, 1988). Although they did not illustrate the

differences which may have existed between the chemosensitivities of the cells depending on the *in vitro* culture substrata, they did show a good correlation between the *in vitro* and *in vivo* sensitivities to a panel of chemotherapeutic agents.

I wished to test the hypothesis that suggested that our general lack of success with the solid tumour screening models may be a direct result of the unphysiological and restrictive nature of the *in vitro* environment. Consequently, the main experiments presented in this thesis (see section 6) aimed to determine the effects that collagen gel culture may have on not only the growth and differentiation of HT-29 human adenocarcinoma cells, but also the effects that it may have on the chemosensitivity of these cells to a panel of chemotherapeutic agents.

Firstly, it was necessary to determine the maximal differentiation which was achievable by the HT-29 cells used in our laboratory. This was achieved by growing the cells as xenografts in nude mice (see section 4). It was hoped that the nude mice would provide the most physiological environment possible and would lead to the optimum level of differentiation of the HT-29 cells. The xenografts grew as poorly differentiated tumours which were highly heterogeneous containing a small proportion (approximately 11%) of well differentiated intestinal-like cells (see table 1). These results were in contrast with those of earlier studies which described HT-29 xenografts as being well differentiated (Hajdu and Fogh, 1978). It should be noted that their data was obtained sooner after derivation of the HT-29 cell line from the primary tumour than our present data and that the continued *in vitro* culture may explain the increased anaplasticity observed during our experiments. Studies performed by Richman and Bodmer (1988) have shown that the xenografts produced by their HT-29 cells were rich in mucus-containing signet ring cells, with extracellular lakes of mucinous material and few columnar cells as assessed by immuncytochemistry. Therefore, the degree of cellular and structural differentiation achieved by HT-29 varies from strain to strain and is also dependent upon the site of implantation as suggested by my preliminary results (see section 4).

The morphological data presented in section 4 served as the optimum achievable differentiation for the HT-29 cells, against which all of the future morphological observations would be compared. The degree of differentiation and the proportion of differentiated cells *in vitro* was not expected to exceed that observed *in vivo*.

Previous studies into the role that the components of the ECM may play in regulating the differentiation of HT-29 cells showed little differentiation-inducing properties. Phillips *et al.* (1988) showed that the culture of HT-29 cells on glass or inert filter inserts coated with laminin, fibronectin, collagen type I and collagen type IV did not enhance the differentiation induced by the absence of glucose in the media. The culture of HT-29 cells within collagen type I gels resulted in the formation of solid, slightly irregular spheroids and it was only the culture on a mesenchymal cell lawn and as a xenograft which resulted in an enhancement of the differentiation status of the cells (Richman and Bodmer, 1988). A later study by Pignatelli and Bodmer (1989) showed that the HT-29 cells which they used for their studies did not bind well to the collagen and this may explain their earlier lack of success in inducing differentiation by collagen. The HT-29 cells ($HT\text{-}29_2$) used in the studies described in this thesis (section 6) attached readily to collagen type I and as a consequence of their culture on the collagen formed tighter cell-cell contacts which was demonstrated by the extensive degree of cell clumping observed following the enzymic digestion of the collagen. $HT\text{-}29_2$ cells grew readily on the collagen gels, with only a slightly longer lag phase observed for cells which had been continuously subcultured on the collagen (i.e. seeded from cells already growing on collagen), compared to $HT\text{-}29_2$ cells cultured on plastic and collagen gels (i.e. seeded from plastic-derived cells). There was no overt indication of mass differentiation of the cells cultured on collagen (either Non-PPC or PPC) as this would have resulted in a reduction in cell proliferation and consequently a reduction in the population doubling time (see section 6.III). However, this was anticipated because the data from the xenograft work (see Section 4.I) demonstrated that only a small proportion of the cells underwent profound morphological differentiation (up to 11%) and that this small proportion of cells would not have effected the population doubling time significantly.

Light microscopic observations of the cells cultured on the various substrata showed that the cells cultured on collagen displayed a more cuboidal appearance compared to the cells cultured on plastic substrata (figure 42). These results were confirmed by SEM (figure 43) and the TEM observations showed that the culture of HT-29_2 cells on collagen gels resulted in the formation of more differentiated cells arranged around intercellular lumen. These differentiated cells frequently displayed characteristics of enterocytic differentiation, such as tight junctions, microvilli and desmosomes (figures 44 and 45). The permanent subculturing of HT-29_2 cells on collagen (i.e. PPC) did not enhance the differentiation observed above that of the cells seeded onto the collagen from cells cultured on plastic (i.e. Non-PPC).

Only the permanent subculturing of HT-29_2 cells on collagen (i.e. PPC) was shown to enhance the activity of the brush border associated hydrolytic enzyme, alkaline phosphatase above that determined for the cells cultured on plastic substrata (figure 49). However, the localization of this enzyme was not confined to apical microvilli-lined membranes as in the normal gut, but was found all around the plasma membrane of cells showing few morphological feature of intestinal differentiation (figures 51 and 52). The secretion of CEA was also only enhanced above that of the control cells cultured on plastic, in cells permanently subcultured on released collagen gels (i.e. PPC R)(figure 56). It must be borne in mind that the biochemical results may have been dampened to some extent by the majority of anaplastic undifferentiated cells not showing elevations in the marker being studied.

The analysis of aminopeptidase activity in HT-29_2 cells cultured on different substrata by both a biochemical and an immunocytochemical method produced contradictory results. Those from the biochemical assay showed that enzyme activity was enhanced with time to produce higher activities in the HT-29_2 cells cultured on collagen (figure 60) compared to those cultured on plastic substrata. However, the immunocytochemical data suggested that even after prolonged cell culture (up to 28 days after seeding) there was no enhancement of aminopeptidase expression above that observed for the control cells cultured on plastic substrata (see section 6.VI). It was therefore not possible to draw firm conclusions from this series of experiments.

Collagen gel culture did not lead to changes in the expression of sucrase-isomaltase (see section 6.VII), although the immunocytochemical assay may not have been sensitive enough to detect low levels of activity. Further studies using immunogold labeling and transmission electron microscopy may be useful to confirm these results, as would the use of a biochemical microassay for the detection of sucrase-isomaltase activity.

The experiments discussed up to this point have been concerned with assessing the effects that culturing HT-29_2 cells on collagen type I gels may have on their growth, morphological and biochemical differentiation. It would appear from these experiments that HT-29_2 cells were capable on undergoing limited differentiation (up to 11%) when grown as a xenograft in nude mice and that the culture on collagen type I gels was able to induce similar morphological changes indicative of differentiation, with a similar degree of heterogeneity. There were also moderate enhancements in the activities of the brush-border enzymes alkaline phosphatase and aminopeptidase, with an increase in CEA secretion by cells cultured continuously on collagen gels (only released gels for the CEA secretion). However, there was no increase in sucrase-isomaltase expression as detected by an immunocytochemical technique. The secretion of CEA was the only parameter measured which showed a difference between the inductive properties of non-released and released collagen gels. There were no significant differences observed between cells cultured on non-released and released collagen gels for any of the other parameters studied. This would imply that being able to feed from the basolateral surface (which was possible for the cells cultured on the released collagen gels) did not prove to be more inductive than exposure to the collagen alone (i.e. non-released gel culture). Interesting results obtained by culturing HT-29_2 cells on inert filter inserts (section 6.X) suggested that being provided with an environment which allowed polarization without an added ECM was inductive for differentiation. Further studies to determine the mechanism of this induction and the composition of any ECM laid down by the cells need to be performed. By considering these results as a whole, it may be suggested that the *in vitro* environment provided by the culture of HT-29_2 cells on collagen type I gels is more representative of the *in vivo* environment than that provided by the standard *in vitro* environment of a plastic substrata.

This then left the key question to be answered . Did this more physiological environment lead to an alteration in the chemosensitivity of HT-29_2 cells to a panel of five chemotherapeutic agents? The results obtained for the experiments which aimed to answer this question would suggest that the culture of HT-29_2 cells on collagen type I gels does not alter their chemosensitivity to either 5-FU, TCNU, NaB, DMF, or SRI 62-834 (see section V.III). It is likely that the unaltered chemosensitivity of HT-29_2 cells in response to their culture on collagen type I gels was a result of the small proportion of cells undergoing intestinal differentiation. Again, as with the biochemical assays any response by a small proportion of the cells may have been masked by that of the majority of the cells which remained anaplastic and undifferentiated. It would be of great interest to determine the chemosensitivity of the HT-29_2 xenografts to the five agents tested *in vitro* and to see if the *in vitro* chemosensitivity correlated well with that determined *in vivo*. This would show whether or not cells which had achieved the ultimate degree of differentiation also had an altered sensitivity to the test agents. It may be that the HT-29 cell line was not the most appropriate cell line to have used to study the role of the *in vitro* environment because of its limited capacity to differentiate even *in vivo*. It should be noted that previous *in vitro* studies by other research groups had shown that these cells were capable of profound differentiation in response to glucose deprivation and treatment with differentiating agents (see section 1.II.iv). However, the particular strain of HT-29 (HT-29_2) cells used in these studies were not as easy to induce to differentiate by those methods employed by others and consequently proved difficult to induce by culture on collagen type I. The question that was asked with regards to the *in vitro* environment being able to alter chemosensitivity may have been better answered by using a cell line which more readily differentiated *in vitro*. A cell line which readily differentiates *in vitro*, such as the CaCo-2 colon adenocarcinoma cell line may be more appropriate for such experiments.

It may also be worthwhile to experiment with the type of ECM component used, because collagen type I is not the collagen which epithelial cells would usually be exposed to *in vivo*. The use of a complete basement membrane, for example EHS may prove to be a better inducer of differentiation, but again the ability of the HT-29_2 cells may be

inherently blocked in the majority of cells thus preventing extensive differentiation. It remains to be elucidated as to whether any other ECM component or complete basement membrane can induce a more profound differentiation of the HT-29 cell line.

Alternatively, the derivation of new human colon adenocarcinoma cell lines from primary tumours directly onto ECM components or complete basement membranes would prevent the detrimental effects that the culture on plastic or glass surfaces has on the maintenance of epithelial differentiation. These cell lines may prove to be more representative of the *in vivo* tumour from which they were derived, with respect their morphological and functional differentiation, and their chemosensitivity. Their use as part of the early screening panels of human tumour cell lines for the selection of novel antineoplastic agents could lead to the more accurate selection of clinically useful agents for the treatment of solid tumours. For the above reasons I would like to suggest that the derivation and development of newly derived human malignant colon cell lines onto ECM components is worthy of thorough investigation.

REFERENCES

REFERENCES

Adams, J.C., and Watt, F.M. (1989). Fibronectin inhibits the terminal differentiation of human keratinocytes. Nature (Lond.), **340**, 307-309.

Adibi, S.A., and Mercer, D.W. (1973). Protein digestion in human intestine as reflected in luminal, mucosal, and plasma amino acid concentrations after meals. J.Clin.Invest., **52**, 1586-1594.

Agris, P.L., and Campbell, I.D. (1982). Proton nuclear magnetic resonance of intact friend leukemia cells: Phosphorylcholine increase during differentiation. Science (Washington, DC), **216**, 1325-1327.

Ahnen, D.J., Kinoshita, K., Nakane, P.K., and Brown, W.R. (1987). Differential expression of carcinoembryonic antigen and secretory component during colonic epithelial cell differentiation and in colonic carcinomas. Gastroenterology, **93**, 1330-1338.

Akiyama, S.K., Nagata, K., and Yamada, K.M. (1990). Cell surface receptors for extracellular matrix components. Biochem.Biophys. Acta, **1031**, 91-110.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D. (1983). Molecular Biology of the Cell. Garland PublishingInc., New York and London, 549-609.

Alley, M.C., Scudiero, D.A., Monks, A., Hursey, M.L., Czerwinski, M.J., Fine, D.L., Abbott, B.J., Mayo, J.G., Shoemaker, R.H., and Boyd, M.R. (1988). Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res., **48**, 589-601.

Au, F.C., Stein, B., and Tang, C-K. (1986). Carcinoembryonic antigen level in colonic lesions. Am.J.Surg., **151**, 61-64.

Augeron, C., and Laboisse, C.L. (1984). Emergence of permanently differentiated cell clones in a human colonic cancer cell line in culture after treatment with sodium butyrate. Cancer Res., **44**, 3961-3969.

Bancroft, J.D., and Stevens, A. (1982). Theory and practice of histological techniques. Churchil Livingstone, New York, 113.

Barcellos-Hoff, M.H., Aggeler, J., Ram, T.G., and Bissell, M.J. (1989). Functional differentiation and alveolar morphogenesis of primary mammary epithelial cell cultures on reconstituted basement membrane. Development, **105**, 223-235.

Bazill, G.W., and Dexter, T.M. (1989). An antagonist to platelet activating factor counteracts the tumoricidal action of alkyl lysophospholipids. Biochem. Pharmacol., **38**, 374-377.

Beauchemin, N., Benchimol, S., Cournoyer, D., Fuks, A., and Stanners, C.P. (1987). Isolation and characterisation of full-length functional c-DNA clones for human carcinoembryonic antigen. Mol. Cell Biol., **7**, 3221-3230.

Bellot, F., Luis, J., El Battari, A., Secchi, J., Cau, P., Marvaldi, J., and Pichon, J. (1985). Extracellular material secreted by human colonic adenocarcinoma cell lines promotes spreading in serum-free medium and induces neurite outgrowth of PC-12 cells. Int. J. Cancer, **36**, 609-615.

Benchimol, S., Fuks, A., Jothy, S., Beauchemin, N., Shirota, K., and Stanners, C.P. (1989). Carcinoembryonic antigen, a human tumour marker, functions as an intercellular adhesion molecule. Cell, **57**, 327-334.

Bergmeyer, H.U. (editor) (1974). Methods of enzymatic analysis. Volume 1. Verlag Chemie Weinheim Academic Press Inc., New York, 496.

Bernard, C. (1959). De la matiere glycogene consideree comme condition de developpement de certains tissus chez le foetus, avant l'apparition de la fonction glycogenique du foie. C.r.Lebd.Seanc.Acad.Sci. Paris, **48**, 673-683.

Bill, C.A., Hickman, J.A., and Gescher, A. (1986). Effects of N-Methylformamide (NMF) on the glutathione status and cell cycle of TLX5 murine lymphoma cells *in vitro*. Br.J.Cancer, **54**, 168.

Bissell, M.J. (1981). The differentiated status of normal and malignant cells or how to define a "normal" cell in culture. Int. Rev.Cytol., **70**, 27-100.

Bissell, M.J., Hall, G., and Parry, G. (1982). How does the extracellular matrix direct gene expression ?. J.Theoret. Biol., **99**, 31-68.

Bitensky, J.C.L., and Poulter, R.B.L. (1969). A guide to practical histochemistry. Oliver and Boyd Ltd., Edinburgh, 229-230.

Blair, S.D., Theodorou, N.A., Begent, R.H.J., Dawson, P.M., Salmon, M., Riggs, S., Kelly, A., Boxer, G., Southall, P., and Gregory, P. (1990). Comparison of anti-fetal colonic microvillus and anti-CEA antibodies in peroperative radioimmunolocalisation of colorectal cancer. Br.J.Cancer, **61**, 891-894.

Blais, A., Bissonnette, P., and Berteloot, A. (1987). Common characteristics for Na^+ - dependent sugar transport in Caco-2 cells andhuman fetal colon. J.Membr.Biol., **99**, 113-125.

Bodmer, W.F.(1988). Somatic cell genetics and cancer. Cancer Surv., **7**, 239-250.

Bodmer,W.F., Bailey, C.J., Bodmer, J., Bussey, H.J.R., Ellis, A., Gorman, P., Lucibello, F.C., Murday, V.A., Rider, S.H., Scambler, P., Sheer, D., Solomon, E., and Spurr, N.K. (1987). Localization of the gene for familial adenomatous polyposis on chromosome 5. Nature (Lond.), **328**, 614-616.

Boffa, L.C., Vidali, G., Mann, R.S., and Allfrey, V.G. (1978). Suppression of histone deacetylation in vivo and in vitro by sodium butyrate. J.Biol. Chem., **253,** 3364-3366.

Bordes, M., Michaels, R., and Martin, F. (1973). Detection by immunofluorescence of carcinoembryonic antigen in colonic carcinoma , other malignant or benign tumours, and non-cancerous tissues. Digestion, **9**, 106-115.

Borenfreund, E., Steinglass, M., Korngold, G., and Bendich, A. (1975). Effect of dimethyl sulfoxide and dimethylformamide on the growth and morphology of tumor cells. Ann. N.Y.Acad.Sci., **243**, 164-171.

Bradford, M.M., (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72,** 248-254.

Brattain, M.G., Levine, A.E., Chakrabarty, S., Yeoman, L.C., Willson, J.K.V., and Long, B.H. (1984). Heterogeneity of human colon carcinoma. Cancer Metastasis Rev., **3**, 177-191.

Breitman, T.R., Selonick, S.E., and Collins, S.J. (1980). Induction of differentiation of the human promyelocytic leukaemia cell line (HL-60) by retinoic acid. Proc. Natl. Acad. Sci., USA, **77**, 2936-2940.

Burgess. D.R. (1976). Structure of the epithelial-mesenchymal interface during early morphogenesis of the chick duodenum. Tissue Cell, **8**, 147-158.

Burkitt, D.P. (1971). Epidemiology of cancer of the colon and rectum. Cancer (Phila.), **28**, 3-13.

Buroker, T.R., Moertel, C.G., Fleming, T.R., Everson, L.K., Cullinan, S.A., Mailliard, J.A., Marschke, R.F., Klaassen, D.J., Laurie,J.A., and Moon, M.D. (1985). A controlled evaluation of recent approaches to biochemical modulation or enhancement of 5-fluorouracil therapy in colorectal carcinoma. J. Clin. Oncol. , **3**, 1624-1631.

Candido, E.P.M., Reeves, R., and Davie, J.R. (1978). Sodium butyrate inhibits histone deacetylation in cultured cells. Cell, **14**, 105-113.

Chambard, M., Gabrion, J., and Mauchamp, J. (1981). Influence of collagen gel on the orientation of epithelial cell polarity : Follicle formation from isolated thyroid cells and from preformed monolayers. J. Cell Biol., **91**, 157-166.

Chastre, E., Emami, S., Rosselin, G., and Gespach, C. (1985).Vasoactive intestinal peptide receptor activity and specificity during enterocyte-like differentiation and retrodifferentiation of the human colonic cancerous subclone HT29-18. FEBS Lett., **188**, 197-204.

Chen, L-H., and Bissell, M.J. (1989). A novel regulatory mechanism for whey acidic protein gene expression. Cell Reg., **1**, 45-54.

Cheng, H. and Leblond, C.P. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. Am.J. Anat., **141**, 461-561.

Chou, J.Y. (1979). Regulation of the induction of alkaline phosphatase in choriocarcinoma cells by sodium butyrate. In Vitro, **15**, 789-795.

Christmann, J.K., Weich, N., Schoenbrun, B., Schneiderman, N., and Acs, G. (1980). Hypomethylation of DNA during differentiation of Friend erythroluekemia cells. J. Cell. Biol., **86**, 366-370.

Chung, Y.S., Song, I.S., Erickson, R.H., Sleisenger, M.H., and Kim, Y.S. (1985). Effects of growth and sodium butyrate on brush border membrane-associated hydrolases in human colorectal cancer cell lines. Cancer Res., **45**, 2976-2982.

Coffey, R.J.Jr., Goustin, A.S., Soderquist, A.M., Shipley, G.D., Wolfshohl, J., Carpenter, G., and Moses, H.L. (1987). Transforming growth factor alpha and beta expression in human colon cancer lines : implications for an autocrine model. Cancer Res., **47**, 4590-4594.

Collins, S.J., Gallo, R.C., and Gallagher, R.E. (1977). Continuous growth and differentiation of human myeloid leukemic cells in suspension culture. Nature (Lond.), **270**, 347-349.

Collins, S.J., Ruscetti, F.W., Galllagher, R.E., and Gallo, R.C. (1978). Terminal differentiation of human promyelocytic cells induced by dimethylsulfoxide and other polar compounds. Proc.Natl. Acad.Sci., USA, **75**, 2458-2462.

Cordeiro, R.F., and Savarese, T.M. (1984). Reversal by L-cysteine of the growth of inhibitory and glutathione-depleting effects of N-methylformamide and N,N-dimethylformamide. Biochem. Biophys. Res. Commun., **122**, 798-803.

Cordeiro, R.F., and Savarese, T.M. (1986). Role of glutathione depletion in the mechanism of action of N-methylformamide and N,N-dimethylformamide in a cultured human colon cancer carcinoma cell line. Cancer Res., **46**, 1297-1305.

Culouscou, J.M., Garrouste, F., Remacle-Bonnet, M., Bettetini, D., Marvaldi, J., and Pommier, G. (1988). Autocrine secretion of a colorectum-derived growth factor by HT-29 human colon adenocarcinoma cell line. Int. J.Cancer, **42**, 895-901.

Culouscou, J.M., Remacle-Bonnet, M., Garrouste, F., Marvaldi, J., and Pommier, G. (1987). Simultaneous production of IGF-I and EGF competing growth factors by HT29 human colon cancer line. Int.J.Cancer, **40**, 646-652.

Cummings, J.H. (1981). Short-chain fatty acids in the human colon. Gut, **22**, 763-779.

Dahlqvist, A. (1968). Assay of intestinal dissaccharidases. Anal. Biochem., **22**, 99-107.

D'Anna, J.A., Tobey, R.A., and Gurley, L.R. (1980). Concentration-dependent effects of sodium butyrate in chinese hamster cells : cell-cycle progression, inner-histone acetylation, histone H2 dephosphorylation, and induction of an H2-like protein. Biochemistry, **19**, 2656-2671.

Davidson, B.R., Sams, V.R., Styles, J., Dean, C., and Boulos, P.B. (1989). Comparative study of carcinoembryonic antigen and epithelial membrane antigen expression in normal colon, adenomas and adenocarcinomas of the colon and rectum. Gut, **30**, 1260-1265.

Day, R.S. III, Ziollowski, C.H.J., Scudiero, D.A., Meyer, S.A., Lubiniecki, A.S., Girardi, A.J., Galloway, S.M., and Bynum, G.D. (1980). Defective repair of alkylated DNA by human tumour and SV40-transformed human cell strains. Nature (Lond.), **288**, 724-727.

De Haan, J.B., Gevers, W., and Parker, M.I. (1986). Effects of sodium butyrate on the synthesis and methylation of DNA in normal cells and their transformed counterparts. Cancer Res., **46**, 713-716.

Denk, H., Tappeiner, G., Eckerstorfer, R., and Holner, J.H. (1972). Carcinoembryonic antigen (CEA) in gastrointestinal and extragastrointestinal tumours and its relationship to tumour-cell differentiation. Int. J. Cancer, **10**, 262-272.

Dexter, D.L. (1977). N,N-Dimethylformamide-induced morphological differentiation and reduction of tumorigenicity in cultured mouse rhabdomyosarcoma cells. Cancer Res., **37**, 3136-3140.

Dexter, D.L., Barbosa, J.A. and Calabresi, P. (1979). N,N-Dimethylformamide-induced alteration of cell culture characteristics and loss of tumorigenicity in cultured human colon carcinoma cells. Cancer Res., **39**, 1020-1025.

Dexter, D.L., Crabtree, G.W., Stoeckler, J.D., Savarese, T.M., Ghoda, L.Y., Rogler-Brown, T.L., Parks, R.E., and Calabresi, P. (1981). N,N-Dimethylformamide and sodium butyrate modulation of the activities of purine-metabolizing enzymes in cultured human colon carcinoma cells. Cancer Res., **41**, 808-812.

Dexter, D.L., Spremulli, E.N., Matook, G.M., Diamond, I., and Calabresi, P. (1982). Inhibition of the growth of human colon cancer xenografts by polar solvents. Cancer Res., **42**, 5018-5022.

Dibner, M.D., Ireland, K.A., Koerner, L.A., and Dexter, D.L. (1985). Polar solvent-induced changes in membrane lipid lateral diffusion in human colon cancer cells. Cancer Res., **45**, 4998-5003.

Double, J.A., Ball, C.R., and Cowan, P.N. (1975). Transplantation of adenocarcinomas of the colon in mice. J. Natl. Cancer. Inst., **54**, 271-275.

Eilber, F.R. (1985). Principles of cancer surgery. In : Cancer Treatment, 2nd Edition. Haskell, C.M.(Editor), W.B. Saunders Company, Philadelphia, 7-13.

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

Eliyahu,D., Raz, A., Gruss, P., Givol, D., and Oren, M. (1984). Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature (Lond.), **312**, 646-649.

Emerman, J.T., Bartley, J.C. and Bissell, M.J. (1981). Glucose metabolite patterns as markers of functional differentiation in freshly isolated and cultured mouse mammary epithelial cells. Exp. Cell Res., **134**, 241-250.

Emerman, J.T., Burwen, S.J., and Pitelka, D.R. (1979). Substrate properties influencing ultrastructural differentiation of mammary epithelial cells in culture. Tissue Cell, **11**, 109-119.

Emerman, J.T., and Pitelka, D.R. (1977). Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. In Vitro, **13**, 316-328.

Erlichman, C., Fines, C., Wong, A., and Elhakim, T. (1988). A randomized trial of fluorouracil and folonic acid in patients with metastatic colorectal carcinoma. J. Clin. Oncol., **6**, 469-475.

Fahey, J.L., and Liu, B.C-S. (1988). Cancer and the immune response. In : Cancer the outlaw cell. 2nd edition. Lafond. R.E. (editor), American Chemical Society, Washington DC., 143-158.

Fantini, J., Abadie, B., Tirard, A., Remy, L., Ripert, J.P., El Battari, A., and Marvaldi, J. (1986). Spontaneous and induced dome formation by two clonal cell populations derived from a human adenocarcinoma cell line, HT29. J.Cell Sci., **83**, 235-249.

Fantini, J., Rognoni, J-B., Culouscou, J-M., Pommier, G., Marvaldi, J., and Tirard, A. (1989). Induction of polarized apical expression and vectorial release of carcinoembryonic antigen (CEA) during the process of differentiation of HT29-D4 cells. J. Cell. Physiol., **141**, 126-134.

Farber, E., and Cameron, R. (1980). The sequential analsis of cancer development. Adv. Cancer. Res., **31**, 125-226.

Fearon, E.R., Hamilton, S.R., and Vogelstein, B.(1987). Clonal analysis of human colorectal tumors. Science (Washington, DC), **238**, 193-197.

Fearon, E.R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell, **61**, 759-767.

Fearon, E.R., Cho, K.R., Nigro, J.M., Kern, S.E., Simons, J.W., Ruppert, J.M., Hamilton, S.R., Preisinger, A.C., Thomas, G., Kinzler, K.W. and Vogelstein, B. (1990). Identification of a chromosome 18q gene that is altered in colorectal cancers. Science (Washington, DC), **247,** 49-56.

Finlay, C.A., Hinds, P.W., Levine, A.J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. Cell, **57**, 1083-1093.

Fogh, J., Fogh, J.M., and Orfeo, T. (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. J. Natl. Cancer Inst., **59**, 221-226.

Fogh, J., and Giovanella, B.C. (1978). The nude mouse in experimental and clinical research. Academic Press, New York.

Fogh, J., and Trempe, G. (1975). New human tumor cell lines. In : Human tumor cells "in vitro". Fogh, J. (editor). Plenum Publishing Corporation, New York, 115-141.

Frais, F. (1972). Practical biochemistry, an introductory course. Butterworths, London, 129-131.

Friend, C., Scher, W., Holland, J.G., and Stato, T. (1971). Hemoglobin synthesis in murine virus-induced leukemic cells in vitro : stimulation of erythroid differentiation by dimethylsulfoxide. Proc.Natl. Acad.Sci.,USA, **68**, 378-382.

Gallagher, R.E., Collins, S.J., Trujillo, J., McCredie, K., Ahearn, M., Tsai, S., Metzgar, R., Avlakh, G., Ting, R., Ruscetti, F., and Gallo, R. (1979). Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. Blood, **54**, 713-733.

Galons, J-P., Fantini, J., Vion-Dury, J., Cozzone, P.J., and Canioni, P. (1989). Metabolic changes in undifferentiated and differentiated human colon adenocarcinoma cells studied by multinuclear magnetic resonance spectroscopy. Biochimie, **71**, 949-961.

Galons, J-P., Fantini, J., Vion-Dury, J., Cozzone, P.J., and Canioni, P. (1990). Effect of VIP on the glycogen metabolism of human colon adenocarcinoma cells studied by ^{13}C nuclear magnetic resonance spectroscopy. Int.J.Cancer, **45**, 168-173.

Gastrointestinal Tumor Study Group (1989). The modulation of fluorouracil with leucovorin in metastatic colorectal carcinoma : A prospective randomized phase III trial. J. Clin. Oncol., **7**, 1419-1426.

Gilbert, C.W., and Lajtha, L.G. (1965). The importance of cell population kinetics in determining response to irradiation of normal and malignant tissue. In : Cellular radiation biology. The University of Texas M.D. Anderson Hospital Tumor Institute, 74.

Gold, P., and Freedman, S.O. (1965). Demonstration of tumor specific antigens in human colonic carcinomata by immunological tolerance and absorbance techniques. J. Exp. Med., **121**, 439-462.

Goldenberg, D.M., Sharkey, R.M., and Primus, F.J. (1976). Carcinoembryonic antigen in histopathology : Immunoperoxidase staining of conventional tissue sections. J. Natl. Cancer Inst., **57**, 11-22.

Goldin, A. (1983). Animal models for cancer chemotherapy. In : Cancer Treatment and Research, Volume 7, Cancer Chemotherapy I. Muggia, F.M. (Editor), Martinus Nijhoff Publ., The Hague, 65 -102.

Goldin, A., Serpick, A.A., and Mantel, N. (1966). A commentary : Experimental screening procedures and clinical predictability value. Cancer Chemother.Rep., **50**, 173-218.

Grand, R.J., Watkins, J.B., and Torti, F.M. (1976). Development of the gastrointestinal tract. Gastroenterology, **70**, 790-810.

Greenberg, G., and Hay, E.D. (1988). Cytoskeleton and thyroglobulin expression change during transformation of thyroid epithelium to mesenchyme-like cells. Development, **102**, 605-622.

Gregg, C.T. (1972). In : Growth, nutrition and metabolism of cells in culture. Volume 1. Rothblat, G.H., and Cristofalo, V.T. (editors). Academic Press, New York, 83-136.

Griffin, M.J., Price, G.H., and Bazzell, K.L. (1974). A study of adenosine 3':5'-cyclic monophosphate, sodium butyrate, and cortisol as inducers of HeLa alkaline phosphatase. Arch. Biochem.Biophys., **164**, 619-623.

Griswold, D.P., and Corbett, T.H. (1978). Use of experimental models in the study of approaches to treatment of colorectal cancer. In : Gastrointestinal Tract Cancer. Lipkin, M.D., and Good, R.A. (Editors), Plenum Medical Book Company, New York, 399-418.

Grobstein, C. (1953). Morphogenetic interaction between embryonic mouse tissues separated by a membrane filter. Nature (Lond.), **172**, 869-871.

Grobstein, C. (1955). Tissue interactions in the morphogenesis of mouse embryonic rudiments in vitro. In : Aspects of synthesis and order in growth. Rudnik, D. (editor). Princeton University Press, Princeton. 233-256.

Gustafsson, M., Sundqvist, T., and Magnusson, K-E. (1988). Lateral diffusion of the secretory component (SC) in the basolateral membrane of the human colon carcinoma cell line HT29 assessed with fluorescence recovery after photobleaching. J. Cell. Physiol. **137**, 608-611.

Haeuptle, M-T., Suard Y.L.M., Bogenmann, E., Reggio, H., Racine, L., and Kraehenbuhl, J-P. (1983). Effect of cell shape change on the function and differentiation of rabbit mammary cells in culture. J.Cell Biol., **96**, 1425-1434.

Haffen, K., Kedinger, M., and Lacroix, B. (1986). Cytodifferentiation of the intestinal villus epithelium. In : Molecular and Cellular Basis of Digestion. Desnuelle, P., Sjostrom, H., and Noren, O., (Editors). Elsevier Science Publishers, New York, 311-322.

Haffen, K., Kedinger, M., and Simon-Assmann, P. (1987). Mesenchyme-dependent differentiation of epithelial progenitor cells in the gut. J.Pedr.Gastro.Nutr., **6**, 14-23.

Hager, J.C., Gold, D.V., Barbosa, J.A., Fligiel, Z., Miller, F., and Dexter, D.L. (1980). N,N-Dimethylformamide-induced modulation of organ- and tumour-associated markers in cultured human colon carcinoma cells. J. Natl.Cancer Inst., **64**, 439-446.

Hajdu, S.I., and Fogh, J. (1978). The nude mouse as a diagnostic tool in human tumor cell research. In : The nude mouse in experimental and clinical research. Fogh, J., and Giovanella, B.C. (Editors). Academic Press, New York, 235-266.

Hamburger, A.W., and Salmon, S.E. (1977a). Primary bioassay of human myeloma stem cells. J.Clin.Invest., **60**, 846-854.

Hamburger, A.W., and Salmon, S.E. (1977b). Primary bioassay of human tumor stem cells. Science (Washington, DC), **197**, 461-463.

Harris, A.L., Karran, P., and Lindahl, T. (1983). O^6-Methylguanine-DNA-methyltransferase of human lymphoid cells : structural and kinetic properties and absence in repair deficient cells. Cancer Res., **43**, 3247-3252.

Hauri, H-P., Sterchi, E.E., Bienz, D., Fransen, J.A.M., and Marxer, A. (1985). Expression and intracellular transport of microvillus membrane hydrolases in human intestinal epithelial cells. J.Cell Biol., **101**, 838-851.

Hay, E.D., and Meier, S. (1976). Stimulation of corneal differentiation by interaction between cell surface and extracellular matrix. Dev. Biol., **52**, 141-157.

Heidelberger, C., Chaudhuri, N.K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R.J., Pleven E., and Scheiner, J. (1957). Fluorinated pyrimidines, a new class of tumor-inhibiting compounds. Nature (Lond.), **179**, 663-666.

Heintzelman, M.B., and Mooseker, M.S. (1990). Assembly of the brush border cytoskeleton : Changes in the distribution of microvillar core proteins during enterocyte differentiation in adult chicken intestine. Cell Motil.Cytoskel., **15**, 12-22.

Herz, F., Schermer, A., Halwer, M., and Bogart, L.H. (1981). Alkaline phosphatase in HT-29, a human colon cancer cell line : influence of sodium butyrate and hyperosmolality. Arch.Biochem.Biophys., **210**, 581-591.

Hildebrand-Zanki, S.U., and Kern, D.H. (1984). A new bioassay for *in vitro* drug stability. In : Human Tumor Cloning. Salmon, S.E., and Trent, J.M. (editors), Grune and Stratton, Orlando, Florida. 451-458.

Horwitz, A., Duggan, K., Greggs, R., Decker, C., and Buck, C.(1985). The cell substrate attachment (CSAT) antigen has properties of a receptor for laminin and fibronectin. J.Cell Biol., **101**, 2134-2144.

Houlihan, W.J., Lee, M.L., Munder, P.G., Nemecek, G.M., Handley, D.A., Winslow, C.M., Happy, J., and Jaeggi, C. (1987). Antitumor activity of SRI 62-834, a cyclic ether analog of Et-18-OCH_3. Lipids, **22**, 884-890.

Hubbard, S.C., and Ivatt, R.J. (1981). Synthesis and processing of asparagine-linked oligosaccharides. Annu.Rev.Biochem., **50**, 555-583.

Huberman, E., Heckman, C., and Langenbach, R. (1979). Stimulation of differentiated functions in human melanoma cells by turmor promoting agents and dimethylfsulfoxide. Cancer Res., **39**, 2618-2624.

Huet, C., Sahuquillo-Merino, C., Coudrier, E., and Louvard, D. (1987). Absorptive and mucus-secreting subclones isolated from a multipotent intestinal cell line (HT-29) provide new models for cell polarity and terminal differentiation. J.Cell Biol., **105**, 345-357.

Hynes, R.O. (1987). Integrins : a family of cell surface receptors. Cell, **48**, 549-554.

Ishidate, M., Sakurai, Y., and Izums, M. (1955). N-Bis-(ß-chloroethyl) amino acids and related compounds as tumor growth retarding agents. J.Am.Pharm.Assoc.,**44**, 132-135.

Jakel, H-P., Wobus, A.M., Bloch, C.H., Jakel, N., and Schoneich, J. (1983). A micromethod for the determination of alkaline phosphatase in mammalian cells. Biomed. Biochim. Acta, **42**, 1123-1128.

Jones, P.A. and Taylor, S.M. (1980). Cellular differentiation, cytidine analogs and DNA methylation. Cell, **20**, 85-93.

Kaye, G.I., Lane, N., and Pascal, P.R. (1968). Colonic pericryptal fibroblast sheath : replication, migration, and cytodifferentiation of mesenchymal cell system in adult tissue. II. Fine structural aspects of normal rabbit and human colon. Gastroenterology, **54**, 852-865.

Kedinger, M., Haffen, K., and Simon-Assmann, P. (1986). Control mechanisms in the ontogenesis of villus cells. In : Molecular and cellular basis of digestion. Desnuelle, P., Sjostrom, H., and Noren, O., (editors). Elsevier Science Publishers, New York, 323-334.

Kedinger, M., Simon-Assmann, P., and Haffen, K. (1987). Growth and differentiation of intestinal endodermal cells in a coculture system. Gut, **28**, 237-241.

Kedinger, M., Simon-Assmann, P., Lacroix, B., and Haffen, K. (1983). Role of glucocorticoids on the maturation of brush border enzymes in fetal rat gut endoderm. Experientia, **39**, 1150-1152.

Kellokumpu, I., Ekblom, P., Scheinin, T.M., and Andersson, L.C. (1985). Malignant transformation in human colorectal mucosa as monitored by distribution of laminin, a basement membrane glycoprotein. Acta.path.microbiol.immunol.scand.,**93**,285-291.

Kenny, A.J., and Maroux, S. (1982). Topology of microvillar membrane hydrolases of kidney and intestine. Physiol.Rev., **69**, 91-128.

Kerbel, R.S., and Davies, A.J.S. (1982). Facilitation of tumour progression by cancer therapy . The Lancet,**11**,977-978.

Kim, Y.S., Nicholson, J.A., and Curtis, K.J. (1974). Intestinal peptide hydrolases : Peptide and amino acid absorption. Med.Clin.N.Am., **58**,1397-1412.

Kim, Y.S., Tsao, D., Siddiqui, B., Whitehead, J.S., Arnstein, P., Bennett, J., and Hicks, J. (1980). Effect of sodium butyrate and dimethyl sulfoxide on biochemical properties of human colon cancer cells. Cancer (Phila.), **45**, 1185-1192.

Kimhi, YU., Palfrey, C., Spector, I., Barak, Y., and Littauer, U.Z. (1976). Maturation of neuroblastoma cells in the presence of dimethylsulfoxide. Proc.Natl.Acad.Sci.USA, **73**, 462-466.

Koh, S-W.M. (1989). The chick retinal pigment epithelium grown on permeable support demonstrates functional polarity. Exp. Cell Res., **181**, 331-347.

Kohn, K.W. (1977). Interstrand crosslinking with DNA by 1,3-bis (2-chloroethyl)-1-nitrosurea and other 1-(2-haloethyl)-1-nitrosureas. Cancer Res., **37**, 1450-1454.

Kroes, W.G.M., Slater, R.M., van den Berg, F.M., Schipper, M.E.I., and Walboomers, J.M.M. (1987). Cytogenetic studies on colon carcinoma. Cancer Genet. Cytogenet., **28**, 34.

Kruh, J. (1982). Effects of sodium butyrate, a new pharmacological agent, on cells in culture. Mol.Cell. Biochem., **42**, 65-82.

Kubota, Y., Kleinman, H.K., Martin, G.R., and Lawley, T.J. (1988). Role of laminin and basement membrane in the morphological differentiation of human endofthelial cells into capillary-like structures. J.Cell Biol., **107**, 1589-1598.

Lacroix, B., Kedinger, M., Simon-Assmann, P., and Haffen, K. (1985). Enzymatic response to glucocorticoids of the chick intestinal endoderm associated with various mesenchymal cell types. Biol. Cell, **54**, 235-240.

Lacroix, B., Kedinger, M., Simon-Assmann, P., Rousset, M., Zweibaum, A., and Haffen, K. (1984). Developmental pattern of brush border enzymes in the human fetal colon. Correlation with some morphogenic events. Early Hum. Dev., **9**, 95-103.

Land, H., Chen, A.C., Morgenstern, J.P., Parada, L.F., and Weinberg, R.A. (1986). Behaviour of myc and ras oncogenes in transformation of rat embryo fibroblasts. Mol.Cell Biol., **6**, 1917-1925.

Land, H., Parada, L.F., and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature (Lond.), **304**, 596-602.

Lane, D.P., and Crawford, L.V. (1979). T antigen is bound to a host protein in SV40-transformed cells. Nature (Lond.), **278**, 261-263.

Langdon, S.P., and Hickman, J.A. (1987). Alkylformamides as inducers of tumour cell differentiation - a minireview. Toxicology, **43**, 239-249.

Lazenby, C.M., Thompson, M.G., and Hickman, J.A. (1990). Elevation of leukemic cell intracellular calcium by the ether lipid SRI 62-834. Cancer Res., **50**, 3327-3330.

Lazo, J.S., Ingber, D.E., and Sartorelli, A.C. (1978). Enhancement of experimental lung metastases by cultured B16 melanoma cells treated with (±)-1,2-Bis(3,5-dioxopiperazin-1-yl) propane (ICRF-159). Cancer Res., **38**, 2263-2270.

Leavitt, J., Barrett, J.C., Crawford, B.D., and Ts'O, P.O.P. (1978). Butyric acid suppression of the *in vitro* neoplastic state of syrian hamster cells. Nature (Lond.), **271**, 262-265.

Le Bivic, A., Hirn, M., and Reggio, H. (1988). HT-29 cells are an *in vitro* model for the generation of cell polarity in epithelia during embryonic differentiation. Proc.Natl.Acad.Sci. USA, **85**, 136-140.

Leder, A., and Leder, P. (1975). Butyric acid, a potent inducer of erythroid differentiation in cultured erythroleukemic cells. Cell, **5**, 319-322.

Le Douarin, N., Bussonnet, C., and Chaumont, F. (1968). Etude des capacites de differenciation et du role morphogene de l'endoderme pharyngien chez l'embryon d'oiseau. Ann.Embryol.Morphol., **1**, 29-39.

Lee, E.Y-H., Lee, W-H., Kaetzel, C.S., Parry, G., and Bissell, M.J. (1985). Interaction of mouse mammary epithelial cells with collagenous substrata : Regulation of casein gene expression and secretion. Proc.Natl. Acad.Sci., USA, **82**, 1419-1425.

Lee, E.Y-H., Parry, G., and Bissell, M.J. (1984). Modulation of secreted proteins of mouse mammary epithelial cells by the extracellular matrix. J.Cell Biol., **98**, 146-155.

Leiter, J., and Schneiderman, M.A. (1959). Screening data from the Cancer Chemotherapy National Service Centre Screening Laboratories. Cancer Res., **19**, 31-193.

Lennarz, W., (1980). Editor. The biochemistry of glycoproteins and proteoglycans. Plenum Press, New York, 1-160.

Lev, R., and Griffiths, W.C. (1982). Colonic and small intestinal alkaline phosphatase. A histochemical and biochemical study. Gastroenterology, **82**, 1427-1435.

Lewis, P.R., and Knight, D.P. (1977). Staining methods for sectioned material. In : Practical methods in electron microscopy. Volume 5 Part 1, Glauert, A.M. (editor). Elsevier Biomedical Press, North Holland, 1-325.

Liu, E., Hjelle, B., Morgan, R., Hecht, F., and Bishop, J.M. (1987). Mutations of the Kirsten *ras* oncogene in human preleukaemia. Nature (Lond.), **330**, 186-188.

Lord, J.M., Bunce, C.M., and Brown, G. (1988). The role of protein phosphorylation in the control of cell growth and differentiation. Br. J. Cancer, **58**, 549-555.

Lotan, R. (1980). Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. Biochem. Biophys. Acta, **605**, 33-91.

Lotan, R., Francis, G.E., Freeman, C.S. and Waxman, S. (1990). Meeting report : Differentiation therapy. Cancer Res., **50**, 3453-3464.

Low, M.G., Ferguson, M.A.J., Futerman, A.H., and Silman, I. (1986). Covalently attached phosphatidylinositol as a hydrophobic anchor for membrane proteins. Trends Biochem.Sci., **11**, 212-215.

Luis, J., Martin, J.M, El Battari, A., Fantinin, J., Giannellini, F., Marvaldi, J., and Pichon, J. (1987). Cycloheximide induces accumulation of vasoactive intestinal peptide (VIP) binding sites at the cell surface of a human colonic adenocarcinoma cell line (HT29-D4). Evidence of the presence of an intracellular pool of VIP receptors. Eur.J. Biochem., **167**, 391-396.

Mackillop, W.J., Ciampi, A., Till, J.E., and Buick, R.N. (1983). A stem cell model of human tumor growth. Implications for tumor cell clonogenic assays. J.Natl.Cancer Inst., **70**, 9-16.

Magnusson, K-E., Gustafsson, M., Holmgren, K., and Johansson, B. (1990). Small intestinal differentiation in human colon carcinoma HT29 cells has distinct effects on the lateral diffusion of lipids (ganglioside GM_1) and proteins (HLA class 1, HLA class 2, and neoplastic epithelial antigens) in the apical cell membrane. J. Cell. Physiol., **143**, 381-390.

Magnusson, K-E., Johansson, B., and Sundqvist, T. (1987). Rapid lateral diffusion of lectin-labelled glycoconjugates in the human colonic adenocarcinoma cell line HT29. Eur. Biophys.J.,**15**, 141-145.

Marsh, M.N., and Trier, J.S. (1974a). Morphology and cell proliferation of subepithelial fibroblasts in adult mouse jejunum. I. Structural features. Gastroenterology, **67**, 622-635.

Marsh, M.N., and Trier, J.S. (1974b). Morphology and cell proliferation of subepithelial fibroblasts in adult mouse jejunum. II. Radioautographic studies. Gastroenterology, **67**, 636-645.

Marvaldi, J., Mangeat, P., Aitahmed, O., Coeroli, C., and Marchis-Mouren, G. (1979). Activation of cyclic AMP-dependent protein kinases in human gut adenocarcinoma (HT29) cells in culture. Biochem. Biophys. Acta, **588**, 12-19.

Maskens, A.P., Rahier, J.T., Meersseman, F.P., Dujardin-Loits, R-M., and Haot, J.G. (1979). Cell proliferation of pericryptal fibroblasts in the rat colon mucosa. Gut, **20**, 775-779.

Mathan, M., Hermos, J.A., and Trier, J.S. (1972). Structural features of the epithelio-mesenchymal interface of rat duodenal mucosa during development. J. Cell Biol., **52**, 577-588.

Mathan, M., Moxey, P.C., and Trier, J.S. (1976). Morphogenesis of fetal rat duodenal villi. Am.J. Anat., **146**, 73-92.

Mayer, R.J., O'Connell, M.J., Tepper, J.E., and Wolmark, N. (1989).Status of adjuvant therapy for colorectal cancer. J. Natl. Cancer Inst., **81**, 1359-1364.

McComb, R.B., Bowers, G.N., and Posen, S. (1979). Alkaline phosphatase. Plenum Publishing Corp., New York. 27-153.

McCormack, S.A., and Johnson, L.R. (1989). Putrescine uptake and release by colon cancer cells. Am. J. Physiol., **256**, G868-G877.

McCue, P.A., Gubler, M.L., Sherman, M.I., and Cohen, B.N. (1984). Sodium butyrate induces histone hyperacetylation and differentiation of murine embryonal carcinoma cells. J.Cell Biol., **98**, 602-608.

McMillan, T.J., and Hart, I.R. (1986). Enhanced experimental metastatic capacity of a murine melanoma following pre-treatment with anticancer drugs. Clin.Exp. Met., **4**, 285-292.

McMillan, T.J., and Hart, I.R. (1987). Can cancer chemotherapy enhance the malignant behaviour of tumours ? Cancer Met.Rev., **6**, 503-520.

McMillan, T.J., Rao, J., and Hart, I.R. (1986). Enhancement of experimental metastasis by pretreatment of tumour cell with hydroxurea. Int. J. Cancer, **38**, 61-65.

Menard, D., and Pother, P. (1987). Differential distribution of digestive enzyme in isolated epithelial cells from developing human fetal small intestine and colon. J. Pediatre. Gastroenterol.Nutr., **6**, 509-516.

Menko, A.S., and Boettiger, D. (1987). Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. Cell, **51**, 51-57.

Michalopoulos, G., and Pitot, H.C. (1975). Primary cultures of parenchymal liver cells on collagen membranes. Exp. Cell Res., **94**, 70-78.

Milas, L., Tofilon, P., and Iwakawa, M. (1988). Maturational agents alone or in combination with cytotoxic agents in therapy of tumor metastases. Adv.Exp.Med.Biol., **233**, 277-383.

Moertel, C.G., Schutt, A.J., Hahn, R.G., and Reitemeier, R.J. (1975). Therapy of advanced colorectal cancer with a combination of 5-fluorouracil, methyl-1,3-cis (2-chloroethyl)-1-nitrosurea, and vincristine. J. Natl. Cancer Inst., **54**, 69-71.

Mooseker, M.S. (1985). Organization, chemistry, and assembly of the cytoskeleton apparatus of the intestinal brush border. Ann. Rev. Cell Biol., **1**, 209-241.

Morita, A., Tsao, D., and Kim, Y.S. (1982). Effect of sodium butyrate on alkaline phosphatase in HRT-18, a human rectal cancer cell line. Cancer Res., **42**, 4504-4545.

Nigro, J.M., Baker, S.J., Preisinger, A.C., Jessup, J.M, Hostetter, R., Cleary, K., Bigner, S.H., Davidson, N., Baylin, S., Devilee, P., Glover, T., Collins, F.S., Weston, A., Modall, R., Harris, C.C., and Vogelstein, B. (1989). Mutations in the p53 gene occur in diverse human tumour types. Nature (Lond.), **342**, 705-708.

Noseda, A., White, J.G., Godwin, P.L., Jerome, W.G., and Modest, E.J. (1989). Membrane damage in leukemic cells induced by ether and ester lipids : an electron microscopic study. Exp.Mol.Pathol., **50**, 69-83.

Nover, L. (1982) Molecular basis of cell differentiation. In : Cell differentiation. Molecular basis and problems. Nover, L., Luckner, M., and Parthier, B. (editors). Springer Verlag, Berlin. 99-254.

Nowell, P.C. (1976). The clonal evolution of tumor cell populations. Science (Washington, DC), **194**, 23-28.

O'Brien, M.J., Zamcheek, M., Burke, B., Kirkham, S.E., Saravis, C.A., and Gottlieb, L.S. (1981). Immunocytochemical localisation of carcinoembryonic antigen in benign and malignant colorectal tissues. Am.J. Clin. Pathol., **75**, 283-290.

Ogier-Denis, E., Codogno, P., Chantret, I., and Trugnan, G. (1998). The processing of asparagine-linked oligosaccharides in HT-29 cells is a function of their state of enterocytic differentiation. J. Biol. Chem., **263**, 6031-6037.

Ohkura, Y., Yamaguchi, M., and Miyamoto, S. (1980). An ultramicro phosphorimetric assay for alkaline and acid phosphatase activities. Chem. Pharm. Bull., **28**, 3127-3130.

Oikawa, S., Nakazato, H., and Kozaki, G. (1987a). Primary structure of human carcinoembryonic antigen (CEA) deduced from cDNA sequence. Biochem.Biophys.Res.Commun., **142**, 511-518.

Oikawa, S., Imajo, S., Noguchi, T., Kozaki, G., and Nakazato, H. (1987b). The carcinoembryonic antigen (CEA) contains multiple immunoglobulin-like domains. Biochem.Biophys.Res.Commun., **144**, 634-642.

Olsson, L., Due, C., and Diamant, M. (1985) Treatment of human cell lines with 5-azacytidine may result in profound alterations in clonogenicity and growth rate. J.Cell Biol., **100**, 508-513.

Omary, M.B., and Kagnoff, M.F. (1987). Identification of nuclear receptors for VIP on a human colonic adenocarcinoma cell line. Science (Washington, DC), **238**, 1578-1581.

Paget, S.(1889). The distribution of secondary growths in cancer of the breast. Classical Article. Cancer Metastasis Rev., **8**, 98-101, 1989.

Parker, M.I., Judge, K., and Gevers, W. (1982). Loss of type I procollagen gene expression in SV40-transformed human fibroblasts is accompanied by hypermethylation of these genes. Nucleic Acids Res., **10**, 5879-5891.

Parry, G., Lee, E.Y-H., and Bissell, M.J. (1982). Modulation of the differentiated phenotype of cultured mouse mammary epithelial cells by collagen substrata. In : Extracellular Matrix. Hawkes, S.P., and Wang, J. (editors). Academic Press, New York, 303-308.

Paxton, R.J., Mooser, G., Pande, H., Lee, T.D., and Shively, J.E. (1987). Sequence analysis of carcinoembryonic antigen : identification of glycosylation sites and homology with the immunoglobulin supergene family. Proc.Natl.Acad.Sci., USA, **84**, 920-924.

Petrelli, N., Herrera, L., Rustum, y., Burke, P., Creaven, P., Stulc, J., Emrich, L.J., and Mittelman, A. (1987). A prospective randomized trial of 5-fluorouracil versus 5-fluorouracil and high-dose leucovorin versus 5-fluorouracil and methotrexate in previously untreated patients with advanced colorectal carcinoma. J. Clin. Oncol., **5**, 1559-1565.

Phillips, R.M., Bibby, M.C., and Double, J.A. (1990). A clinical appraisal of the predictive value of in vitro chemosensitivity assays. J.Natl.Cancer Inst., **82**, 1457-1468.

Phillips, T.E., Huet, C., Bilbo, P.R., Podolsky, D.K., Louvard, D., and Neutra, M.R. (1988). Human intestinal goblet cells in monolayer culture : characterization of a mucus-secreting subclone derived from HT29 colon adenocarcinoma cell line. Gastroenterology, **94**, 1390-1403.

Pierce, G.B. (1961). Teratocarcinomas. Can. Cancer Conf., **4**, 119-137.

Pierce, G.B., and Dixon, F.J. (1959). Testicular teratomas. 1. Demonstation of teratogenesis by metamorphosis of multipotential cells. Cancer (Phila.), **12**, 573-583.

Pierce, G.B., Dixon, F.J., and Verney, E.L.(1960). Teratocarcinogenic and tissue-forming potentials of cell types comprising neoplastic embryoid bodies. Lab. Invest., **9**, 583-602.

Pierce, G.B., and Speers, W.C. (1988). Tumors as caricatures of the process of tissue renewal : Prospects for therapy by directing differentiation. Cancer Res., **48**, 1996-2004.

Pierce, G.B., and Verney, E.L. (1961). An *in vitro* and *in vivo* study of differentiation in teratocarcinomas. Cancer (Phila.), **14**, 1017-1029.

Pierson, H.F., Fisher, J.M., and Rabinovitz, M. (1985). Modulation by taurine of the toxicity of taumustine, a compound with antitumor activity. J.Natl. Cancer Inst., **75**, 905-909.

Pignatelli, M., and Bodmer, W.F. (1988). Genetics and biochemistry of collagen binding-triggered glandular differentiation in a human colon carcinoma cell line. Proc.Natl.Acad.Sci.USA, **85**, 5561-5565.

Pignatelli, M., and Bodmer, W.F. (1989). Integrin-receptor-mediated differentiation and growth inhibition are enhanced by transforming growth factor-ß in colorectal tumour cells grown in collagen gel. Int. J. Cancer, **44**, 518-523.

Pinto, M., Appay, M-D., Simon-Assmann, P., Chevalier, G., Dracopoli, N., Fogh, J., and Zweibaum, A. (1982). Enterocytic differentiation of cultured human colon cancer cells by replacement of glucose by galactose in the medium. Biol. Cell, **44**, 193-196.

Podlecki, D.A., Smith, R.M., Kao, M., Tsai, P., Huecksteadt, T., Brandenburg, D., Lasher, R.S., Jarett, L., and Olefsky, J.M. (1987). Nuclear translocation of the insulin receptor. A possible mediator of insulins long term effects. J. Biol.Chem., **262**, 3362-3368.

Potten, C.S., and Morris, R.J. (1988). Epithelial stem cells *in vivo*. J.Cell Sci. Suppl., **10**, 45-62.

Prasad, K.N., and Sinha, P.K. (1976). Effect of sodium butyrate on mammalian cells in culture : A review. In Vitro, **12**, 125-132.

Prizont, R., Whitehead, J.S., and Kim, Y.S. (1975). Short chain fatty acids in rats with jejunal blind loops. Gastroenterology, **69**, 1254-1264.

Puddington, L., Lively, M.O., and Lyles, D.S. (1985). Role of the nuclear envelope in synthesis, processing, and transport of membrane glycoproteins. J.Biol. Chem., **260**, 5641-5647.

Rabenandrasana, C., Baghdiguian, S., Marvaldi, J., and Fantini, J. (1990). CD4 molecules are restricted to the basolateral membrane domain of *in vitro* differentiated human colon cancer cells (HT29-D4). FEBS Lett., **265,** 75-79.

Ramming, K.P., and Haskell, C.M. (1985). Colorectal malignancies. In : Cancer Treatment, 2nd Edition. Haskell, C.M.(Editor), W.B. Saunders Company, Philadelphia, 275-307.

Raper, S.E., Burwen, S.J., Barker, M.E., and Jones, A.L. (1987). Translocation of epidermal growth factor to the hepatocyte nucleus during rat liver regeneration. Gastroenterology, **92**, 1243-1250.

Reale, E., and Luciano, L. (1967). Kritische Elektronmikro-skopische Studien uber die Lokalisation der Aktivat Alkalischer Phosphatase in Haupstruck der Niere von Mausen. Histochemie, **8**, 302-314.

Reichmann, A., Martin, P., and Levin, B. (1981). Chromosomal banding patterns in human large bowel cancer. Int. J. Cancer, **28**, 431-440.

Remy, L., Marvaldi, J., Rua, S., Secchi, J., and Lechene de la Porte, P. (1984). The role of intracellular lumina in the repolarization process of a colonic adenocarcinoma cell line. Virchows Archive (Cell.Pathol.), **46**, 297-305.

Richards, F., Case, L.D., White, D.R., Muss, H.B., Spurr, C.L., Jackson, D.V., Cooper, M.R., Zekan, P., Cruz, J., Stuart, J.J., Capizzi, R.L., McCulloch, J.H., McFarland, J.A., Kincaid, W.R., Harding, R.W., Pope, E., McMahan, R., and Wells, H.B. (1986). Combination chemotherapy (5-fluorouracil, methyl-CCNU, mitomycin C) versus 5-fluorouracil alone for advance previously untreated colorectal carcinoma. A phase III study of the Piedmont Oncology Association. J. Clin. Oncol., **4**, 565-570.

Richards, F.M., Watson, A., and Hickman, J.A. (1988) Investigation of the effects of heat shock and agents which induce a heat shock response on the induction of differentiation of HL-60 cells. Cancer Res., **48**, 6715-6720.

Richman, P.I., and Bodmer, W.F. (1988). Control of differentiation in human colorectal carcinoma cell lines : epithelial-mesenchymal interactions. J.Pathol., **156**, 197-211.

Riggs, M.G., Whittaker, R.G., Neumann, J.R., and Ingram, V.M. (1977). n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. Nature (Lond.), **268**, 462-464.

Roberts, A.B., Thompson, N.L., Heine, U., Flanders, C., and Sporn, M.B. (1988). Transforming growth factor-beta : possible role in carcinogenesis. Brit.J.Cancer, **57**, 594-600.

Rodriguez-Boulan, E., and Nelson, W.J. (1989). Morphogenesis of the polarized epithelial cell phenotype. Science (Washington, DC), **245**, 718-725.

Rodriguez-Boulan, E., Paskiet, K.T., and Sabatini, D.D. (1983). Assembly of enveloped viruses in Madin-Darby canine kidney cells : polarized budding from single attached cells and from clusters of cells in suspension. J.Cell Biol., **96**, 866-874.

Rodriguez-Boulan, E., and Sabatini, D.D. (1978). Asymmetric budding of viruses in epithelial monolayers : A model system of epithelial cell polarity. Proc.Natl. Acad. Sci. USA, **75**, 5071-5075.

Rognum, T., Elgjo, K., Brandtzaeg, P., Orjasaeter, H., and Bergan, A. (1982). Plasma carcinoembryonic antigen concentrations and immunohistochemical patterns of epithelial marker antigens in patients with large bowel carcinoma. J.Clin. Pathol., **35**, 922-933.

Roncari, G., and Zuber, H. (1968). Thermophilic aminopeptidases from bacillus stearothermophilus. 1. Isolation, specificity, and general properties of the thermostable aminopeptidase. Int.J. Protein Res., **1**, 45-61.

Rousset, M., Dussaulx, E., Chevalier, G., and Zweibaum, A. (1980). Growth-related glycogen levels of human intestine carcinoma cell lines grown *in vitro* and *in vivo* in nude mice. J. Natl. Cancer Inst., **65**, 885-889.

Rousset, M., Robine-Leon, S., Dussaulx, E., Chevalier, G., and Zweibaum, A. (1979). Glycogen storage in foetal and malignant epithelial cells of the human colon. Front.gastrointest.Res., **4**, 80-85.

Ruley, H.E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature (Lond.), **304**, 602-606.

Salas, P.J.I., Vega-Salas, D.E., Hochman, J., Rodrguez-Boulan, E., and Edidin, M. (1988). Selective anchoring in the specific plasma membrane domain. A role in epithelial polarity. J.Cell Biol., **107**, 2363-2376.

Salmon, S.E. (1984). Human tumor colony assay and chemosensitivity testing. Cancer Treat. Rep., **68**, 117-125.

Sato, K., Morris, H.P., and Weinhouse, S. (1973). Characterization of glycogen synthetases and phosphorylases in transplantable hepatomas. Cancer Res., **33**, 724-733.

Scher, W., Preisler, H.D., and Friend, C. (1973). Hemoglobin synthesis in murine virus-induced leukemic cells in vitro. III. Effects of 5-bromo-2'-deoxyuridine, dimethylformamide and dimethylsulfoxide. J. Cell. Physiol., **81**, 63-70.

Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J. and Crane, R.K. (1973). Purification of the human intestinal brush border membrane. Biochem. Biophys. Acta, **323**, 98-112.

Scudiero, D.A., Meyer, S.A., Clatterbuck, B.E., Mattern, M.R., Ziolkowski, C.H.J., and Day, R.S. III. (1984). Sensitivity of human cell strains having different abilities to repair O^6-methylguanine in DNA to inactivation by alkylating agents including chloroethylnitrosureas. Cancer Res., **44**, 2467-2474.

Schmidhauser, C., Bissell, M.J., Myers, C.A., and Casperson, G.F. (1990). Extracellular matrix and hormones transcriptionally regulate bovine ß-casein 5'sequences in stably transfected mouse mammary cells. Proc. Natl. Acad.Sci., USA, **87**, 9118-9122.

Semenza, G., Sebastio, G., Hunziker, W., Malo, C., Menard, D., and Auricchio, S. (1988). Biosynthesis of human small intestinal sucrase-isomaltase. In : Brush border membranes. Lentze, M.J., and Sterchi, E.E. (editors). Thierme Verlag, Stuttgart. 43-51.

Selby, P., Buick, R.N., and Tannock, I. (1983). A critical appraisal of the "Human tumor stem-cell assay". N. Engl. J. Med., **308**, 129-134.

Shuster, J., Thomson, D.M.P., Fuks, A., and Gold, P. (1980). Immunologic approaches to the diagnosis of malignancy. Prog. Exp. Tumor Res.. **25**, 89-139.

Silverberg, E., and Lubera, J.A. (1989). Cancer statistics, 1989. Cancer Abstracts, **39**, 3-20.

Simon-Assmann, P., Bouziges, F., Daviaud, D., Haffen, K., and Kedinger, M. (1987). Synthesis of glycosaminoglycans by undifferentiated and differentiated HT29 human colonic cancer cells. Cancer Res., **42**, 4478-4484.

Sinha, D.K., and White, C.J. (1988). Collagen gel culture of rat mammary tumor cells as an assay for determination of therapeutic efficacy of chemotherapeutic agents. In Vitro Cell. Devel. Biol., **24**, 525-529.

Skovbjerg, H. (1982). High-molecular weight pro-sucrase-isomaltase in human fetal intestine. Pediatr.Res., **16**, 948-949.

Slamon, D.J., de Kernion, J.B., Verma, I.M. and Cline, M.J. (1984) Expression of cellular oncogenes in human malignancies. Science (Washington, DC), **224**, 256-262.

Smith, A.M., and MacDonald, D.J. (1983). Enzyme immunoassay for carcinoembryonic antigen. Clin. Chem., **29**, 2019-2021.

Sporn, M.B., and Todaro, G.J. (1980). Autocrine secretion and malignant transformation of cells. N. Engl. J. Med., **303**, 878-880.

Spremulli, E.N., and Dexter, D.L. (1984). Polar solvents : A novel class of antineoplastic agents. J. Clin. Oncol., **2**, 227-241.

Streuli, C.H., and Bissell, M.J. (1990). Mammary epithelial cells, extracellular matrix and gene expression. In : Breast Cancer : Cellular and Molecular Biology. Lippman, M.E., and Dickson, R. (editors), Kluwer Academic Publishers. In press.

Streuli, C.H., Ram, T.G., Aibarian, D.C., and Bissell, M.J. (1988). Basement membrane involvement in mammary gland differentiation. J.Cell Biol. **107**, 663a.

Takenaga, K. (1986). Modification of the metastatic potential of tumor cells by drugs. Cancer Met. Rev., **5**, 67-75.

Tanaka, M., Levy, J., Terada, M., Breslow, R., Rifkind, R.A., and Marks, P.A. (1975). Induction of erythroid differentiation in murine virus infected erythroleukemia cells by highly polar compounds. Proc. Natl. Acad. Sci. USA, **72**, 1003-1006.

Tanaka, Y., Bush, K.K., Eguchi, T., Ikekawa, N., Taguchi, T., Kobayashi, Y., and Higgins, P.J. (1990). Effects of 1,25-dihydroxyvitamin D_3 and its analogs on butyrate-induced differentiation of HT29 human colonic carcinoma cells and on the reversal of the differentiated phenotype. Arch. Biochem.Biophys., **276**, 415-423.

Tew, K.D., Dean, S.W., and Gibson, N.W. (1987). The effect of a novel taurine nitrosurea, 1-(2-chloroethyl)-3-[2-(dimethylaminosulfonyl) ethyl]-1-nitrosurea (TCNU) on cytotoxicity, DNA crosslinking and glutathione reductase in lung carcinoma cell lines. Cancer Chemo.Pharm., **19**, 291-295.

Tew, K.D., Kyle, G., Johnson, A., and Wang, A.L. (1985). Carbamoylation of glutathione reductase and changes in cellular and chromosome morphology in a rat cell line resistant to nitrogen mustards but collaterally sensitive to nitrosureas. Cancer Res., **45**, 2326-2333.

Tofilon, P.J., Vines, C.M., and Bill, C.A. (1989). Enhancement of radiation-induced DNA double-strand breaks and micronuclei in human colon carcinoma cells by N-methylformamide. Radiat.Res., **119**, 166-175.

Toole, B.P., and Trelstad, R.L. (1971). Hyaluronate production and removal during corneal development in the chick. Dev. Biol., **26**, 24-35.

Triadou, N., and Zweibaum, A. (1985). Maturation of sucrase-isomaltase complex in human fetal small and large intestine during gestation. Pediatr. Res., **19**, 136-138.

Tsao, D., Morita, A., Bella, A. Luu, P., and Kim, Y.S. (1982). Differential effects of sodium butyrate, dimethylsulfoxide, and retinoic acid on membrane associated antigens, enzymes and glycoproteins of human rectal adenocarcinoma cells. Cancer Res., **42**, 1052-1058.

Valone, F.H., Friedman, M.A., Whittlinger, P.S., Drakes, T., Eisenburg, P.D., Malec, M., Hannigan, J.F., and Browne, B.W. (1989). Treatment of patients with advanced colorectal carcinomas with fluorouracil alone, high-dose leucovorin plus fluorouracil, or sequential methotrexate, fluorouracil, and leucovorin: A randomized trial of the Northern California Oncology Group. J. Clin. Oncol., **7**, 1427-1436.

van den Berg, F.M., Tigges, A.J., Schipper, M.E.I., den Hartog-Jager, F.C.A., Kroes, W.G.M., and Walboomers, J.M.M. (1989). Expression of the nuclear oncogene p53 in colon tumours. J.Pathol., **157**, 193-199.

Van Dongen, G.A.M.S., Braakhuis, B.J.M., Leyva, A., Hendriks, H.R., Kipp, B.B.A., Bagnay, M., and Snow, G.B. (1989). Anti-tumor and differentiation-inducing activity of N,N-dimethylformamide (DMF) in head-and-neck cancer xenografts. Int. J. Cancer, **43**, 285-292.

Vega-Salas, D.E., Salas, P.J.I., Gundersen, D., and Rodriguez-Boulan, E. (1987). Formation of the apical pole of epithelial (Madin-Darby canine kidney) cells : Polarity of an apical protein is independent of tight junctions while segregation of a basolateral marker requires cell-cell interactions. J. Cell Biol., **104**, 905-916.

Venditti, J.M., Wesley, R.A., and Plowman, J. (1984). Current NCI preclinical antitumour screening *in vivo* : Results of tumour panel screening, 1976-1982 and future directions. Adv.Pharm.Chem., **20**, 1-20.

Vichi, P., and Tritton, T.R. (1989). Stimulation of growth of human and murine cells by Adriamycin. Cancer Res., **49**, 2679-2682.

Vidali, G., Boffa, L.C., Bradbury, E.M., and Allfrey, V.C. (1978). Butyrate suppression of histone deacetylation of multiacetylated formes of histones H3 and H4 and increased DNase I sensitivity of the associated DNA sequences. Proc. Natl. Acad. Sci. USA., **75**, 2239-2243.

Vlodavsky, I., and Gospodarowicz, D. (1981). Respective roles of laminin and fibronectin in adhesion of human carcinoma and sarcoma cells. Nature (Lond.), **289**, 304-306.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, B.A., Leppert, M., Nakamura, Y., White, R., Smits, A.M.M., and Bos, J.L.(1988). Genetic alterations during colorectal-tumor development. N. Engl. J. Med., **319**, 525-532.

Watt, F.M. (1986). The extracellular matrix and cell shape. Trends Biochem. Sci., **11**, 482-485.

Weinburg, R.A., (1989). Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. Cancer Res., **49**, 3713-3721.

Weinhouse, S. (1980). New dimensions in the biology of cancer. Cancer (Phila.), **45**, 2975-2980.

Weiss, R.B., and Issel, B.F.(1982). The Nitrosureas : Carmustine (BCNU) and lomustine (CCNU). Cancer Treat.Rev., **9**, 313-330.

Wice, B.M., Reitzer, L.J., and Kennell, D. (1981). The continuous growth of vertebrate cells in the abscence of sugar. J. Biol. Chem., **256**, 7812-7819.

Wice, B.M., Trugnan, G., Pinto, M., Rousset, M., Chevalier, G., Dussaulx, E., Lacroix, B., and Zweibaum, A. (1985). The intracellular accumulation of UDP-N-acetylhexosamines is concomitant with the inability of human colon cancer cells to differentiate. J. Biol. Chem., **260**, 139-146.

Williams, A.F. (1987). A year in the life of the immunoglobulin superfamily. Immunology Today, **8**, 298-303.

Wigley, C.B., Paraskeva, C., and Coventry, R. (1986). Elevated production of growth factor by human premalignant colon adenomas and a derived epithelial cell line. Br. J. Cancer, **54**, 799-805.

Wolf, D., and Rotter, V. (1985). Major deletions in the gene encoding the p53 tumor antigen cause lack of p53 expression in HL-60 cells. Proc. Natl. Acad. Sci.USA, **82**, 790-794.

Wolpert, L. (1988). Stem Cells : a problem in asymmetry. J. Cell Sci., Supplement **10**, 1-9.

Woods, A., Hook, M., Kjellen, L., Smith, C.G., and Rees, D.A. (1984). Relationship of heparan sulfate proteoglycans to the cytoskeleton and extracellular matrix of cultured fibroblasts. J.Cell Biol., **99**, 1743-1753.

Wright, N.A. (1978). The cell population kinetics of repopulating cells in the intestine. In : Stem cells and tissue homeostasis. Lord, B.I., Potten, C.S., and Cole, R.J. (editors), Cambridge University Press, Cambridge, 335-358.

Wright, N.A., and Alison, M. (1984a). The theory of renewing cell populations. In : The biology of epithelial cell populations. Volume 1, Clarendon Press, Oxford, 33.

Wright, N.A., and Alison, M. (1984b). The theory of renewing cell populations. In : The biology of epithelial cell populations. Volume 1, Clarendon Press, Oxford, 62.

Wright, N.A., and Alison, M. (1984c). Introduction. In : The biology of epithelial cell populations. Volume 1, Clarendon Press, Oxford, 3.

Wright, N.A., and Alison, M. (1984d). Cell lineage in the gastrintestinal tract. In : The biology of epithelial cell populations. Volume 2, Clarendon Press, Oxford, 598.

Wright, N.A., and Irwin, M. (1982). The kinetics of villus cell populations in the mouse small intestine. I. Normal villi : the steady state requirement. Cell Tissue Kinet., **15**, 595-609.

Yong, V.W., Horie, H., and Kim, S.U. (1988). Comparison of six different substrata on the plating efficiency, differentiation and survival of human dorsal root ganglion neurons in culture. Dev. Neurosci., **10**, 222-230.

Zubrod, C.G.(1972). Chemical control of cancer. Proc. Natl. Acad. Sci.USA, **69**, 1042-1047.

Zuk, A., Matlin, K.S. and Hay, E.D. (1989). Type I collagen gel induces Madin-Darby canine kidney cells to become fusiform in shape and lose apical-basal polarity. J.Cell Biol. **108**, 903-919.

Zupi, G., Marangolo, M., Arancia, G., Greco, C., Lavdonio, N., Iosi, F., Formisano, G., and Malorni, W. (1988). Modulation of the cytotoxic effect of 5-fluorouracil by N-methylformamide on a human colon carcinoma cell line. Cancer Res., **48**, 6193-6200.

Zweibaum, A., Hauri, H-P., Sterchi, E., Chantret, I., Haffen, K., Bamat, J., and Sordat, B. (1984). Immunohistological evidence obtained with monoclonal antibodies, of small intestina brush border hydrolases in human colon cancers and foetal colons. Int. J. Cancer, **34**, 591-598.

Zweibaum, A., Pinto, M., Chevalier, G., Dussaulx, E., Triadou, N., Lacroix, B., Haffen, K., Brun, J-L., and Rousset, M. (1985). Enterocytic differentiation of a subpopulation of the human colon tumor cell line HT-29 selected for growth in sugar-free medium and its inhibition by glucose. J. Cell. Physiol., **122**, 21-29.

Zweibaum, A., Triadou, N., Kedinger, M., Augeron, C., Robine-Leon, S., Pinto, M., Rousset, M., and Haffen, K. (1983). Sucrase-isomaltase : A marker of foetal and malignant epithelial cells of the human colon. Int.J.Cancer, **32**, 407-412.

APPENDIX 1

Supporting data as described and discussed in Section 6.VIII (The effects of collagen gel culture on chemosensitivity to a panel of chemotherapeutic agents).

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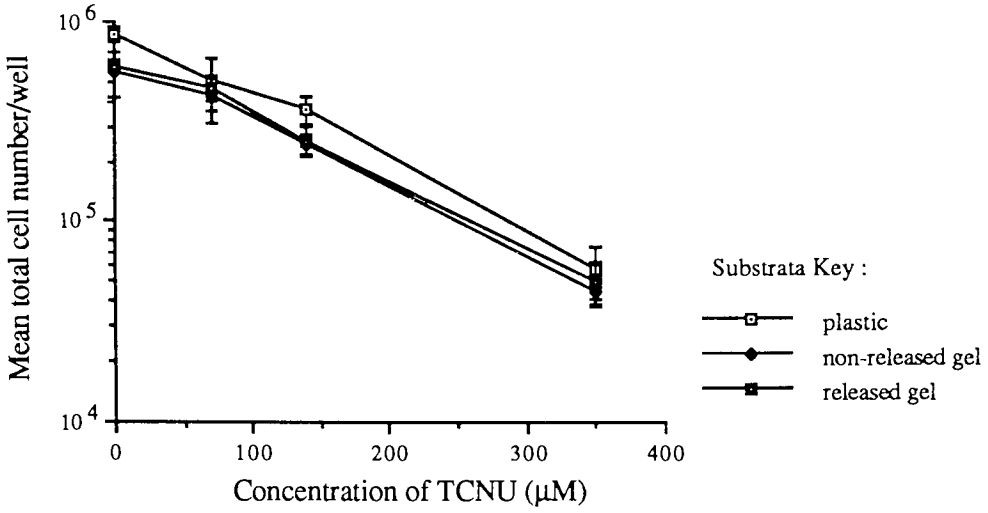

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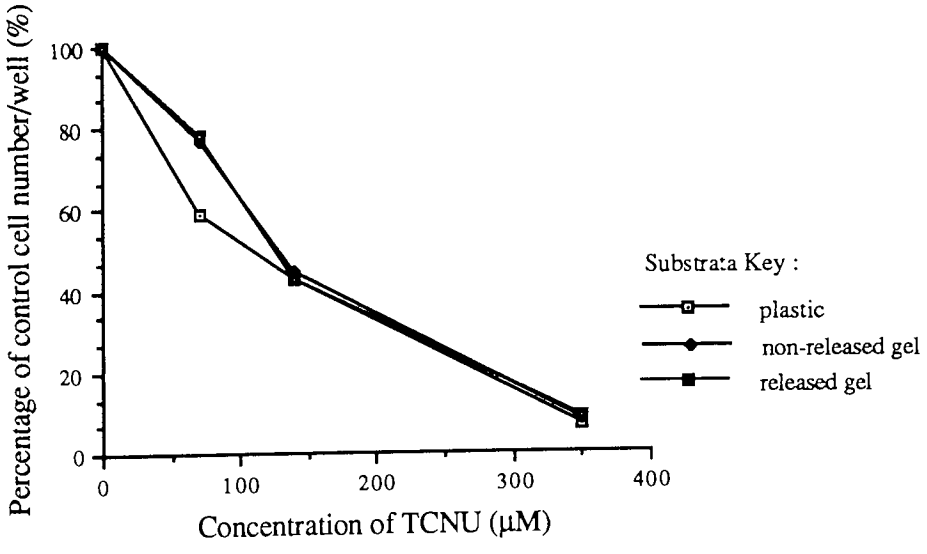
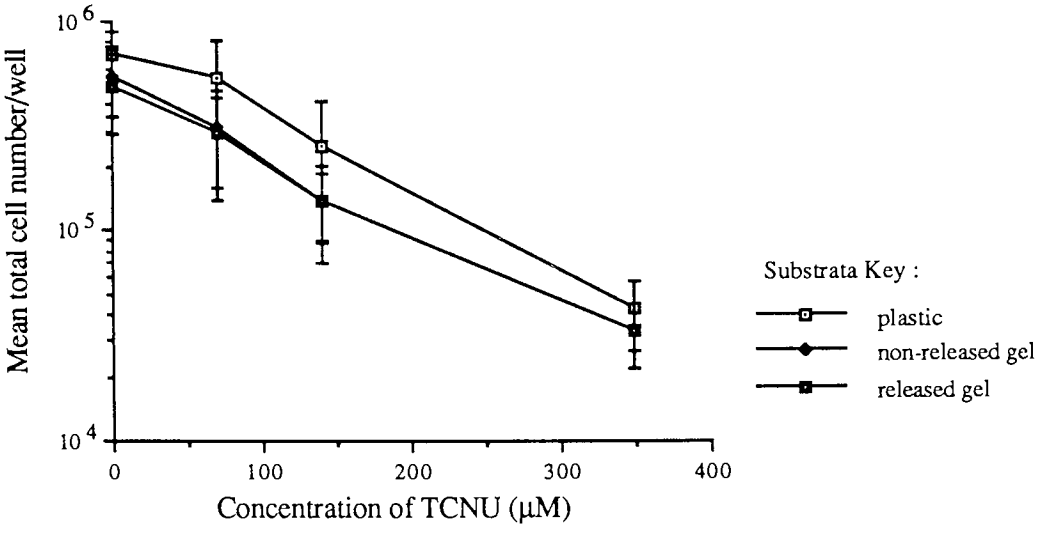


Figure 72 : Concentration-response curves for TCNU toxicity to HT-29_2 cells cultured on plastic substrata and non-released (NR), and released (R) non-passaged collagen gels (Non-PPC).
Results expressed as a) mean total cell number/well following treatment for 6 days, and b) cell number expressed as a percentage of the control cell number/well (%) following treatment for 6 days. (n = 3 ; ± sd).

a



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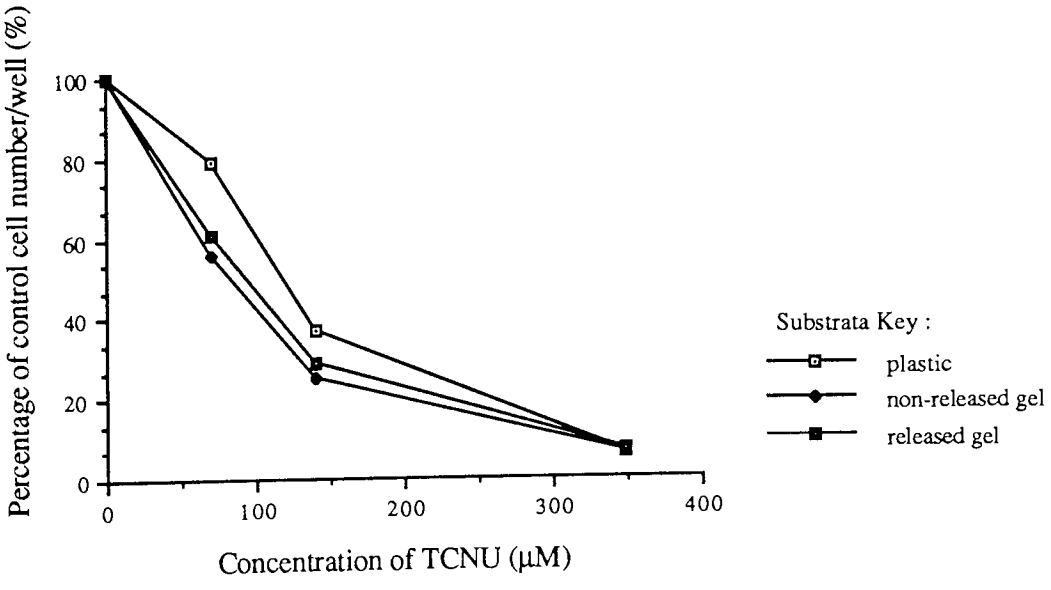
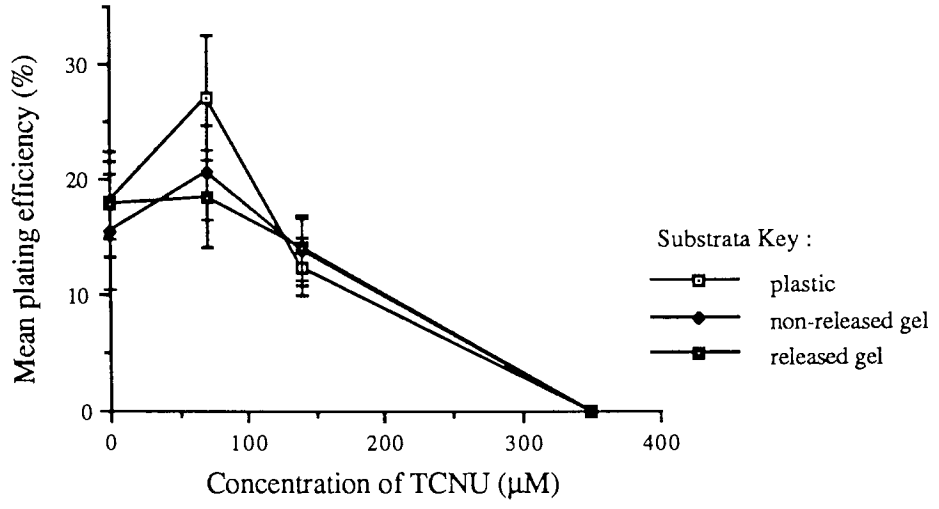


Figure 73 : Concentration-response curves for TCNU toxicity to HT-29$_2$ cells cultured on plastic substrata and non-released (NR), and released (R) passaged collagen gels (PPC). Results expressed as a) mean total cell number/well following treatment for 6 days, and b) cell number expressed as a percentage of the control cell number/well (%) following treatment for 6 days. (n = 3 ; ± sd).

a



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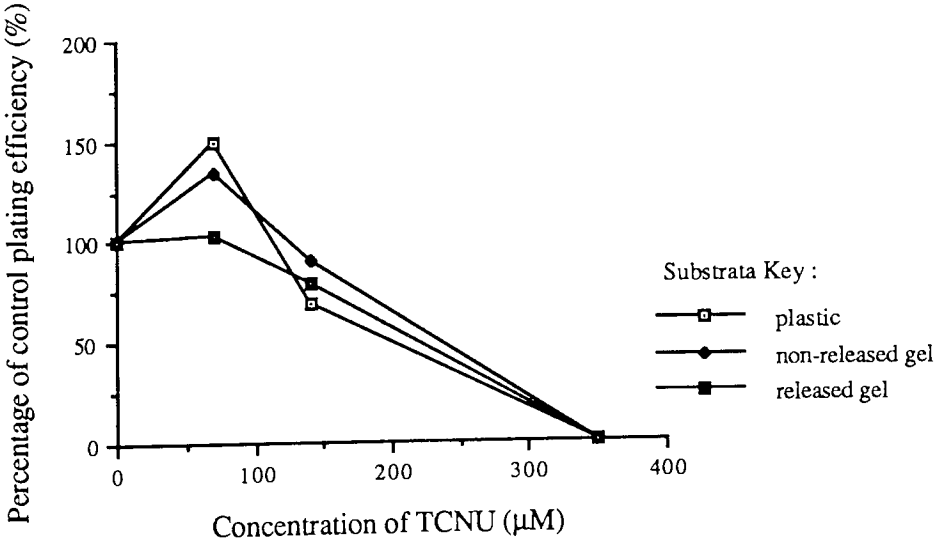
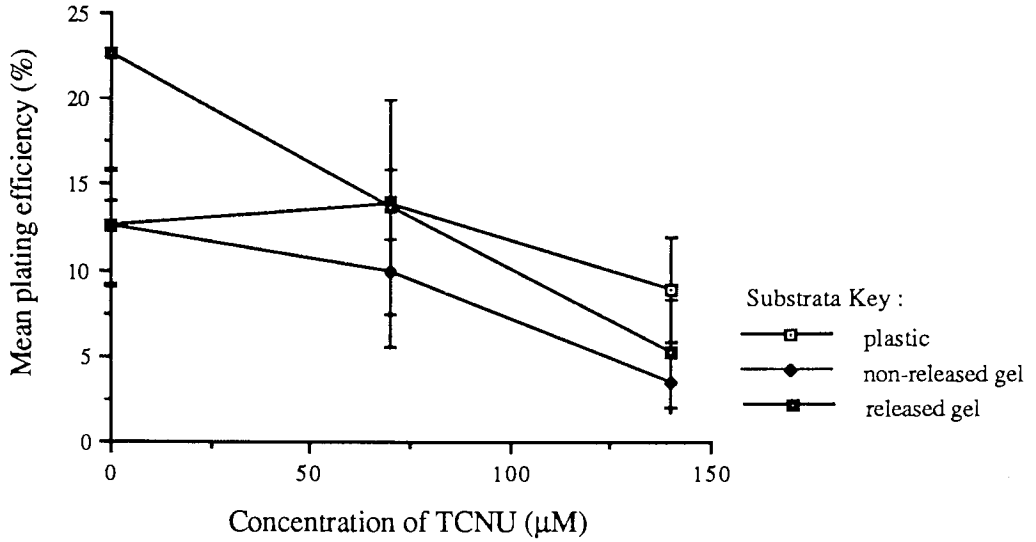


Figure 74 : The effect of TCNU on clonogenic potential of HT-29$_2$ cells cultured on plastic substrata and non-released (NR), and released (R) non-passaged collagen gels (Non-PPC).
Results expressed as a) mean plating efficiency (%) of remaining cells following treatment for 6 days, and b) mean plating efficiency as expressed as percentage of control (%) following treatment for 6 days. (n = 18 ; ± sd).

a



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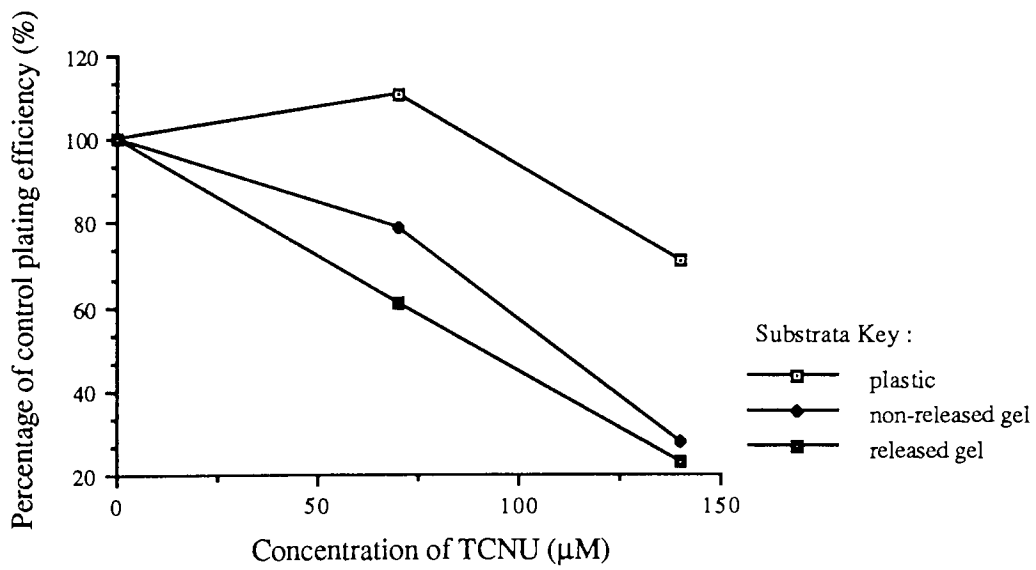
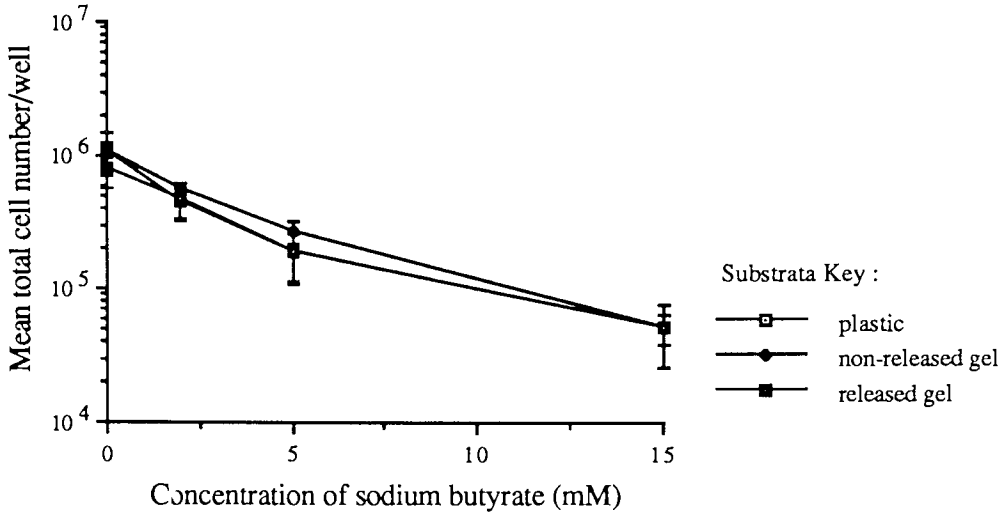


Figure 75 : The effect of TCNU on clonogenic potential of HT-29_2 cells cultured on plastic substrata and non-released (NR), and released (R) passaged collagen gels (PPC). Results expressed as a) mean plating efficiency (%) of remaining cells following treatment for 6 days, and b) mean plating efficiency as expressed as percentage of control (%) following treatment for 6 days. (n = 18 ; ± sd).

a



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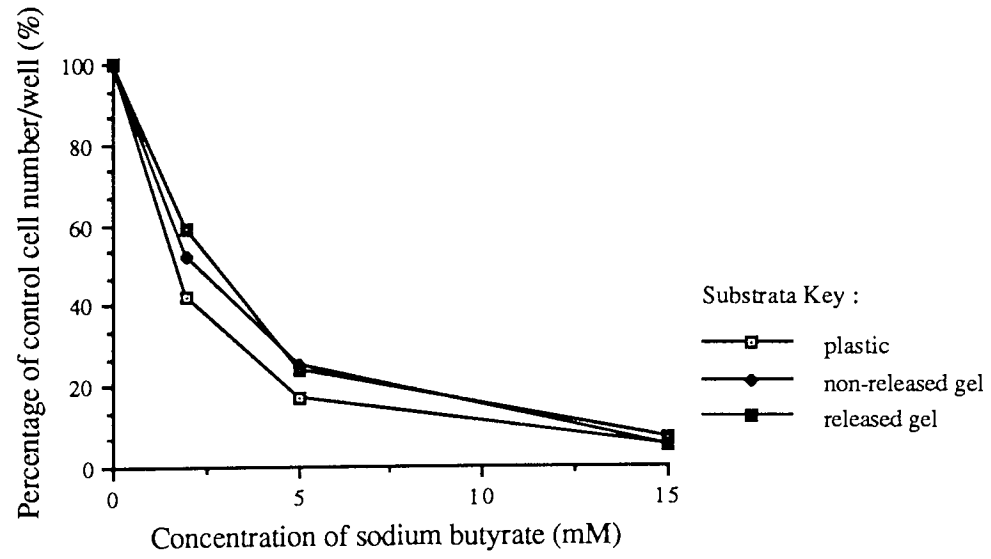
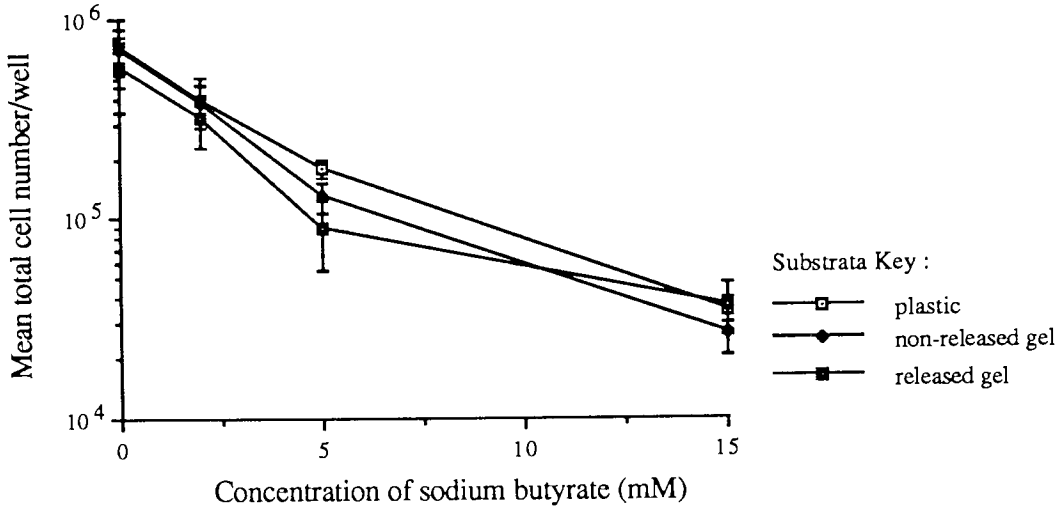


Figure 76 : Concentration-response curves for NaB toxicity to HT-29$_2$ cells cultured on plastic substrata and non-released (NR), and released (R) non-passaged collagen gels (Non-PPC).
Results expressed as a) mean total cell number/well following treatment for 6 days, and b) cell number expressed as a percentage of the control cell number/well (%) following treatment for 6 days. (n = 3 ; ± sd).

a



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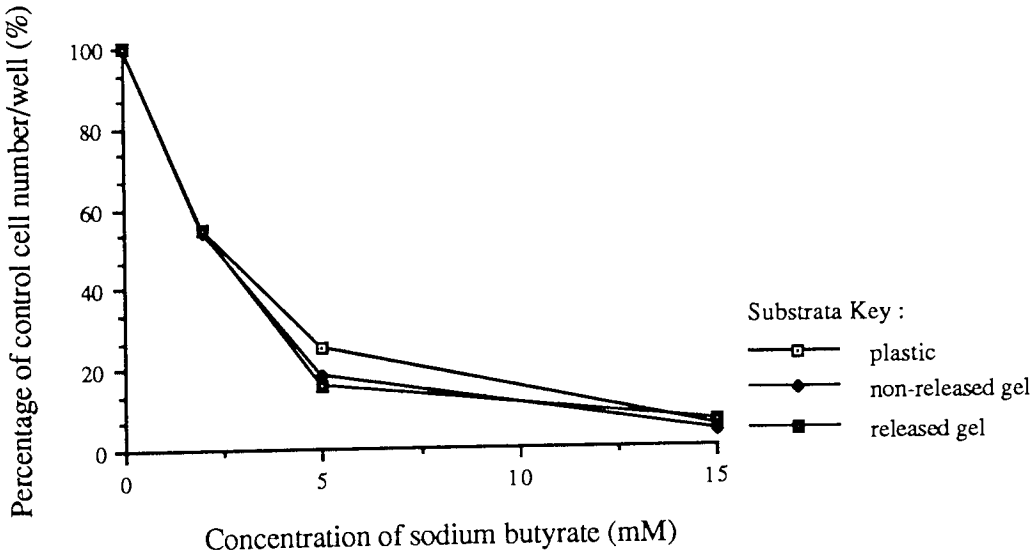
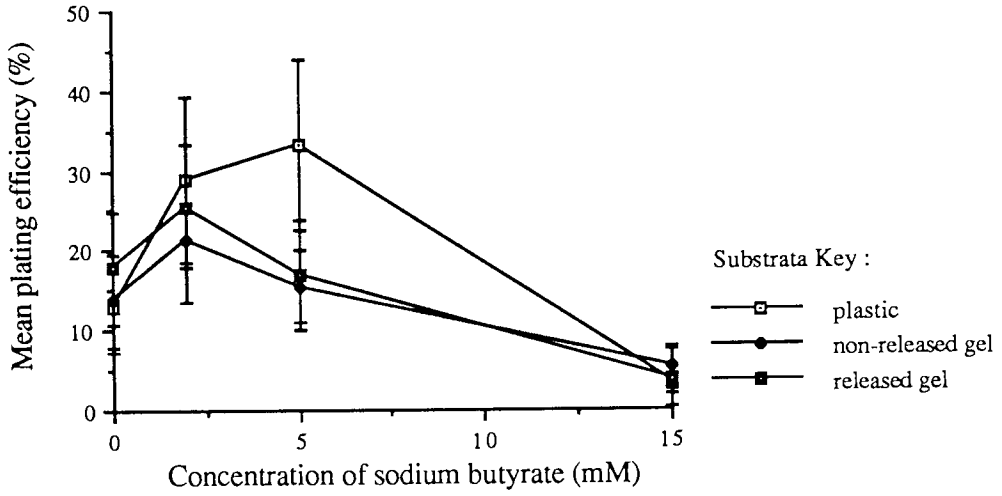


Figure 77 : Concentration-response curves for NaB toxicity to HT-29_2 cells cultured on plastic substrata and non-released (NR), and released (R) passaged collagen gels (PPC). Results expressed as a) mean total cell number/well following treatment for 6 days, and b) cell number expressed as a percentage of the control cell number/well (%) following treatment for 6 days. (n = 3 ; ± sd).

a



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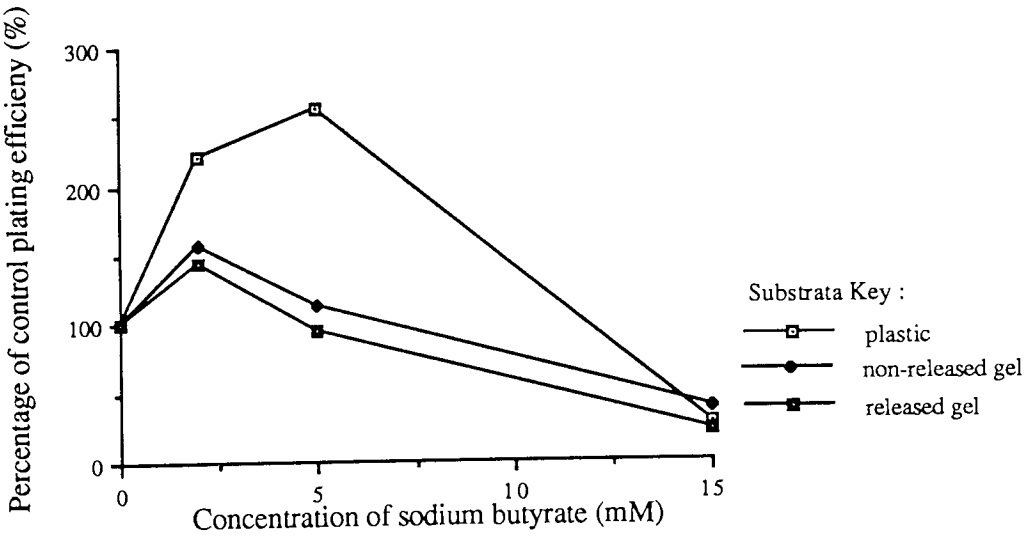
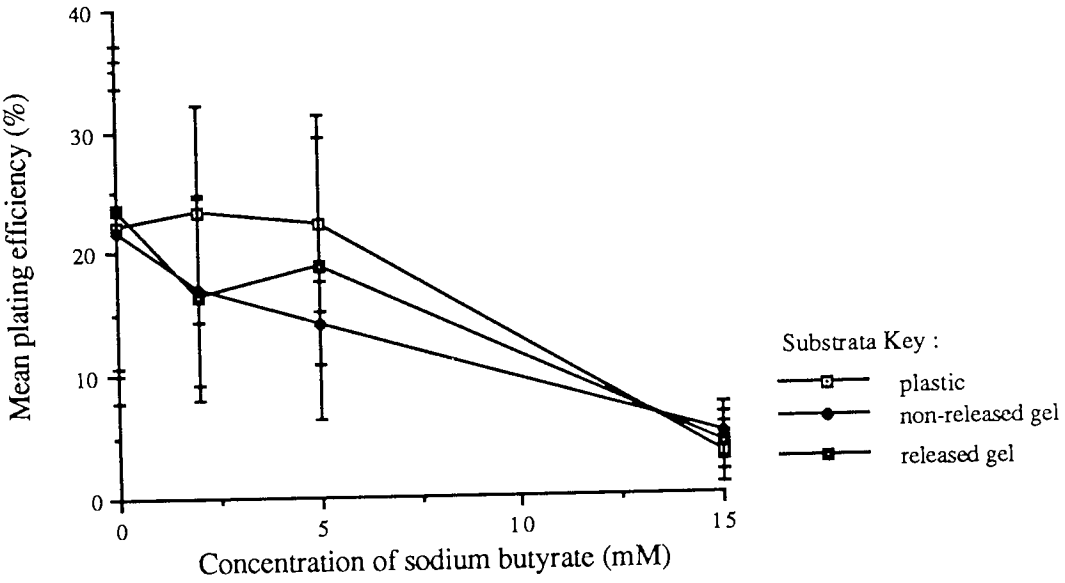


Figure 78 : The effect of NaB on clonogenic potential of HT-29$_2$ cells cultured on plastic substrata and non-released (NR), and released (R) non-passaged collagen gels (Non-PPC).
Results expressed as a) mean plating efficiency (%) of remaining cells following treatment for 6 days, and b) mean plating efficiency as expressed as percentage of control (%) following treatment for 6 days. (n = 18 ; $\pm$sd).

a



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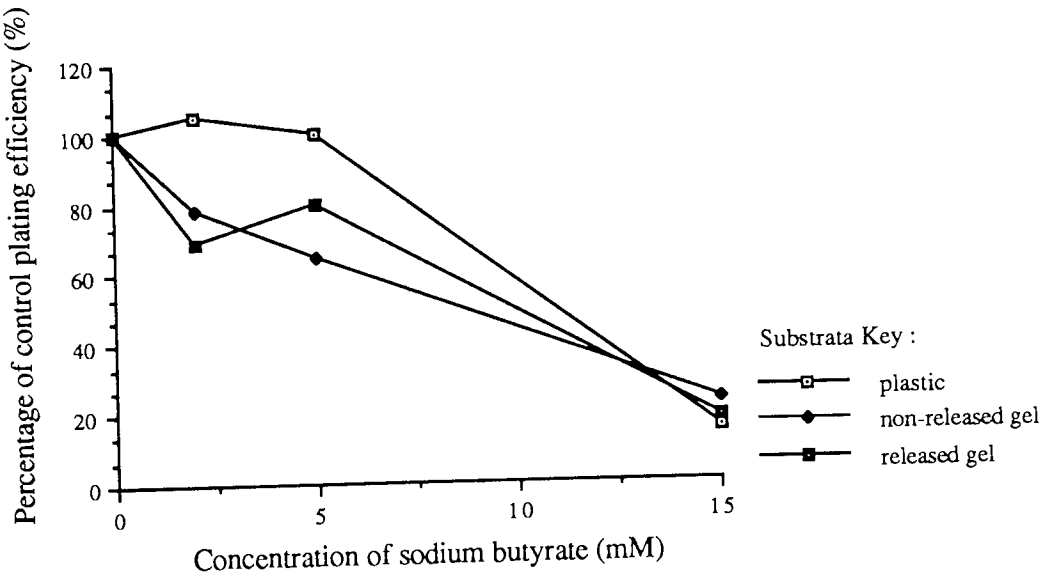
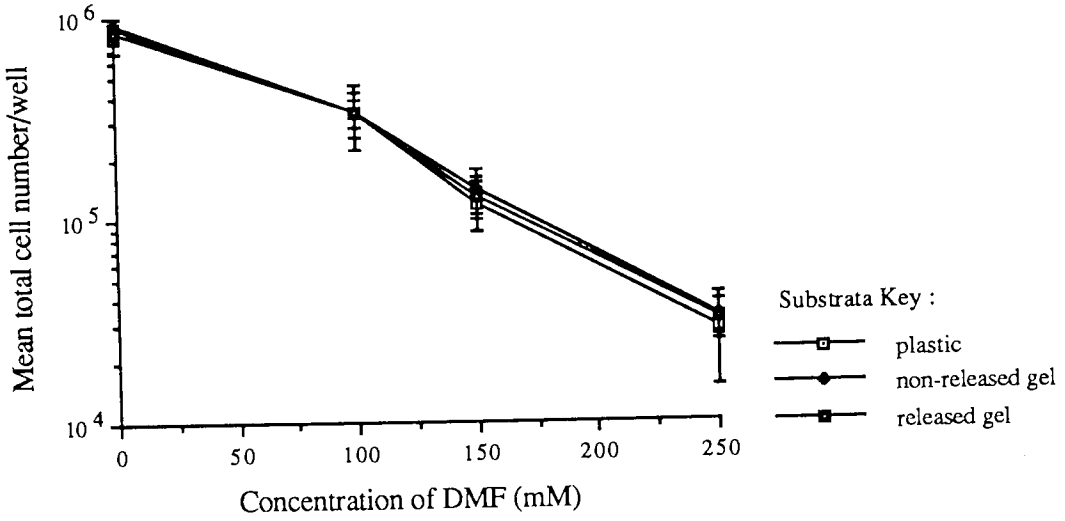


Figure 79 : The effect of NaB on clonogenic potential of HT-29$_2$ cells cultured on plastic substrata and non-released (NR), and released (R) passaged collagen gels (PPC). Results expressed as a) mean plating efficiency (%) of remaining cells following treatment for 6 days, and b) mean plating efficiency as expressed as percentage of control (%) following treatment for 6 days. (n = 18 ; $\pm$sd).

a



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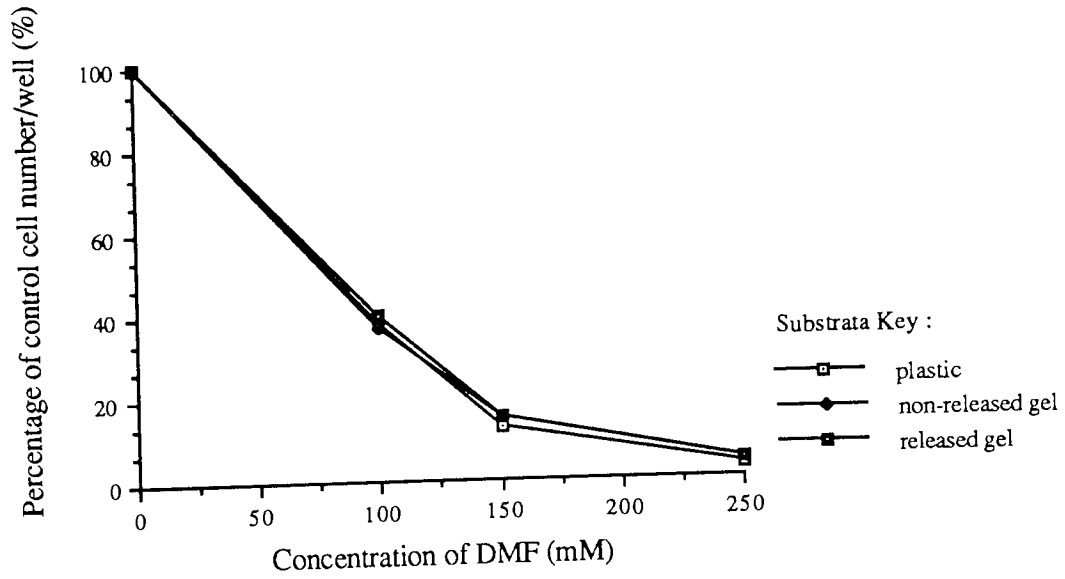
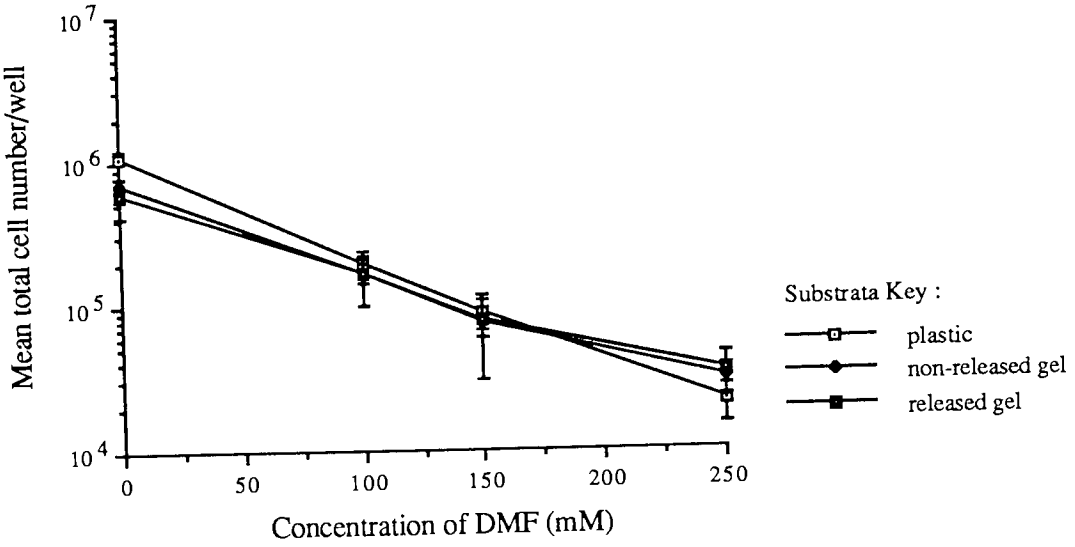


Figure 80 : Concentration-response curves for DMF toxicity to HT-29_2 cells cultured on plastic substrata and non-released (NR), and released (R) non-passaged collagen gels (Non-PPC).
Results expressed as a) mean total cell number/well following treatment for 6 days, and b) cell number expressed as a percentage of the control cell number/well (%) following treatment for 6 days. (n = 3 ; ± sd).

a



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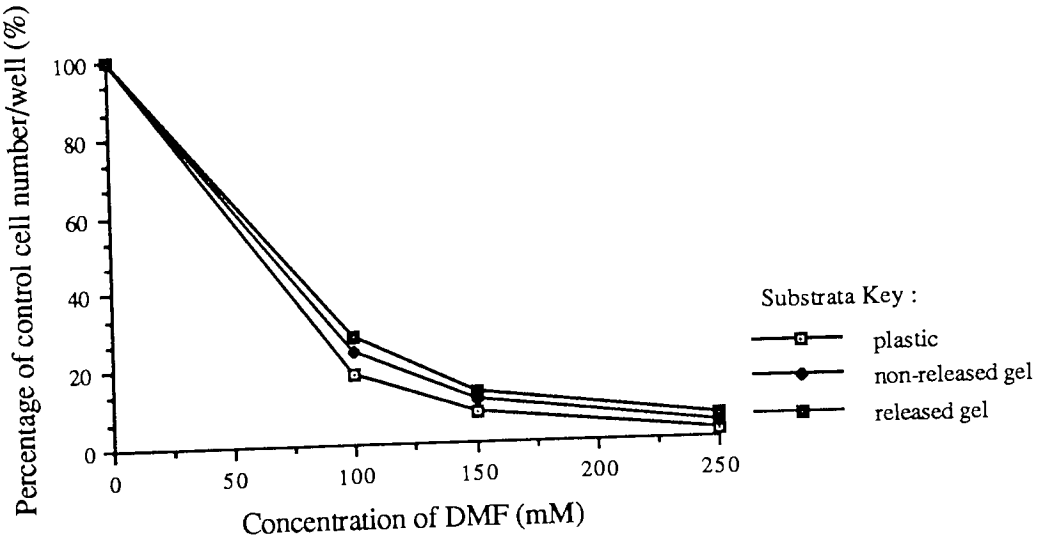

Figure 81 : Concentration-response curves for DMF toxicity to HT-29_2 cells cultured on plastic substrata and non-released (NR), and released (R) passaged collagen gels (PPC). Results expressed as a) mean total cell number/well following treatment for 6 days, and b) cell number expressed as a percentage of the control cell number/well (%) following treatment for 6 days. (n = 3 ; ± sd).

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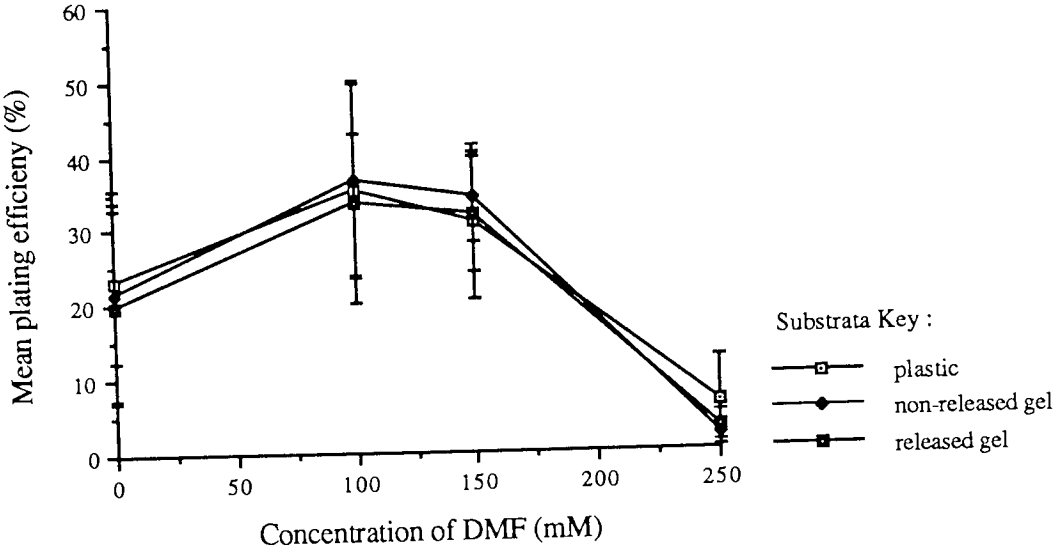

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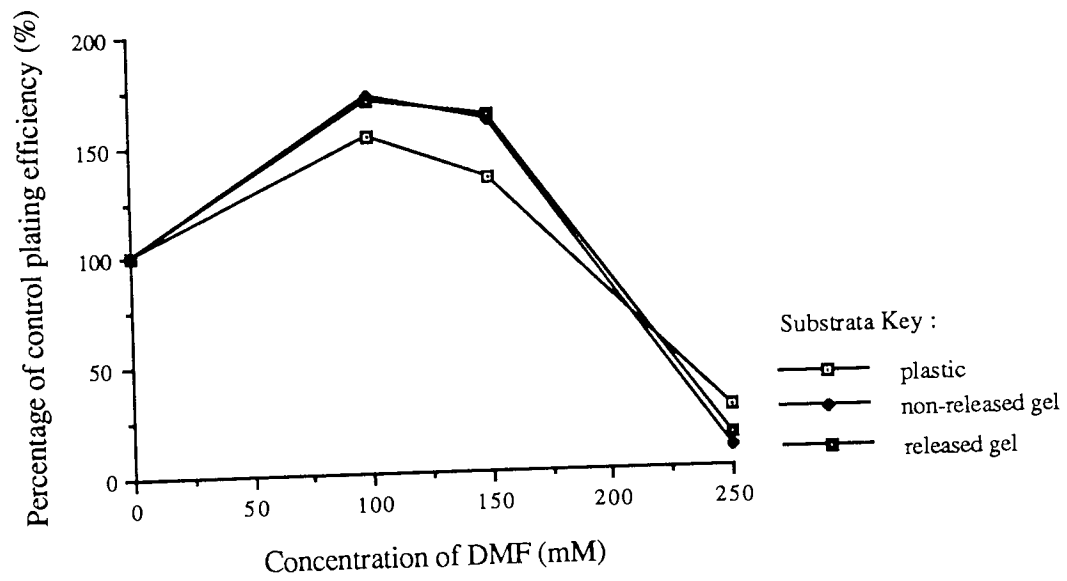

Figure 82 : The effect of DMF on clonogenic potential of HT-29$_2$ cells cultured on plastic substrata and non-released (NR), and released (R) non-passaged collagen gels (Non-PPC).
Results expressed as a) mean plating efficiency (%) of remaining cells following treatment for 6 days, and b) mean plating efficiency as expressed as percentage of control (%) following treatment for 6 days. (n = 18 ; $\pm$sd).

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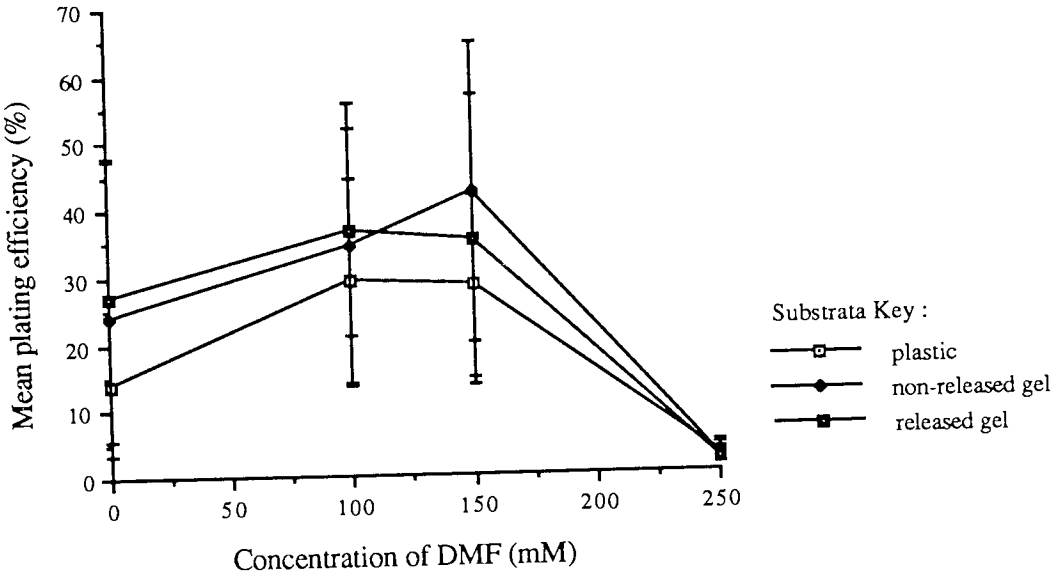

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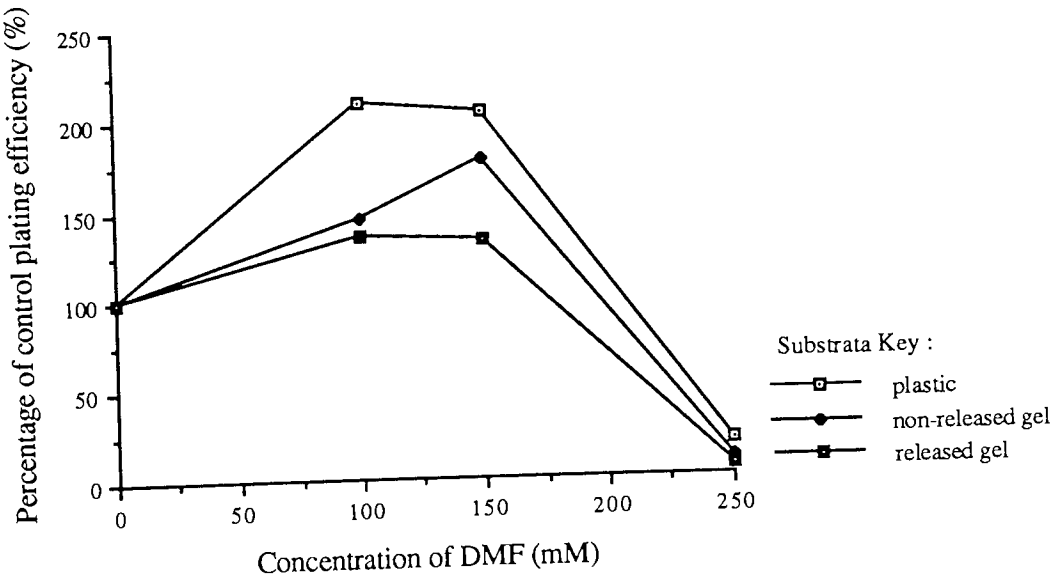

Figure 83: The effect of DMF on clonogenic potential of HT-29_2 cells cultured on plastic substrata and non-released (NR), and released (R) passaged collagen gels (PPC). Results expressed as a) mean plating efficiency (%) of remaining cells following treatment for 6 days, and b) mean plating efficiency as expressed as percentage of control (%) following treatment for 6 days. (n = 18 ; $\pm$ sd).