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~~Establishment and~~ Characterisation of Subpopulations from
HCA-7, a Heterogeneous Human Colonic
^{Carcinoma}
~~Adenocarcinoma~~ Cell Line.

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Thesis Submitted for the Degree
of
Doctor of Philosophy

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February, 1990.

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ABSTRACT.

Cellular heterogeneity within colorectal carcinomas is a well acknowledged phenomenon. The HCA-7 cell line was derived from a primary human colonic adenocarcinoma and displays a cellular heterogeneity at early passage. Two subpopulations have previously been isolated from the parent line and have been shown to differ in morphology and transport properties. This study describes the isolation of a further nine subpopulations from the parent line and their characterisation.

Early passage HCA-7 cells were used for the isolation of the subpopulations, using manual methods, which have retained their characteristic morphologies for at least 21 passages in vitro. The subpopulations were shown to differ from each other and the parent line in their in vitro growth rates, antigen expression, anchorage independent growth, modal chromosome numbers and electrolyte transport properties.

All the cell lines were found to be tumourigenic in nude mice. Differences were observed between the cell lines in the time taken to form tumours, the structural organisation of the tumours and the degree of differentiation of the cells present therein.

The possibility of interactions occurring between the subpopulations was investigated firstly by mixing cocultures of two cell types. Here a divergence in the growth rate was observed from that predicted from the growth rates of the individual components. Non-contact mediated interactions between the cells was assessed by conditioned media experiments and by the use of transwells. These results suggested that a soluble factor is involved in subpopulation interactions which alter the growth rate of one or other of the two cell types observed.

This study has produced an extensive range of subpopulations isolated from a single primary human colonic carcinoma, which have different biological properties. The parent line and subpopulations will be invaluable tools in the investigation of the mechanisms involved in the generation and maintenance of heterogeneity within colorectal tumours.

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ACKNOWLEDGEMENTS.

I would first and foremost like to express my gratitude to Dr. Susan Kirkland for the opportunity to work on the HCA-7 cell line and her continued interest, encouragement and help throughout this work .

I also wish to thank Professor Nicholas Wright for the opportunity to work in his department and thanks must also go to the Cancer Research Campaign and the Imperial Cancer Research Fund for their funding of this study.

Many, many thanks go to Dr Gordon Stamp for his painstaking classification of the xenograft tumours.

I also wish to thank Dr Lesley MacVinish for her invaluable help with the electrolyte transport studies and to Professor Alan Cuthbert for his advice and for allowing me to carry out these studies in his laboratories at Cambridge.

I am indebted to the following people who gave freely of their advice and time; Yvonne Price for the processing and staining of the numerous xenograft tumours, Vivian Emons for processing and cutting of EM sections and Steve Aldington for his patience and help with the production of the photographs.

I would finally like to thank my family and friends who have always been there when needed.

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INTRODUCTION.

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Colorectal Carcinoma.

Colorectal cancer is the most common gastrointestinal cancer in the western world and is second only to lung cancer in incidence. In 1984 there were over 24,000 new cases of colorectal cancer reported in England and Wales alone (figures taken from Cancer Statistics Registrations, 1984). Each year an approximate 17,000 deaths occur as a result of colorectal neoplasms.

The majority of large bowel cancers are adenocarcinomas with gland formation. Staging of colorectal adenocarcinomas is performed by a histologist, in most cases using the Duke's classification (Duke, 1932). This classification was further extended by Astler and Coller in 1954 whereby stage A tumours had penetrated the mucosa and submucosa, B1 the muscularis mucosae, B2 the muscularis propria and serosa, C1 as B1 with lymph node metastases, C2 as B2 with lymph node metastases and D showing distant metastases.

Prognosis of colorectal cancer is related to some pathological features (Sternberg, 1980) including depth of penetration of the tumour, grading and lymphatic involvement. In a study by Scott and co-workers (1987) it was shown that, in a group of 264 colorectal cancer patients, the 5 year survival rate decreased significantly from 93% for patients with Duke's A classification to 0% for patients with Duke's D carcinomas. This decrease in survival rate, in relation to Duke's staging, had also been noted by Eisenberg *et al.* (1982) who also noted different 5 year survival rates related to the anatomical location of the tumour.

Most colorectal carcinomas are thought to arise from pre-existing adenomas. Morson *et al.* (1983) presented some evidence to support the existence of an adenoma - carcinoma sequence. Firstly, in addition to the carcinoma, one third of all operational specimens of colorectal carcinoma contain one or more adenomas and approximately 7% of this group develop a second or metachronous tumour in the remaining bowel. Secondly, 75% of patients with spontaneous carcinomas have associated adenomas (Heald and Bussey,

1975). Thus it is assumed that most colorectal carcinomas arise from benign adenomas, however the events leading to this change are unclear. That an increased number of aneuploid cells are present in colorectal carcinomas, compared to normal epithelium and benign polyps, was noted as far back as 1960 (Stich *et al.*, 1960). However it is only over the past decade, with the advent of new cytogenetic methods, that it has become possible to identify the chromosomal abnormalities occurring in colorectal carcinoma. Specific genetic alterations in sporadic colorectal carcinomas have been noted on many chromosomes (Vogelstein *et al.*, 1989), however it is the alterations on chromosome 17, shown to be the most common, which has gained an increasing interest in recent years (Reichmann *et al.*, 1981 ; Fearon *et al.*, 1987). The involvement of oncogenes in the development of colorectal carcinoma has also received much attention. One important genetic alteration is that of the ras-gene mutation, which has been demonstrated to occur comparatively early in colorectal tumourigenesis (Vogelstein *et al.*, 1988 ; Baker *et al.*, 1989). However it has now been suggested that mutations of the nuclear oncogene p53, which encodes for a nuclear tumour antigen, is involved in colorectal neoplasia (Baker *et al.*, 1989 ; Van Der Berg *et al.*, 1989). This p53 gene had previously been located on the short arm of chromosome 17 (Isobe *et al.*, 1986) adding weight to the involvement of chromosome 17p in the later events of the neoplastic process. More recently, tumours demonstrating allelic deletions of the 17p chromosome were examined to observe whether they contained mutant p53 genes in the allele which was retained (Nigro *et al.*, 1989). From this work it was concluded that mutations of this p53 gene do play a role in the development of many common human malignancies including colorectal tumours.

An increased risk of colorectal carcinoma is present in patients with a family history of the disease. There are cases with a genetic predisposition to the disease such as familial polyposis coli (familial adenomatous polyposis) and inherited non-polyposis colorectal cancer syndrome, both of which have an

autosomally dominant mode of inheritance. It has recently been demonstrated that the primary genetic defect in familial adenomatous polyposis is located on chromosome 5, position q21-q22 (Bodmer et al., 1987).

Despite many successful advances in the therapy of other types of cancer, colorectal cancers are still one of the least responsive to the current modes of treatment. Therefore to enable us to develop a more effective treatment of the disease we must first increase our understanding of the biology of colorectal cancers and of the normal colorectal epithelium from which they arise.

Normal Colorectal Epithelium.

Structure.

The lining of the large intestine, known as the mucosa, is covered by a continuous sheet of a single layer of epithelial cells. The mucosa also includes the lamina propria, an underlying layer of stroma. Beneath the mucosa lies a strip of smooth muscle known as the muscularis mucosae (Fig. 1). However it is the single layer of epithelial cells which are involved in the functions of the large intestine. The intestinal mucosa has a highly organised structure which, in the large intestine, consists of a flat surface interspersed with many tubular invaginations or crypts (Fig. 2).

The epithelial cells lining each crypt can be positionally separated into compartments, see Fig. 2 (Wright and Alison, 1984). Compartment 4 contains the functional cells which, in the main, carry out the various functions of the large intestine to be discussed later. These functional cells arise from stem cells situated in the base of the crypt (compartment 1) and in the next compartment 2, they are found to proliferate. These cells then migrate up the crypt towards the luminal surface and on the way they lose their proliferative capacity (compartment 3) to become mature and functional cells.

Cellular Function of the Large Intestine.

The main functions of the large intestine are to absorb water and also secrete mucous in order to facilitate the movement and excretion of faeces.

To enable the large intestine to carry out these functions, four main cell types are found in the epithelial layer including columnar, mucous, entero-endocrine, and undifferentiated cells (Shamsuddin *et al.*, 1982).

Columnar Cells.

Two types of columnar cell are present in the colorectal epithelium, absorptive columnar and secretory columnar. These cells which make up the greater

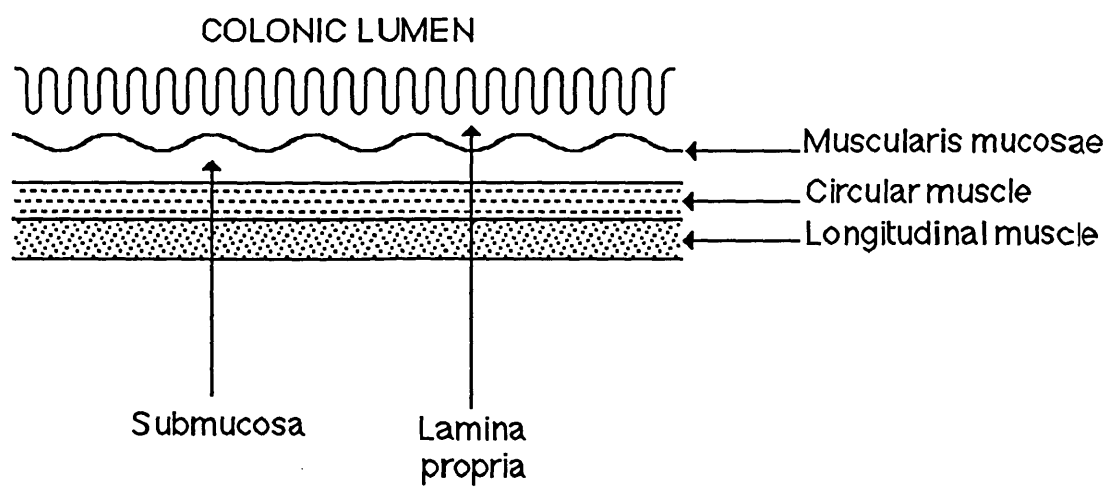


Fig. 1. Diagrammatic representation of the mucosa and submucosa of the human large intestine.

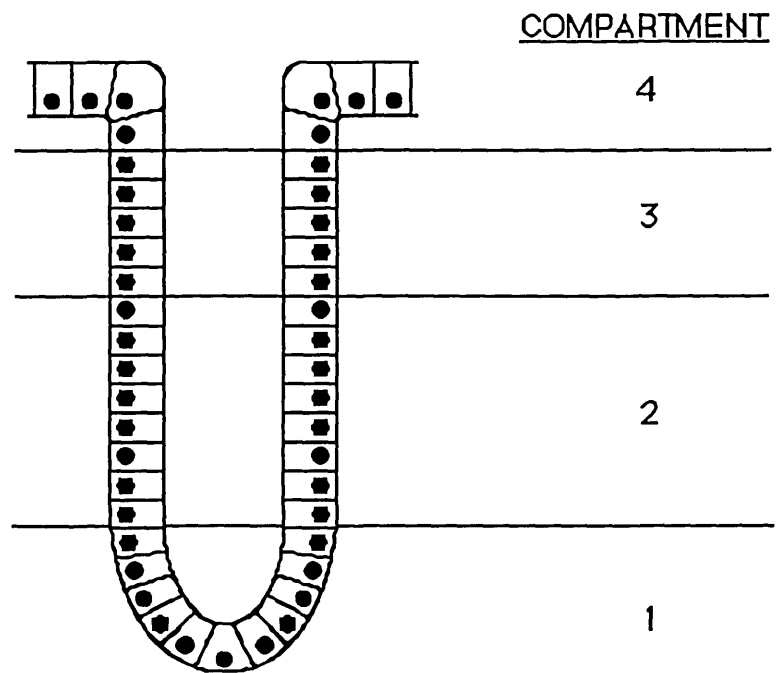


Fig2. Diagrammatic representation of the colonic crypt demonstrating the four compartments which represent the functional state of the cells.

portion of colonic epithelial cells are highly polarised with an abundance of well formed apical microvilli. The absorptive columnar cells are generally found covering the luminal surface of the colon between the crypts and also in the upper portion of the crypts (Shamsuddin *et al.*, 1982). They absorb sodium and water by an electrogenic sodium ion transport mechanism (Binder, 1983). Chloride secretion also occurs in the large intestine as a result of an electrogenic chloride transport process, however this secretion has been shown to take place in the colonic crypt (Welsh *et al.*, 1982).

Goblet Cells.

Goblet cells are characterised by the presence of large vacuoles containing mucous and are situated in the lower part of the crypt and increase in number towards the luminal surface. Mucous is continually secreted into the lumen of the colon to aid the movement of faeces.

Both mature goblet and columnar cells arise from undifferentiated cells in the crypt base, which undergo maturation as they travel up the crypt. Immature goblet and columnar cells are therefore often seen in the lower and mid portions of the crypt.

Enteroendocrine Cells.

Colonic endocrine cells are recognised by their many electron dense neurosecretory granules. Their function is the secretion of peptide hormones which are important in the overall function of the large intestine. In contrast to goblet and columnar cells, endocrine cells are located mainly in the lower part of the crypt towards the crypt base.

Undifferentiated Cells.

Undifferentiated cells are also present in the crypt of the large intestine and do not contain organelles which may indicate any differentiation. These cells are located in the basal area of the crypt and often lie on the basement

membrane. Undifferentiated cells can show the beginnings of differentiation towards mucous and endocrine cells (Shamsuddin et al., 1982).

Cell Lineage in the Colonic Mucosa.

As stated earlier, the differentiated cell types present in the colonic mucosa originate from the stem cells located in the base of the crypt. All these differentiated cell types are thought to originate from multipotential stem cells. This theory, known as the unitarian hypothesis (Chang and Leblond, 1971 ; Cheng and Leblond, 1974), has been supported by evidence obtained from several studies in the normal rodent gastrointestinal tract.

Cheng and Leblond (1974) discovered that columnar cells in the crypt base of the mouse small intestine phagocytose neighbouring non-viable cells, which results in the presence of a large phagosome in the cytoplasm of the columnar cell. They then selectively killed cells sensitive to tritiated thymidine in the crypt base that were then phagocytosed by the surrounding, viable columnar cells which as a result contained labelled phagosomes. These labelled phagosomes were later found to be present in mucous cells, villous columnar cells and paneth cells.

More recent work to support the unitarian hypothesis was obtained using mouse embryo aggregation chimaeras. In this study, immunocytochemical evidence demonstrated that individual crypts of the small and large intestine of adult mice, are derived from a single progenitor cell (Ponder et al., 1985). However the factors which commit the stem cell to a particular path of differentiation are as yet unknown.

Colorectal Carcinoma Cell Lines.

Establishment.

In order to study in more detail the different cell types present in the large intestine, it was considered, by investigators, that to establish an in vitro system where cells could be easily examined and manipulated, would alleviate some of the problems encountered when studying the tissue *in situ*. With regard to the large intestine, attempts have been made to establish long term cultures of normal colorectal epithelium. However, these in the main have proved unsuccessful with few exceptions (Danes and Sutano, 1982), although the short term culture of normal gut cells is now an established procedure (Quaroni et al., 1989). It was clear however that the establishment of immortal cell lines could be achieved if a carcinoma, originating from the tissue under investigation, were used instead of its normal counterpart. Colorectal carcinomas have been shown to retain a certain amount of the differentiated features of the normal human colorectal mucosa. Seiler et al. (1984) showed that several well differentiated colorectal carcinomas contained both absorptive cells and immature mucous cells. Endocrine cells have also been discovered in colorectal carcinomas (Ulich et al., 1983 : Smith and Haggitt, 1984), though these are generally found in the more differentiated tumours (Smith and Haggitt, 1984).

However it was unclear at the outset whether or not cells of a carcinoma would retain any of these differentiated properties when transferred into culture. It was with the aim of studying normal colorectal mucosa and colorectal carcinomas, that many investigators set out to attempt to establish immortal cell lines from colorectal carcinomas.

Much perseverance by many groups of workers have resulted in the establishment of several cell lines from rodent colonic carcinomas (Brattain et al., 1980 ; Borman et al., 1982 ; Gerharz et al., 1987) and primary carcinoma and adenoma tissue and metastases from patients with colorectal carcinomas.

Name of cell line.	Tissue of Origin.	Primary Tumour.	Grade.	Reference.
HCT-8	Primary	Colonic adenocarcinoma		Tompkins et al, 1974
HRT-18	Primary	Rectal adenocarcinoma		Tompkins et al, 1974.
LoVo	Subclavicular metastasis	Colonic adenocarcinoma		Drewinko et al, 1974.
LS-174T	Primary	Colonic adenocarcinoma	M/W differentiated	Tom et al, 1976
Co-115	Primary	Colonic adenocarcinoma	Duke's C	Carrell et al, 1976
SW Series	Primary and metastases	Colorectal adenocarcinomas	Duke's A-C	Leibovitz et al, 1976
HT-29	Primary	Colorectal carcinoma		Fogh et al, 1977
Caco-2	Primary	Colorectal carcinoma		Fogh et al, 1977
S-7512	Subcutaneous metastasis	Rectal adenocarcinoma		Machida et al, 1977
COLO Series	Primary, ascite and blood	Colonic carcinoma		Semple et al, 1978
COLO Series	Primary	Sigmoid carcinoma	P differentiated	Qiunn et al, 1979
WiDr	Primary	Rectosigmoid adenocarcinoma		Noguchi et al, 1979
T84	Lung metastasis	Colonic carcinoma		Murakami and Masui, 1980
DLD-1	Primary	Colonic carcinoma		Dexter et al, 1981
HCT-116	Primary	Colonic carcinoma		Brattain et al, 1981

HCT-C	Primary	Colonic carcinoma		Brattain et al, 1983
LS-123	Primary	Colonic adenocarcinoma	Duke's B	Rutzky et al, 1983
OM-1	Omental Metastasis	Colonic adenocarcinoma	W differentiated	Spremulli et al, 1983
HOT-3	Ovarian Metastasis	Colonic adenocarcinoma	W differentiated	Spremulli et al, 1983
KMS-4	Metastatic lymph node	Polyposis coli		Namba et al, 1983
VACO Series	Primary and metastases	Colorectal carcinomas	Duke's C and D	McBain et al, 1984
PC / AA	Colonic adenoma	Polyposis coli		Paraskeva et al, 1984
PC / JW	Colonic carcinoma	Polyposis coli		Paraskeva et al, 1984
HCA-7	Primary	Colonic adenocarcinoma	Duke's B	Kirkland, 1985
LIM 1215	Involved omentum	Inherited colorectal cancer		Whitehead et al, 1985
HCA-2	Primary	Sigmoid adenocarcinoma	Duke's C	Kirkland and Bailey, 1986
HRA-16	Primary	Rectal adenocarcinoma	Duke's B	Kirkland and Bailey, 1986
HRA-19	Primary	Rectal adenocarcinoma	Duke's B	Kirkland and Bailey, 1986
HCA-24	Primary	Colonic adenocarcinoma	Duke's B	Kirkland and Bailey, 1986
HCA-26	Primary	Sigmoid adenocarcinoma	Duke's C	Kirkland and Bailey, 1986
LIM 1863	Primary	Ileocaecal valve carcinoma	P differentiated	Whitehead et al, 1987

5583 Sand E	Primary	Colonic adenocarcinoma	Duke's B	Verstijnen et al, 1987
MIP-101	Liver metastasis	Colonic adenocarcinoma	Duke's D	Niles et al, 1987
NCI-H Series	Primary and metastases	Colonic adenocarcinomas	W- undifferentiated	Park et al, 1987
VACO Series	Primary and metastases	Colonic adenocarcinoma	Duke's B	Willson et al, 1987
RW-2982	Omentum	Rectosigmoid adenocarcinoma	M/W differentiated	Tibbetts et al, 1988
RW-7213	Primary	Sigmoid adenocarcinoma	M/W differentiated	Tibbetts et al, 1988
S/AN	Primary	Colonic villous adenoma		Paraskeva et al, 1989
S/RG	Primary	Colonic tubular adenoma		Paraskeva et al, 1989
RCM-1	Primary	Rectal adenocarcinoma	W differentiated	Kataoka et al, 1989
CoCM-1	Primary	Colonic adenocarcinoma	M differentiated	Kataoka et al, 1989

Table 1. Cell lines derived from patients with sporadic and inherited colorectal carcinomas and adenomas.

P = poorly ; M = moderately ; W = well.

Table 1 names the human colorectal tumour-derived cell lines obtained over several years and indicates their site of origin, whether from the primary tumour or its metastases. Cultures have been successfully established from patients with tumours of a wide range of histological gradings, which are indicated in Table 1 where available. The majority of resulting cell lines were established from sporadic carcinomas where the patient had no history of colorectal carcinoma. However, long term cultures have been obtained from primary tumours or metastatic lesions of patients with familial polyposis coli (Namba *et al.*, 1983 ; Paraskeva *et al.*, 1984) and inherited non polyposis colorectal cancer syndrome (Whitehead *et al.*, 1985). Since the advent of this method of study, it has been shown that several of these cell lines retain some of the functional properties displayed by normal colorectal epithelium and by colorectal carcinomas.

Differentiated Features of Human Colorectal Carcinoma Cell Lines.

Several colorectal carcinoma cell lines have been shown to exhibit some of the differentiated features of normal intestinal epithelium. Chantret *et al.* (1988) surveyed twenty cell lines which differed in their expression of such differentiated characteristics. Other studies have also shown the presence of many differentiated features in human colorectal carcinoma cell lines.

Absorptive Cells.

At least three of the cell lines shown in Table 1 have displayed a capacity for fluid and electrolyte transport. Fluid transport occurring from the apical to the basolateral side of cells in a monolayer culture can be indicated by the formation of domes or hemicysts. Dome formation occurs as a result of a fluid and electrolyte accumulation between cells in the monolayer and the surface on which they attach (Fig. 3.). Many of the colorectal carcinoma cell lines express some of the morphologically differentiated features of columnar cells, forming a polarised monolayer with a well formed brush border. Absorptive

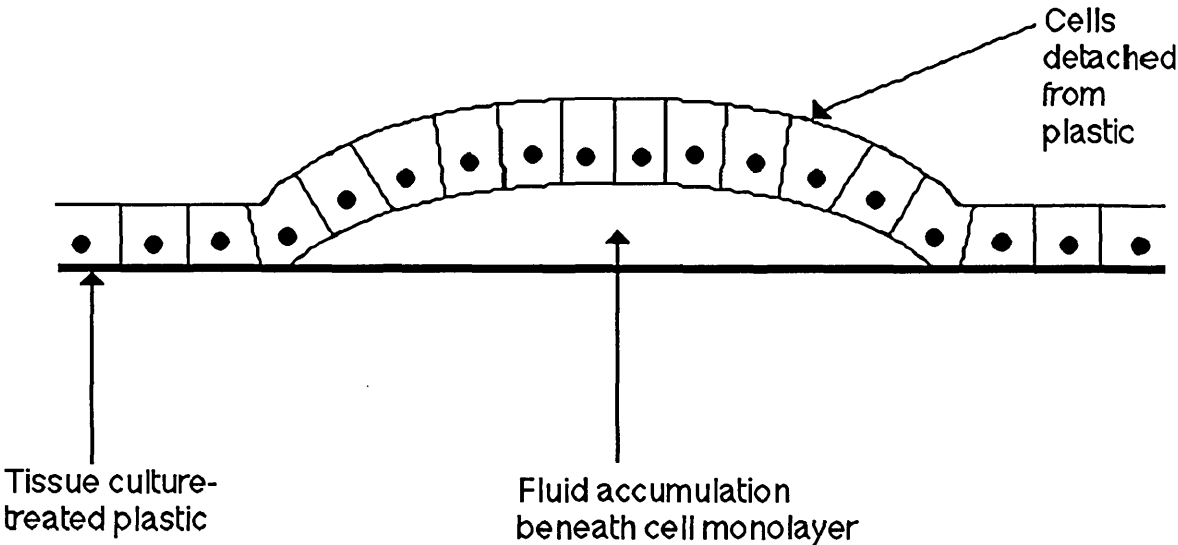


Fig. 3. Diagrammatic representation of a dome formed in monolayer culture.

columnar cells have been detected in the LS174T and SW1222 cell lines by a monoclonal antibody, 1A3, directed against normal colonic epithelial antigenic determinants (Richman and Bodmer, 1987). However, only a few cell lines display a capacity for dome formation.

Dome formation has been noted in Caco-2 cells (Pinto *et al.*, 1983) and fluid transport by these cells was subsequently confirmed by a dome cell micropuncture technique (Grasset *et al.*, 1984). A vectorial electrolyte transport mechanism has also been observed to be in operation in the T84 cell line (Dharmasathaphorn *et al.*, 1984). The HCA-7 cell line also demonstrated dome formation in monolayer culture (Kirkland, 1985) and evidence for fluid transport was reinforced by short circuit current measurements using the Ussing chamber system (Cuthbert *et al.*, 1987). The above cell lines have also been shown to respond to various agents which act as secretagogues in the normal epithelium. For example, vasoactive intestinal polypeptide was found to increase the short circuit currents in monolayers of T84, Caco-2 and HCA-7 (Dharmasathaphorn *et al.*, 1985 ; Grasset *et al.*, 1985 ; Cuthbert *et al.*, 1987). It is postulated that the short circuit current exhibited by these cell lines is due to an electrogenic chloride secretion (Dharmasathaphorn *et al.*, 1985 ; Grasset *et al.*, 1985 ; Cuthbert *et al.*, 1987).

Mucous Cells.

Few cell lines have been shown to spontaneously exhibit the production of mucin or the presence of goblet cells *in vitro*. The LIM 1863 (Whitehead *et al.*, 1987), RW-2982, RW-7213 (Tibbetts *et al.*, 1988) and NCI-H498 (Park *et al.*, 1987) cell lines all grow in culture as three dimensional structures.

Ultrastructural studies showed the presence of mature goblet cells in the LIM 1863 cell line, which secretes mucous into the lumen of the organoid structures (Whitehead *et al.*, 1987). The three dimensional structures of the RW-2982 and RW-7213 cell lines were found to be surrounded by an Alcian

blue-positive coat of mucous (Tibbetts et al., 1988). The NCI-H498 cell line was also found to be surrounded by a 'halo' of mucous which stained positive with the Alcian blue, mucicarmine and Periodic acid Schiff (PAS) stains (Park et al., 1987).

Mucin production has also been observed by cells in monolayer culture. LS174T monolayers show a positive staining with PAS and mucicarmine stains indicating mucin production by the cells (Tom et al., 1976). Mucous has also been demonstrated by electron microscopy, mucicarmine staining and an antibody to normal colonic mucous in monolayers of LIM1215 (Whitehead et al., 1985). An extensive sialomucin and diffuse sulphomucin production was found in 5583-S cells by PAS and high-iron-diamine Alcian blue staining (Verstijnen et al., 1987). The polyposis coli cell line, derived from an adenoma, PC AA (Paraskeva et al., 1984), has been shown to produce enough mucin (detected by the Alcian blue stain) to cover the cell monolayer in culture.

Richman and Bodmer (1987) produced a series of monoclonal antibodies directed against components of the normal human colorectal epithelium. Two of these antibodies, 4D4 and 5D5, were found to be mucous reactive and their distribution was mainly restricted to the colon. In their study they found that the LS174T cell line was reactive to the 4D4 antibody and the SW1222 cell line was reactive to both the 4D4 and 5D5 antibodies, although the reactions were weak. However this does indicate that both cell lines contain mucous constituents found in the normal colonic mucosa.

Enteroendocrine Cells.

Enteroendocrine cells are found in the normal human colorectal epithelium and primary colorectal carcinomas, however they are not a common feature of cell lines established from human colorectal carcinomas although they have been observed in chemically induced rat colonic carcinoma cell lines (Cox and Pierce, 1982). The HRA-19 cell line, established from a primary rectal

adenocarcinoma, has been shown to differentiate into endocrine cells (Kirkland, 1986), in addition to mucous and columnar cells, when grown as xenografts in nude mice (Kirkland, 1988). This cell line has however recently been demonstrated to exhibit endocrine cells in monolayer culture as detected by a monoclonal antibody to chromogranin (S. Kirkland, personal communication). To date this is the only colorectal carcinoma cell line shown to spontaneously exhibit endocrine differentiation in vitro.

Other Differentiated Features.

Colorectal carcinoma cell lines are also capable of expressing antigens which are not usually a feature of the normal adult colorectal epithelium. Several antigens which are normally found in the enterocytes of the foetal colon, but not usually in adult colonocytes, are present in some primary colorectal carcinomas and also cell lines established from such tumours.

Carcinoembryonic antigen (CEA), so called due to the origin of the cell types in which it is found, is one such antigen. It was originally extracted from adult colon cancer tissue and human foetal intestine in 1965 (Gold and Freedman, 1965). The presence of CEA in serum has been noted in patients with colonic carcinoma, though it is not specific for this type of cancer (Steward et al., 1974 ; Vincent et al., 1975 ; Holyoke et al., 1978). However rising levels of serum CEA have suggested a recurrence, after resection, of colonic carcinomas (Minton and Martin, 1978).

Ahnen and co-workers (Ahnen et al., 1987) investigated the expression of CEA during differentiation of normal colonic epithelium and in colonic carcinomas by indirect immunocytochemistry. They suggested from their evidence that the differentiation of colon cancers was not totally analogous to the differentiation of normal colonocytes with respect to the expression of CEA. This indicates that expression of CEA is a differentiated feature of colorectal carcinoma cells though not one found in the normal colon. Almost

all of the cell lines listed in Table 1 have been shown to express this antigen though in widely differing amounts.

Sucrase-isomaltase is a glycoprotein hydrolase normally restricted to the brush border of small intestinal cells (Kenny and Maroux, 1982). Zweibaum and his colleagues investigated the expression of this antigen, using both a monoclonal antibody (Hauri *et al.*, 1985) and an enzyme assay, in human foetal and adult, small intestinal and colonic, mucosae and also in xenografts produced in nude mice from a number of colorectal carcinoma-derived cell lines (Zweibaum *et al.*, 1983). They found that this antigen was expressed by mid gestation foetal colons and also in the xenografts produced by the cell lines HT-29 and Caco-2. From these results they suggested that sucrase-isomaltase was a marker of certain human colonic cancers and that the differentiation patterns of these cancers were related to those of the foetal colon. They have furthered their evidence using frozen sections of primary colorectal carcinomas and discovered that seven of twenty seven cases expressed this antigen in addition to the other small intestinal brush border hydrolase, aminopeptidase N and dipeptidylpeptidase IV (Zweibaum *et al.*, 1984). The expression of this enzyme in primary human colonic carcinoma tissue was later confirmed by Czernichow *et al.*, (1989).

HLA-DR is a class II antigen encoded by the major histocompatibility complex in man (Bodmer and Bodmer, 1978). This antigen is normally restricted in its distribution and is found on the surface of B cells, Langerhans cells, macrophages and endothelial cells (Strominger *et al.*, 1981). Positive staining using a monoclonal antibody directed against this antigen has been observed in the apical cytoplasm of enterocytes of the upper crypt and villi of the human jejunum (Scott *et al.*, 1980). In general, colonic epithelial cells do not express this antigen except in certain pathological conditions such as ulcerative colitis and Crohn's disease (Selby *et al.*, 1983).

However the HLA-DR antigen has been shown to be expressed in tissue sections of colorectal carcinomas of different histological grades (Daar and

Fabre, 1983 ; Rognum et al., 1983). Lampert et al., (1985) discovered that the HCA-7 cell line, derived from a primary human colonic adenocarcinoma also has the ability to spontaneously synthesise the HLA-DR molecule which in this case had mainly an intracytoplasmic location.

Induction of Differentiation in Colorectal Carcinoma Cell Lines.

Several of the cell lines which do not 'spontaneously' express the differentiated features described in the previous sections can be induced to do so under the influence of certain agents or environmental manipulations. One such 'differentiating agent' is the short chain fatty acid salt, sodium butyrate, which is normally present in the lumen of the human colon (Cummings, 1981). Morphological changes have been noted in cells of the SW480, SW620 (Kim et al., 1980), HRT-18 (Tsao et al., 1982), HT-29 (Augeron and Laboisie, 1984) and LIM 1215 (Whitehead et al., 1986) cell lines when sodium butyrate was applied to the monolayers. Other butyrate-induced changes, consistent with a higher degree of differentiation, have been noted in colorectal carcinoma cell lines (Chung et al., 1985). Alkaline phosphatase is a brush border membrane hydrolase normally found in cells of the small intestine. The activity of this enzyme was found to be elevated in the HRT-18 cell line (Morita et al., 1982), the SW480 and SW620 cell lines (Kim et al., 1980) and the LIM1215 cell line (Whitehead et al., 1986) when treated with sodium butyrate. In addition, the carcinoembryonic antigen content of HRT-18 cells (Tsao et al., 1982) and LIM 1215 cells (Whitehead et al., 1986) was also elevated in the presence of sodium butyrate as has been demonstrated in other cell lines (Tsao et al., 1983 ; Friedman et al., 1987 ; Berry and Paraskeva, 1988 ; Miles et al., 1988).

Evidence has been presented by Augeron and Laboisie (1984) that this differentiating agent induced transepithelial transport and also mucous secretion in clones of the normally undifferentiated HT-29 cell line. In contrast the LIM 1215 cell line, which exhibits mucous containing cells under normal

conditions, shows a reduction in the number of this cell type in the presence of sodium butyrate (Whitehead et al., 1986).

The polar solvent, N,N-dimethylformamide has been noted to induce several changes in the DLD-1 and HCT-15 cell lines (Dexter et al., 1979 ; Hager et al., 1980 ; Dexter et al., 1981) producing a less malignant phenotype (Dexter and Hager, 1980). Polyethylene glycol is another differentiating agent which has induced differentiation of the HT-29 cell line (Laboisie et al., 1988).

Enterocytic differentiation in the HT-29 cell line has been demonstrated by replacing glucose with galactose in the medium (Pinto et al., 1982 ; Rousset et al., 1986). More recently a subclone has been isolated from this cell line which, in the absence of glucose, produces a homogeneous monolayer of goblet cells after confluency (Phillips et al., 1988).

Differentiation of cell lines can also be brought about by extracellular matrix components. Gland formation was observed in the LS174T cell line when combined with foetal rat mesenchyme (Fukamachi and Mizuno, 1987).

Substrata derived from well differentiated colon carcinoma cell lines has been shown to induce a more mature behaviour of the MOSER cell line (Boyd et al., 1988). Growth in collagen gel of the SW1222 cell line induces a three dimensional glandular differentiation pattern (Pignatelli and Bodmer, 1988). Recently Daneker et al. (1989) demonstrated that colorectal carcinoma cell lines can vary considerably in their ability to interact with extracellular matrix components, which is related to the degree of differentiation of the cell line.

Malignant Characteristics of Cell Lines.

In addition to retaining some of the differentiated features of normal enterocytes, the afore-mentioned cell lines also, not altogether surprisingly, retain many features of carcinoma cells. The very fact that they are immortal cell lines indicate that there is a lack of growth control and little terminal differentiation in the cells, which is consistent with cells of a primary carcinoma.

One feature of colorectal carcinoma-derived cell lines which has been repeatedly noted is that of chromosomal alterations in the cells. The majority of modal chromosome numbers observed in these cell lines fall in the range of 46-92 with few hypodiploid counts (see Table 1 for references). More detailed karyotypic analyses have been performed on several cell lines and genetic abnormalities have been observed in many of these cells. Perhaps the most common is the presence of an additional chromosome 7 (Brattain et al., 1983 ; Namba et al., 1983 ; McBain et al., 1984 ; Whitehead et al., 1987). Alterations on chromosome 7 have also been noted in primary colorectal carcinomas (Becher et al., 1983 ; Ochi et al., 1983). However changes in chromosome 17, reputed to be involved in colorectal carcinogenesis (Reichmann et al., 1981 ; Fearon et al., 1987) were no more common than any other chromosome in these cell lines. Whether or not these genetic alterations are a product of adaptation to in vitro conditions remains to be seen. Recently a more detailed examination of oncogene expression by eleven colorectal carcinoma-derived cell lines was performed (Trainer et al., 1988). Most of the oncogenes examined, including the p53 nuclear oncogene mentioned earlier, were expressed by at least nine of the eleven cell lines. However, no correlation was found between expression of specific oncogene(s) and the phenotypic characteristics of the cells including tumourigenesis.

The ability of the cells to form tumours as xenografts in immunodeficient hosts is another characteristic of the cancer cell and its apparent unlimited capacity for growth. Of all the cell lines listed in Table 1 that have been tested for tumourigenicity, only the LS123 cell line fails to produce any kind of xenograft tumour growth (Rutzky et al., 1983). The remaining cell lines do, however, differ widely in the time taken to form xenograft tumours and also in the organisation and differentiation of the resulting tumours.

An indication of how tumourigenic a cell line may be is their ability to grow independent of anchorage to a solid substrate. An example of which is where cells are suspended in a soft agar gel (Kimball et al., 1978). Marshall et al.

(1977) described the growth in agar as a trait of malignant transformation of the cells. Again differences are observed in the efficiency of the cell lines to form colonies in soft agar. Differences in anchorage independent growth were also observed between subpopulations of single cell lines. For example, clone A of the DLD-1 cell line has a cloning efficiency, in 0.5% agar, of 77% whereas clone D has a 24% cloning efficiency (Dexter et al., 1981). This was also shown to be the case by Brattain (Brattain et al., 1981) where colony formations in 0.5% agarose were 2.9%, 9.2%, and 2.1% for the subpopulations HCT-116, HCT-116a and HCT-116b respectively. It is, however, difficult to compare the growth in semisolid medium of the cell lines from the literature alone, as different substrates and methods are used for the evaluations.

In the past two decades several cell lines have been established from primary colorectal adenomas and carcinomas and their metastases. Within this collection are several cell lines available for the study of the mechanisms of differentiation of normal colorectal enterocytes (Grasset et al., 1984 ; Cuthbert et al., 1987 ; Whitehead et al., 1987 ; Kirkland, 1988). The majority of these cell lines will also aid the study of colorectal cancer itself and possibly contribute to advancements in the currently available treatments.

Unfortunately few of these cell lines demonstrate the heterogeneity displayed by many primary colorectal carcinomas (Brattain et al., 1981 ; Dexter et al., 1981) which is one of the more poorly understood phenomena exhibited by the disease.

Heterogeneity of Primary Colorectal Carcinomas and Cell Lines.

It is evident to most that tumours arising in different tissues differ greatly in many of their characteristics including their cell biology. Over the years it also became clear that a diversity occurs among tumours of the same tissue of origin and also among the primary tumour and its metastasis. As we look at these single neoplasms, yet another level of heterogeneity becomes apparent and it is often the case to find many morphological variants within a single tumour. The heterogeneity occurring within and between individual tumours and their metastases has received much attention in recent years. The biological consequences and therapeutic implications of such heterogeneity have been discussed and reviewed by many investigators (Calabresi and Dexter, 1982 ; Dexter and Calabresi, 1982 ; Fidler and Hart, 1982 ; Woodruff, 1983 ; Heppner and Miller, 1983 ; Dexter et al., 1984 ; Heppner et al., 1984 ; Heppner et al., 1984 ; Talmadge and Fidler, 1989).

This heterogeneity was first noted by pathologists examining fixed sections of tissue. Colorectal carcinomas also display this wide variation of cell types and structural organisation within a single tumour. Peterson et al. (1978) demonstrated the presence of subpopulations in five primary human colonic carcinomas with the use of flow cytometry.

Clinical Implications.

This type of heterogeneity has serious implications in the treatment of many types of cancer as one cell type may respond effectively to a certain therapy whereas another within the same neoplasm may be insensitive to that agent. Hakansson and Trope (1974) found that differences in sensitivity to cytotoxic drugs were displayed by different sections of a methylcolanthrene-induced sarcoma in mice. Using cell lines derived from human gliomas, Yung et al. (1982) demonstrated heterogeneous responses to cytotoxic drugs between the subclones of each cell line. This phenomenon was also shown in the

work carried out by Heppner et al. (1978) in subpopulations isolated from a single mouse mammary tumour cell line.

This heterogeneity in drug sensitivity has also been observed with cell lines established from human colorectal carcinomas. Differences in response to cytotoxic agents were detected between several different cell lines all of colorectal carcinoma origin (Drewinko and Yang, 1988). At an intratumoural level, five subclones of the clone A subpopulation of the DLD-1 cell line show a great heterogeneity in their responses to etoposide and vincristine, although their responses to methotrexate were similar (Ferguson and Cheng, 1989). It is clear that these discrepancies in drug sensitivity within a tumour may play an important role in the failure of such treatments.

Unfortunately it is not only in the response to cytotoxic drugs that the heterogeneity of colorectal carcinoma cells becomes evident. In a series of experiments, Leith and co-workers have demonstrated that the subpopulations isolated from the DLD-1 parent line can also differ in their response to X-irradiation (Leith et al., 1982) and hyperthermia (Leith et al., 1983). It is therefore imperative that we increase our understanding of tumour cell heterogeneity in order to effect a more successful therapeutic approach to the disease.

Heterogeneity of cell lines.

Following the establishment of cell lines from primary human colorectal carcinomas, it was noted by some workers that a morphological heterogeneity existed within a single cell line. However the number of cell lines displaying this phenomenon represented only a small portion of the total number of cell lines established. The initial cultures of HCT-8 (Rosenthal et al., 1977), HCT-116 (Brattain et al., 1981), DLD-1 (Dexter et al., 1981), HCT-C (Brattain et al., 1983) and HCA-7 (Kirkland, 1985) all showed morphologically distinct subpopulations.

In order to study this heterogeneity in more detail it was deemed necessary to isolate and characterise the morphological variants observed within these cell lines. Several methods have been used to isolate subpopulations from the parent cell lines, such as cloning in soft agar (Dexter et al., 1981), sedimentation in percoll (Brattain et al., 1981) and mechanical isolation (Brattain et al., 1983 ; Cuthbert et al., 1987).

Subpopulations have also been isolated from cell lines by adaptation of the normal conditions used for the maintenance of the parent cell line. In this way many subpopulations of the undifferentiated HT-29 cell line (Fogh et al., 1977) have been established. By replacing glucose with galactose in the medium, a differentiated clone was established from HT-29 (Pinto et al., 1983) and from that clone, subclones with absorptive and mucus-secreting properties were isolated (Huet et al., 1987). Addition of sodium butyrate to the medium enabled a permanently differentiated clone to be isolated from the HT-29 cell line (Augeron and Labois, 1984). Sodium butyrate was also used in the isolation of a subpopulation from the HCA-7 cell line (Cuthbert et al., 1987). Using increased resistance to a cytotoxic agent, in this case mitoxantrone, a subline of the WiDr cell line (Noguchi et al., 1979) was established (Wallace et al., 1987). The subpopulations from each cell line have been shown in various studies to differ greatly in many of their characteristics such as morphology, carcinoembryonic antigen production, in vitro growth properties, karyotype, cloning efficiency in soft agar, histology of xenografts formed in nude mice and resistance to cytotoxic drugs.

However, previously it has only been possible to isolate three subpopulations (Brattain et al., 1981), or two subpopulations (Dexter et al., 1981) from any one untreated human colorectal carcinoma cell line, which must impose some restrictions on the extent to which we can investigate heterogeneity by the study of its individual components.

Generation of heterogeneity.

One of the major questions involved when studying the heterogeneity of carcinomas is that of how and when does heterogeneity originate. This could be answered fairly simply if the tumour were of a polyclonal origin where several cells are transformed simultaneously at the initiation of the tumour. The idea that tumours are polyclonal in origin has been discussed by several workers (Barrett and Tso, 1978 ; Rubin, 1980). However it is the general consensus of opinion that tumours are monoclonal in origin, that is to say they arise from a single transformed cell. Both Nowell (1976) and Fialkow (1979) came to the same conclusions, from the evidence available, that the majority of neoplasms are of unicellular origin. However Fialkow (1979) also reviewed evidence that some tumours were of multicellular origin, including one arising in the colon.

More recent evidence, using molecular probes with which to study X chromosome inactivation in human colonic neoplasms, indicates that all the tumours studied were of monoclonal origin (Fearon et al., 1987). Also utilising X chromosome inactivation, Griffiths et al. (1988) concluded that both normal crypts and induced murine colonic tumours were of clonal origin.

Thus, the majority of investigators work on the theory that the neoplasm is of monoclonal origin and the characteristic heterogeneity must therefore arise from the one transformed cell.

Many hypotheses have been put forward to explain this phenomenon. One of the most discussed theories for the generation of cellular heterogeneity within a single neoplasm is that of genetic instability of the cancer cell. Nowell (1976) postulated that along with transformation of a normal cell to a neoplastic cell, an acquisition of genetic instability occurs which is beyond that observed in normal cells. This genetic instability results in the production of new mutant cells, many of which do not survive. However, he went on to suggest that as the tumour progresses, the cells become more genetically unstable giving rise to more variants. This hypothesis has been supported by

evidence showing that the rate of spontaneous mutation is elevated in fibrosarcoma cells of a metastatic clone compared to cells of a non metastatic clone (Cifone and Fidler, 1981).

Other hypotheses have been presented which do not involve the genome in their explanation of the origin of tumour heterogeneity. Pierce (1974) suggested that cell variants arose from a process akin to normal cell differentiation acting on a neoplastic stem cell. Evidence suggesting the presence of such a stem cell in a mouse mammary tumour cell line has been presented by Hager *et al.* (1981a, 1981b). Frost and Kerbel (1983) hypothesised on the role of hypomethylation of DNA, caused by mutagens, which could result in the production of many cell variants. Somatic hybridisation is yet another epigenetic mechanism postulated to explain the generation of cellular heterogeneity. De Baetselier *et al.* (1981) demonstrated that the hybridisation of non-metastatic murine plasmacytoma cells with normal spleen B lymphocytes resulted in the production of spontaneously metastasising variants.

The role of the host and the environment may be of some significance in the production of variant subpopulations. The presence of host stroma, vascularisation of the tumour and the immunological response of the host may influence the neoplastic cells in some way as to alter their phenotypic characteristics.

These putative mechanisms put forward as explanation of the origin of tumour cell heterogeneity may not be mutually exclusive to each other and it is possible, if not likely, that one or more of these potential mechanisms are responsible for the phenomenon observed.

Maintenance of heterogeneity.

There is much evidence available to demonstrate that heterogeneity exists within a tumour at the time of diagnosis (Peterson, 1979 ; Siracky, 1979). It is also clear from studies using the subpopulations isolated from single parent cell lines that these subcolonies differ greatly in many of their characteristics, including growth. Why then is it not the case that the faster growing subpopulation does not outgrow its contemporaries yielding a homogeneous tumour? Similarly, why is a non-immunogenic cell type not selected to survive whilst the more immunosensitive colonies are eliminated by the hosts immunological defences? It is not clear, at the present time, how these subpopulations of a tumour, with such diverse phenotypic properties, continue in existence to maintain a heterogeneous tumour.

Again several theories have been proposed in an attempt to explain this phenomenon. It remains unclear as to whether or not heterogeneity is a dynamic process, whereby the mechanisms involved in generating diversity continue through the entire life of the tumour producing a continual array of new variants. If however this is not the case, and once diversity has been established within a tumour these initial processes cease, then the question arises as to what mechanisms take over to ensure that this heterogeneity is maintained.

Calabresi et al. (1979) postulated that cellular differentiation may occur within a tumour to produce phenotypically distinct cells with growth properties different from the parent cell thus aiding the maintenance of heterogeneity. The involvement of differentiation in heterogeneity has also been discussed by Markert (1982) and Tsao (1982).

One of the most studied and discussed putative mechanisms for the maintenance of heterogeneity is that of cellular interactions. Heppner and colleagues, using subpopulations isolated from a single mouse mammary tumour cell line (Dexter et al., 1978), have produced evidence of subpopulation interactions. This group have demonstrated interactions

occurring between the cells which affect the growth of the subpopulations both *in vitro* (Heppner *et al.*, 1980) and *in vivo* (Miller *et al.*, 1980). They have also suggested that interaction may also interfere with other characteristics of a particular subpopulation. Using the same subpopulations, they showed that drug sensitivity can also be altered both *in vivo* (Miller *et al.*, 1981), and *in vitro* (Miller *et al.*, 1981 ; Miller *et al.*, 1984). Tofilon *et al.* (1984) also demonstrated cellular interactions affecting both drug sensitivity and growth of spheroids grown from mixtures of rat brain tumour cells.

These type of cellular interactions are also seen to occur between subpopulations of colorectal carcinoma cell lines. Caignard *et al.* (1985) found that the subpopulation from an induced rat colonic carcinoma cell line, which did not produce tumours itself in syngeneic mice, inhibited the growth of tumours from a tumour producing subpopulation isolated from the same cell line. They also postulated that the cells interacted with each other through their effect on the host immune system.

Much work has been performed by Leith and his co-workers on the human colonic carcinoma cell line DLD-1, investigating possible interactions between the two subpopulations isolated from this line, clone A and clone D. After mixing the two subpopulations in a number of different proportions to each other, and injecting them into nude mice, Leith *et al.* (1987) came to two conclusions about the resulting tumours. The first being that the differences in growth of the admixed tumours were dependant on the initial relative percentages of the two clones and secondly that the growth of the mixed tumour could not be predicted from the growth kinetics of the tumours produced from the individual cell clones. They suggested that the results indicated that interactions were occurring within the tumours, which may or may not have been contacted-mediated.

Although much work has been carried out to investigate the role of cellular interactions in the maintenance of heterogeneity, it is still very unclear exactly what mechanisms are involved. It may be that the host stroma or defences are

involved or that interactions are contact-mediated or indeed occur via a soluble factor or messenger. These mechanisms again may not be mutually exclusive to each other and all may have their own part to play.

Aims and Objectives.

Over the past two decades it has become clear that one of the more manipulable systems, although not the most ideal, for the study of the biology of cancer is that of the cultured cell line. These cell lines have now proved to be a great asset in the study of cancer cell heterogeneity and the mechanisms involved in the generation and maintenance of this phenomenon. By the isolation of subpopulations from a heterogeneous cell line, it is possible to identify characteristics which differ between the cell types present within a tumour, giving us a clearer view of the overall 'scenery' of that tumour. This approach could also prove valuable in finding a common denominator possessed by all of the co-existing subpopulations, which may provide us with a potential target for the treatment of the disease.

The HCA-7 cell line is an ideal candidate for increasing our knowledge of the biological heterogeneity observed within carcinomas. The parent cell line was established in 1981 from a mucinous adenocarcinoma of the hepatic flexure of the colon of a 58 year old female. These cells grow as a monolayer on tissue culture-treated plastic and have been shown to form domes, secrete carcinoembryonic antigen and are tumourigenic in nude mice (Kirkland, 1985). The HCA-7 cell line was also found to display an extensive morphological heterogeneity at early passage. Two subpopulations have previously been isolated from this cell line, namely col 1 but and col 3 (Cuthbert et al., 1987), and have been shown to differ not only in their morphology but also in their electrolyte transport properties (Cuthbert et al., 1987).

It is with the HCA-7 cell line that the study of heterogeneity presented in this thesis begins. The first objective in this project was to isolate some of the many morphologically distinct subpopulations observed in the parent cell line and to establish them in culture as individual cell lines in their own right.

Secondly it was the intention to characterise these subpopulations as fully as possible both in vitro and when grown in vivo in nude mice, and to determine

whether or not their characteristic morphologies were maintained in isolation. It was the final aim to investigate whether interactions were occurring between individual HCA-7 subpopulations, which might give some insight into at least one of the putative mechanisms involved in the maintenance of heterogeneity.

MATERIALS AND METHODS.

Tissue Culture.

Isolation of Subpopulations.

Chromosome Analysis.

Histological Staining.

Ultrastructural Morphology.

Growth Curves.

DNA Assay.

Anchorage Independence.

a) Agarose Gels.

b) Bacteriological Dishes.

Transport Properties.

a) Dome Quantification.

b) Short Circuit Current (SCC) Measurements.

Antigenic Properties.

a) Qualitative Analysis.

b) Quantitative Analysis.

In Vivo Characterisation.

Interactions.

a) Co-cultures.

b) Conditioned Media Experiments.

c) Transwells.

Histology.

1. Tissue culture.

Materials.

Dulbecco's modified Eagle's medium	Gibco
Foetal calf serum	Gibco
Kanamycin	Winthrop Laboratories
Amphotericin B	E R. Squibb and Son
Gentamycin	Roussell
Sodium pyruvate	BDH Chemicals
Trypsin	Lorne Diagnostics
Plastic culture flasks and wells	Becton Dickinson
Millipore filters	Flow Laboratories
Glass coverslips	Raymond Lamb

Methods.

All cells were routinely grown as monolayers in tissue culture-treated plastic flasks and wells. Cells were fed daily by a complete medium change with Dulbecco's modified Eagles medium supplemented with 110 µg/ml sodium pyruvate, 100 µg/ml kanamycin and 10% foetal calf serum. The parent line and subpopulations were subcultured weekly. Cells were washed with 5ml versene and then detached from the flask by repeated application of trypsin (3 x crystallised and dialysed : 0.05% w/v in versene). The resulting cell suspension was then centrifuged at 100g for 5 min and the cell pellet resuspended in fresh medium. Cells were seeded out at the appropriate dilution into fresh flasks or wells. Additional antibiotics occasionally used were amphotericin B (2.5 µg/ml) and gentamycin (100µg/ml). All cells were maintained at 37°C. Sealed flasks were kept in a non-gassing incubator and wells open to the air were kept under 8% carbon dioxide in a Heraeus gassing incubator.

The morphologies of the parent line and subpopulations were viewed by phase contrast microscopy using an Olympus OMT-2 inverted microscope.

2. Isolation of subpopulations.

Early passage HCA-7 cells were sparsely seeded into 25cm² plastic flasks at split ratios of 1:100. Resulting small colonies were selected for their differing morphologies and manually isolated. This was achieved by scraping away unwanted cells surrounding the colonies using a drawn-out Pasteur pipette. When the colonies had reached a size of 50 - 100 cells they were individually removed from the flask. Cloning cylinders were placed around each colony and sealed to the plastic with silicone grease (Fig 4). Colonies were then trypsinised off the plastic and initially transferred to single wells of a 96-well plate either alone or onto a layer of lethally irradiated 3T3 cells (4,000 rads from a ¹³⁷Cs source). When confluent, cells were sequentially transferred into single wells of 24-well plates, 6-well plates and then into 25cm² flasks. All the subpopulations obtained in this way were grown as individual cell lines. When sufficient numbers of cells had been obtained, characterisation of the parent line and nine of the subpopulations was performed.

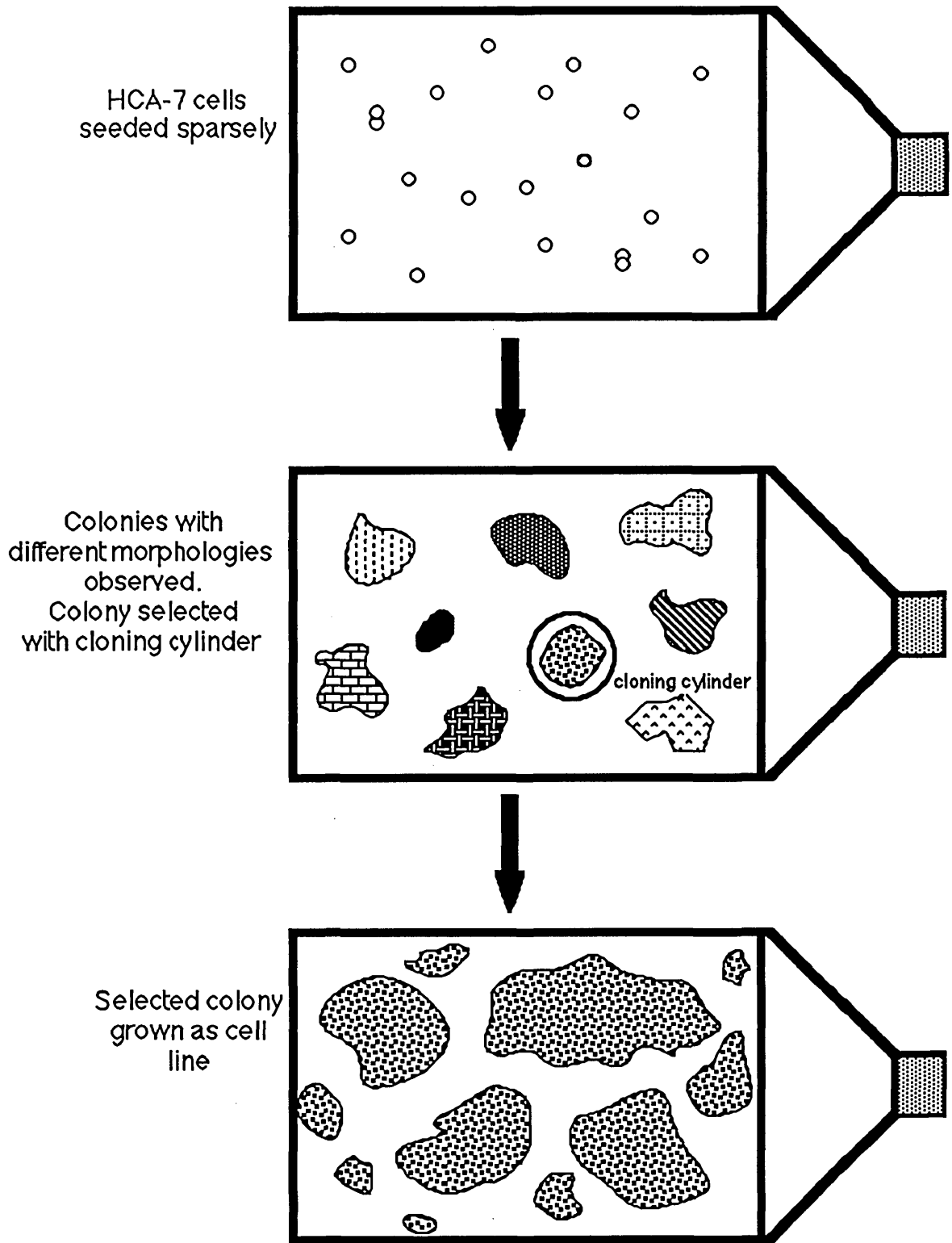


Fig. 4. Method of isolation of subpopulations from flasks of early passage HCA-7 cells.

3. Chromosome Analysis.

Materials.

Demecolcine	Sigma
Methanol	BDH Chemicals Ltd.
Glacial Acetic Acid	BDH Chemicals Ltd.
Giemsa	BDH Chemicals Ltd.
Sorenson's Buffer	Raymond Lamb.

Methods.

The parent line and subpopulations used in this method were all in their exponential growth phase. Cells were incubated with demecolcine (4 $\mu\text{g/ml}$) for 2 hr at 37°C. After this time cells were harvested in the usual manner, centrifuged at 100g for 5 min and resuspended in a hypotonic solution consisting of 1 part normal medium and 3 parts water. This suspension was incubated at 37°C for 10 min and centrifuged as before. The supernatant was aspirated to a volume of approximately 1 ml in which the cells were gently resuspended. A fixative mixture of 1 part glacial acetic acid, 3 parts methanol, was added dropwise to the cell suspension. This was then centrifuged, cells resuspended in 5ml of fresh fixative and left at room temperature for 10 min. This last step was repeated and a final spin at 100g for 2 min was performed. The supernatant was aspirated to 0.5 ml and the cells resuspended in this volume. The suspension was then dropped onto clean, cold glass slides and allowed to air dry. For counting of chromosomes the cells were stained with Giemsa (10% v/v in Sorensens buffer pH 7) for 10 min. Chromosomes from 50 cells of each cell line were counted, using a Leitz Laborlux light microscope, under oil at 100x magnification. From this the modal chromosome numbers were calculated. Normal human foreskin fibroblasts were used as a control.

4. Histological staining.

Monolayers of the parent cell line and subpopulations were grown on 22 x 22mm glass coverslips until confluency was reached. They were then washed three times in phosphate buffered saline (PBS) and fixed with ethanol for 10 min at room temperature. Following fixation monolayers were stained with haematoxylin and eosin, Alcian blue, periodic acid Schiff or the Grimelius silver stain as described in section 12.

5. Ultrastructural Morphology.

Materials.

Glutaraldehyde	Emscope
Osmium tetroxide	Johnson Matthey Chemicals Ltd.
TAAB resin	TAAB laboratories Equipment Ltd.
Methanolic Uranyl Acetate	BDH Chemicals Ltd.
Lead Citrate	BDH Chemicals Ltd.
Grids	Agar Scientific Ltd.

Methods.

For the observation of ultrastructural morphology, cells were grown on 22 x 22mm glass coverslips until confluency. Cells were washed 3 times in 0.1M phosphate buffer and then fixed in glutaraldehyde (2% in 0.1M phosphate buffer) at room temperature for 15min. After further washing in phosphate buffer, the monolayers were post-fixed in osmium tetroxide (1% in Millonig's buffer) for 15 min at 4°C. Following dehydration in alcohol stages the monolayers were embedded in medium mix TAAB resin. A diamond knife on a Reichart Jung Ultracut E ultratome was used to cut 70nm ultrathin cross-sections of the monolayers. These were placed on 100 and 200 mesh hexagonal nickel grids (3.05mm) and stained with methanolic uranyl acetate and lead citrate. The stained sections were then viewed under a Philips CM10 transmission electron microscope.

6. Growth curves.

The growth rates of the parent line and subpopulations were determined in a single experiment. Cells were seeded into 24-well plates so that each well contained the same amount of DNA. This was achieved by measuring the amount of DNA present using a fluorimetric DNA assay and diluting the cells accordingly before plating. Cells were fed daily by a complete medium change and six wells of each line were prepared for DNA assay on days 2, 6, 10, 14 and 18. Wells were washed 3 times with PBS and stored at -20°C until required for assay.

DNA Assay.

Materials.

Protease Type XIV	Sigma
Ribonuclease A	Sigma
Calf thymus DNA Type 1	Sigma
Ethidium bromide	Sigma
Polytron and probes	Northern Media Supply Ltd.

Methods.

All cell numbers throughout this study were estimated using a fluorimetric DNA assay. This method was a modification of that used by Karsten and Wollenberger (1972) and is based on the linear relationship between the fluorescence of ethidium bromide when bound to DNA and the concentration of DNA present in the sample within the range of 0-20 µg/ml. A standard curve was constructed for each assay performed using calf thymus DNA at concentrations ranging from 0-20 µg/ml in PBS. Samples for assay were prepared in the following way. 1.0 ml of pronase (36 µg/ml in PBS) was added to each well and then incubated at 37°C for 15min to detach the cells from the plastic. The remainder of the assay was performed on ice. 800 µl of

PBS was added to each well and the cells were homogenised for 30 sec/well using a Polytron homogeniser with a 7mm probe.

Duplicate aliquots of 800 μ l of standard DNA solutions or the prepared samples were placed in polystyrene tubes for assay. To each tube, 100 μ l ribonuclease (125 μ g/ml in PBS) and 100 μ l pronase (115 μ g/ml in PBS) were added. The tubes were then vortex mixed and incubated in a sliding water bath at 37°C for 1 hour. Following incubation, 500 μ l of ethidium bromide (15 μ g/ml in PBS) was added to each tube and again vortex mixed.

The fluorescence of all the standards and samples was measured on a Perkin Elmer SP2 fluorimeter (540 nm excitation : 590 nm emission). DNA concentrations of the samples were calculated by linear regression analysis of the DNA standards.

7. Anchorage Independence.

Materials.

Sea Prep Agarose.

ICN Biomedicals.

Methods.

a) Agarose Gels.

0.3
2

Soft agarose gels were used for the assessment of the growth of the parent line and subpopulations in a semi-solid medium. A 6% (w/v) solution of sea prep agarose in double distilled water was prepared by boiling in a water bath for 20-30 min. This was stored at 45°C until use. Cells were harvested from flasks by trypsinisation and resuspended in fresh medium. Equal volumes of each cell suspension and agarose solution were mixed giving a final concentration of 3% agarose. Replicate amounts of each cell / agarose mixture were plated out into 6-well plates which were then chilled at 4°C for 10min in order for the gels to set. 2ml of fresh medium were then added to each well. Cells were fed every 3 days by a complete medium change. Growth of colonies was observed under a phase contrast microscope. Gels were also fixed in 10% neutral buffered formalin at room temperature for 30 min and then processed as described in section 12. Sections were then stained with haematoxylin and eosin.

b) Bacteriological Plates.

Non culture-treated dishes were used to determine the growth of the parent line and subpopulations in suspension. Cells were harvested by trypsinisation and resuspended in fresh medium. The required amount of cell suspension was added to bacteriological dishes containing 15ml of fresh medium. Cells were fed by transferring the cell suspension into a universal tube and allowing cell aggregates and colonies to settle. The superfluous medium containing single and dead cells was then aspirated off. The pellet was resuspended in 15ml of fresh medium and returned to the same

bacteriological dish. Growth of the cells was observed by phase contrast microscopy.

8. Transport Properties.

Materials.

Ethanol.	BDH Chemicals Ltd.
Millipore filters	Flow Laboratories
Acetic Acid	BDH Chemicals Ltd
W-P Dual voltage Clamp	W-P Instruments
Lysylbradykinin	Sigma
Forskolin	Calbiochem
Carbechol	Sigma
A23187	Sigma
Piretinide	Sigma

Methods.

a) Dome Quantification.

Quantification of dome formation by the parent line and the subpopulations which displayed this property was performed. Equal numbers of cells were seeded into 6-well plates and fed daily by a complete medium change. At each time point 3 wells of each cell line were washed 3 times in PBS and then prepared for dome quantification. These wells were fixed with ethanol for 10 min at room temperature. They were then washed 3 times with distilled water and stained with Mayer's haematoxylin for 2min. Cells were allowed to blue in tap water, then washed and stored in distilled water. The number of domes present in the entire well was counted.

b) Short Circuit Current (SSC) Measurements.

Preparation of epithelial monolayers.

For the purpose of SSC measurements cells were grown on pervious supports. 0.45µm millipore filters were coated with collagen (0.25% in 0.3% acetic acid). After drying, a silicone washer was attached to the center of the filter, using silastic 743 RTV adhesive. This created a well into which cells

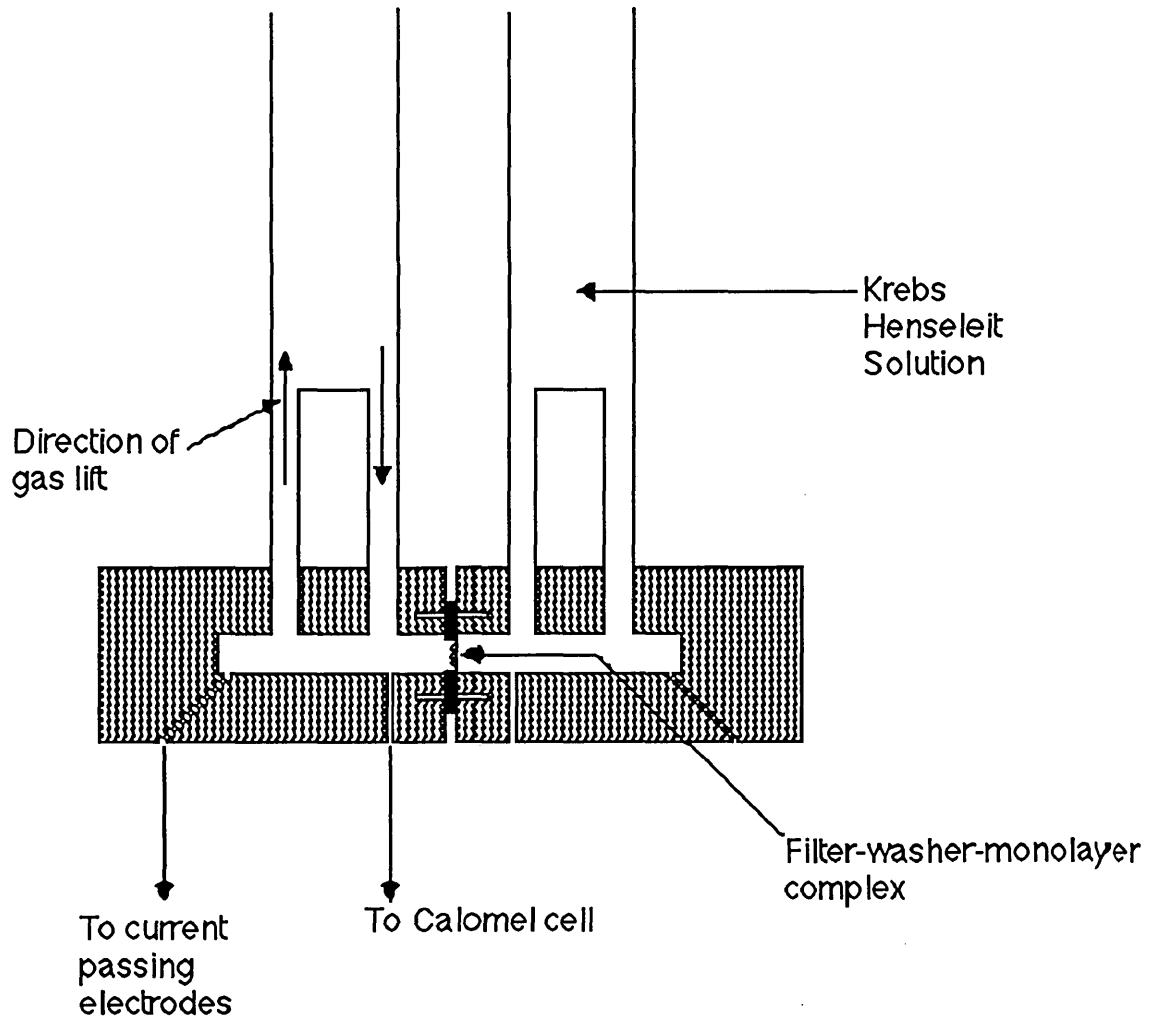


Fig. 5. Ussing chamber used for short circuit current measurements. Kreb's solution is maintained at 37°C by a water jacket and is gassed with 95% oxygen / 5% carbon dioxide.

were seeded (Fig. 6). Units were sterilised by U.V. radiation. Monolayers of the subpopulations were grown to confluency by floating 4 units on 12 ml of normal medium in a 90mm Petri dish. These were kept under 5% CO₂ and medium was changed every 3 days until use.

SSC Measurements.

The Millipore-washer-cell monolayer complexes were clamped between the two halves of the Ussing chamber as shown in Fig. 5. The washer was gripped by a ring of stainless steel pins in one half of the chamber entering holes in the other half, so that the 0.2cm² monolayer was held in the center of a 0.6cm² window.

Initially electrode balance was checked and compensation for fluid resistance adjusted with only a silicone washer in place. Voltage-recording electrodes were apposed to opposite sites of the millipore-washer-cell monolayer complex. These were filled with Krebs Henseleit solution (KHS) that led to calomel cells via KCl-agar bridges. Current-passing electrodes, also filled with KHS, entered the chamber as far from the monolayer as possible and led via KCl-agar bridges to Ag-AgCl current-passing electrodes. Monolayers were short circuited with a W-P dual voltage clamp. SCC was displayed on pen recorders. The clamp was also used to set the potential at a voltage displaced from zero. The resulting current change allowed calculation of trans-epithelial resistance from the Ohmic relationship.

Each side of the monolayer was bathed in 20ml KHS maintained at 37°C by heat exchangers which was circulated through both chambers by use of a gas lift (95% O₂ : 5% CO₂) maintaining the pH at 7.4.

Agents were added directly to the bathing solution in either side of the chamber so that either the apical or basolateral surface of the cells could be exposed to the drug (Fig. 6).

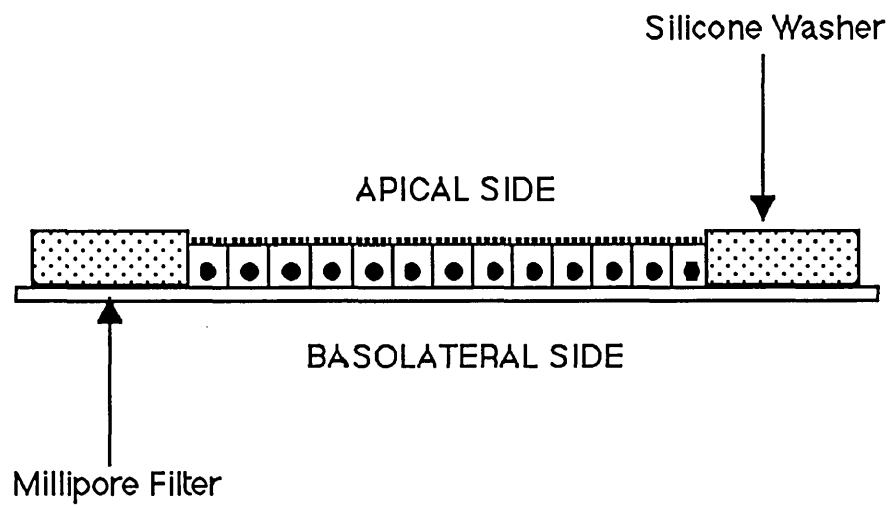


Fig. 6. Millipore - washer - cell monolayer complex. Cells were seeded into the well created by the millipore filter and the silicone washer. Four units were floated on 12 ml of normal medium in a 90mm Petri dish.

9. Antigenic Properties.

Materials.

Mouse monoclonal CR3/43 antibody	Gift from D. Mason, The Radcliffe Infirmary, Oxford.
Mouse monoclonal AUA1 antibody	Gift from A.A. Epenetos, RPMS, London.
1A3, 4D4 and 5D5 Antibodies.	Gift from P Richman, ICRF Lincoln's Inn Fields, London.
Polyclonal anti-sucrase isomaltase antibody	Gift from A. Zweibaum, INSERM U178, Paris
Rabbit polyclonal and mouse monoclonal anti-carcinoembryonic antigen antibody	Dako Ltd.
Hydrogen peroxide	BDH Chemicals
Normal swine or rabbit serum	Dako Ltd
Peroxidase-conjugated swine anti-rabbit or rabbit anti-mouse antibodies	Dako Ltd.
Bovine serum albumin	Sigma
Diaminobenzidine	Aldrich Chemical Company Ltd.
Paraformaldehyde	BDH Chemicals
¹²⁵ I Labelled F(Ab2) sheep anti mouse immunoglobulin	Amersham International plc.
Sodium dodecyl sulphate	Sigma
Gamma counter	LKB

Antibodies.

The majority of antibodies were kind donations from the people mentioned above. Antibodies used for immunocytochemistry were mouse monoclonal CR3/43 (Gatter et al., 1982) directed against the β chain of the HLA-DR

molecule; mouse monoclonal AUA1 (Spurr et al.,1986) recognising an antigen located exclusively on the basolateral domain of certain epithelial cells (Mooi et al.,1986); mouse monoclonals 1A3, 4D4 and 5D5 (Richman and Bodmer 1987) directed against mucous and absorptive components of normal human colorectal epithelium; mouse monoclonal anti-carcinoembryonic antigen (anti-CEA,m) antibody; rabbit polyclonal antibody directed against sucrase isomaltase, a marker of enterocytic differentiation (anti-SI : Zweibaum et al.,1985) and rabbit polyclonal anti-carcinoembryonic antigen (anti-CEA,p) antibody.

a) Qualitative analysis.

The following dilutions and fixatives were used for the qualitative analyses of the antibodies listed below.

<u>Antibody.</u>	<u>Dilution.</u>	<u>Fixative.</u>
CR3/43	1:2	Ethanol
AUA1	1:10	Ethanol
1A3	None	Ethanol
4D4	None	Ethanol
5D5	None	Ethanol
Anti-SI	1:50	3.7% (w/v) paraformaldehyde in PBS.
anti-CEA,p	1:1000	Neutral buffered formalin.

All the above dilutions were made in 0.1% w/v bovine serum albumin (BSA) in distilled water.

Procedure.

Cells were grown on 22 x 22 mm glass coverslips until confluency, then washed 3 times in PBS and fixed for 10 min at room temperature. Following fixation, endogenous peroxidase was blocked with 0.3% (v/v) hydrogen peroxide in PBS (30 min). Normal rabbit serum (for mouse monoclonal antibody application) or normal swine serum (for rabbit polyclonal antibody application) was then applied to the monolayers (1:20 in PBS : 10 min).

Primary antibodies and 0.1% BSA in PBS controls were incubated with cells overnight at 4°C. Cells were then washed 3 times in PBS and exposed to the second layer of peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit antibodies (1:100 in 0.1% BSA in PBS), as appropriate, for 1 hour at room temperature. After washing 3 times in PBS, the monolayers were incubated in diaminobenzidine (0.025% in PBS) with 0.03% hydrogen peroxide for 15 min at room temperature. Non-alcohol fixed cells were then treated with ethanol for 20 sec and all monolayers were counterstained with Mayer's haematoxylin, dehydrated in alcohol stages and mounted.

Quantitative analysis.

CR3/43 and AUA1 were used at a dilution of 1:10, 1A3, 4D4 and 5D5 were all used neat and the anti-CEA,m antibody at a dilution of 1:200. All dilutions were made using PBS.

Procedure.

Cells were seeded into 96-well plates and grown until confluency. They were then washed 3 times with PBS and fixed in ethanol for 10 min. All fixation and incubations were carried out at room temperature. After washing a further 3 times with 200µl PBS, the cells were incubated for 1 hour with 100µl of primary monoclonal antibody. Control cells were incubated with 100µl PBS. Following the primary incubation the wells were given three 10 min washes with 200µl PBS. Cells were then incubated for 1 hour with 100µl ¹²⁵I labelled F(Ab₂) sheep anti-mouse immunoglobulin (5-20 µCi / µg protein), diluted with 10% (v/v) normal human serum in PBS, so that each aliquot contained approximately 400,000 cpm. The wells were each washed again with 200µl PBS (4 x 5min) and the cells were solubilised in two aliquots of 200µl 10% (w/v) sodium dodecyl sulphate. The radioactive emission of each sample was measured in counts per minute using an LKB gamma-counter. Cell numbers present in each well were estimated using the fluorimetric DNA assay described earlier.

10. In vivo Characterisation.

Materials.

Female athymic nude mice	National Institute of Medical Research
Hypnorm	Janssen Pharmaceuticals Ltd.
Hypnovel	Roche
Glutaraldehyde	Emscope
2,2-dimethoxypropane	Emscope
TAAB resin	TAAB Laboratory Equipment Ltd.
Uranyl Acetate	BDH
Lead Citrate	BDH

Methods.

Cells were harvested from 175 cm² tissue culture flasks by trypsinisation and resuspended in a minimum volume of normal medium. Mice were anaesthetised by intraperitoneal injection of 0.1ml anaesthetic mixture (hypnovel:hypnorm:water 1:1:2). Approximately 10⁷ cells of each cell line were injected subcutaneously, in a volume of 0.1ml, into six sites of female athymic nude mice (3-4 weeks old). Mice were maintained in a sterile environment and supplied ad libitum with irradiated food and sterile water. When tumours had reached a size of approximately 0.5 cm in diameter, they were removed and processed for either light or electron microscopy. A sample of colon from each mouse was also removed and histologically examined.

Histology.

For the purpose of light microscopy, tumours were fixed in neutral buffered formalin. After fixation tumours were processed and stained with

haematoxylin and eosin, Alcian blue, periodic acid Schiff and the Grimelius silver stain as described in section 12.

Electron microscopy.

Small pieces of tumour were fixed in 2% glutaraldehyde in 0.01M phosphate buffer for 1 hour at 4°C. They were then post-fixed in Millonig's buffered osmium tetroxide for 1 hour. Following dehydration in acidified 2,2-dimethoxypropane, tissues were embedded in medium mix TAAB resin. 70nm sections, cut with a diamond knife on a Reichart-Jung Ultracut E ultratome, were stained with methanolic uranyl acetate and Reynold's lead citrate. Sections were viewed under a Philips CM10 transmission electron microscope.

Grading of xenografts.

Many differences were observed between the xenograft tumours produced by the different cell lines. In collaboration with G.W.H.Stamp (ICRF, London) tumours were graded according to their degree of differentiation using several histological criteria. Such criteria included abundance and type of mesenchyme, pattern of invasion, cell shape and polarity, abundance of goblet cells, presence of a brush border and size of cell nuclei. By these criteria, tumours were given a grade from 1 to 6, grade 1 being allocated to the most differentiated tumours and grade 6 to the least differentiated.

11. Interactions.

a) Co-cultures.

To observe possible contact-mediated interactions occurring between cell types, co-cultures of two different subpopulations were utilised. Cells were seeded into 24-well plates either alone or in a 1:1 ratio with another subpopulation so that each well contained the same amount of DNA. Cells were fed daily by a complete medium change. At the required time points, replicate wells were washed 3 times with PBS and stored at -20°C until a DNA assay was performed.

b) Conditioned Media Experiments.

The possible involvement of soluble factors in interactions between the parent line and col 3 was investigated using conditioned media. HCA-7 cells or col 3 cells were seeded equally into 24-well plates. At the same time cells of each cell line were seeded separately into 25cm² culture flasks. The wells were fed daily by the 24 hour-conditioned media removed from the flasks containing either HCA-7 or col 3 cells. Medium was prepared before use by mixing equal volumes of conditioned media and fresh medium. These media were then filtered through 0.45µm millipore filters. Filters were initially primed by passing through 5ml foetal calf serum followed by 10 ml fresh medium in an attempt to occupy any sites that might possibly bind active components within the conditioned media.

The control used was that of fresh medium prepared as above. Replicate wells were washed 3 times with PBS on days 2, 6, 10 and 14 and stored at -20°C until required for DNA assay.

c) Transwells.

Transwells allow the co-culture of two cell types without direct physical contact but allow permeation of soluble factors. They consist of tissue culture treated plastic 24-well plates into which polystyrene inserts are placed. These inserts

are smaller wells with a permeable (0.4µm pore diameter) culture treated polycarbonate base, which sit above the base of the 24-well plate (Fig.7). Either HCA-7 cells or col 3 cells were seeded into the 24-well plates so that each well contained replicate amounts of DNA. Cells of HCA-7 or col 3 were also seeded into the transwell inserts placed in fresh 24-well plates. Control transwell inserts contained either medium alone or HRA-19 cells at the same DNA seeding as HCA-7 and col 3. Both the 24-well plates and the transwell inserts were allowed to settle for 48 hours before moving. After this time the transwell inserts were placed into the 24-well plates. Thus the wells containing either HCA-7 cells or col 3 cells had inserts placed over them containing either HCA-7 cells, col 3 cells, HRA-19 cells or fresh medium. The transwell complexes were fed daily by complete medium changes of 1.0ml in the wells and 200µl in the transwell inserts. On day 14 the wells were washed 3 times with PBS and stored at -20°C until required for DNA assay.

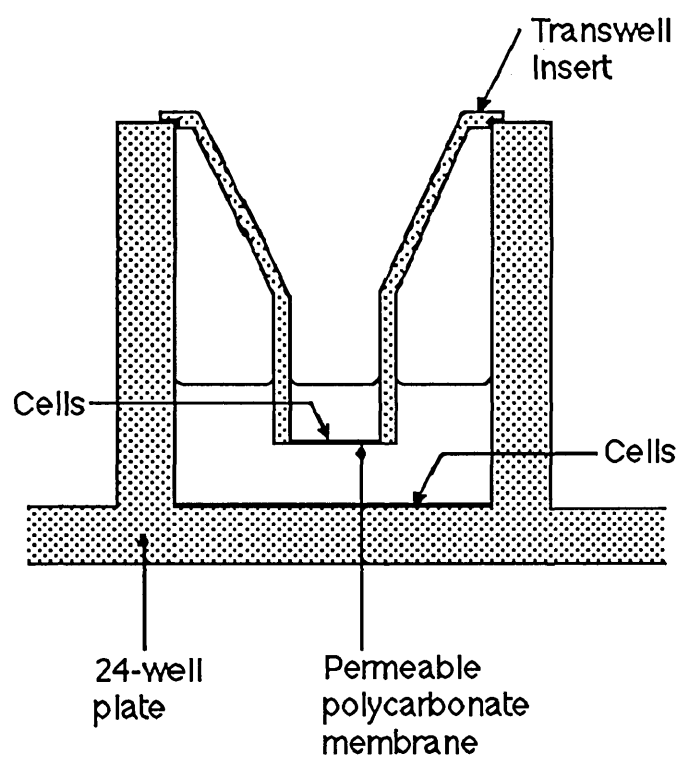


Fig. 7. Illustration of the transwell arrangement. Polystyrene transwell insert sits above the the first layer of cells. Both compartments are fed separately by complete medium changes.

12. Histology.

Materials.

All reagents and dyes were supplied by BDH Chemicals Ltd.

Processing.

After fixation xenograft tissue was processed for embedding using the following protocol :-

Dehydration.

70% alcohol	1.5 hr
90% alcohol	1.5 hr
Absolute alcohol I	1.5 hr
Absolute alcohol II	2 hr
Absolute alcohol III	2 hr
Absolute alcohol IV	2 hr

Link reagent.

Histoclear I	2 hr
Histoclear II	2.5 hr
Histoclear III	2.5 hr
Chloroform	2 hr

Wax

Wax I	1.5 hr
Wax II	1.5 hr

Blocks were then embedded in molten wax, allowed to set and then sectioned at 3-4 μm using an Anglia rotary microtome.

Staining.

Sections were stained following the above processing schedule and after having been dewaxed by heating to 37°C and placing in xylene. The following protocols were used for staining of the xenograft sections and monolayer cultures which were placed in the reagents listed for the time

indicated. All washes were carried out in distilled water unless otherwise stated. All solutions were made up in distilled water.

Haematoxylin and Eosin.

1. Mayer's haematoxylin. 5min.
2. Wash
3. 1% HCl in 70% alcohol. 5 sec.
4. Wash
5. Scott's tap water until blue.
6. Wash
7. 1% eosin. 2min
8. Wash
9. Wash
10. Dehydrate in alcohol stages and mount.

Alcian Blue.

1. 1% Alcian blue in 3% acetic acid. 5 min.
2. Wash
3. 0.2% aqueous neutral red. 2 min.
4. Wash
5. Dehydrate in alcohol stages and mount.

Periodic Acid Schiff.

1. 1% aqueous periodic acid. 5min.
2. Wash
3. Schiff reagent. 10 min.
4. Wash
5. Running tap water. 5 min.
6. Mayer's haematoxylin. 3 min.
7. Wash
8. Scott's tap water until blue.

9. Wash

10. Dehydrate in alcohol stages and mount.

Grimelius Silver Stain.

1. Bouin's picric acid fixative. 30 min.

2. Grimelius silver (3ml 1% AgNO_3 : 10ml acetate buffer pH 5.6 : 87 distilled water) at 60°C. 3 hour. OR overnight at 37°C.

3. Reducer (1% hydroquinone in 5% sodium sulphite) at 60°C. 1 min.

If at this stage the silver reaction is too weak, sections were replaced in silver solution for a further 30 min followed by the reducer for 1 min.

4. Wash

5. 5% Hypo (sodium thiosulphate). 2 min.

6. Wash

7. Dehydrate in alcohol stages and mount

RESULTS.

Isolation of Subpopulations.

Chromosome Analysis.

Morphology.

Phase Contrast Microscopy.

Ultrastructural Morphology.

In Vitro Growth.

Anchorage Independence.

Transport Properties.

Dome Quantification.

Ussing Chamber.

Immunocytochemistry.

Qualitative.

Quantitative.

Xenografts.

Interactions.

Co-cultures.

Conditioned Media.

Transwells.

Isolation.

Using the manual method of isolation described, nine new subpopulations of differing morphology were isolated from the heterogeneous parent cell line, HCA-7. These were designated col 6, col 11, col 17, col 23, col 24, col 26, col 27, col 29 and col 30. These subpopulations have been maintained in culture for at least 21 passages with the exception of col 17 and col 23 which have remained in liquid nitrogen at passage 5. The number of subpopulations now established from HCA-7 totals eleven including the previously isolated col1 but and col 3 (Cuthbert et al., 1987). In this study, characterisation was performed on cells of HCA-7, col 1 but, col 3, col 6, col 11, col 24, col 26, col 27, col 29 and col 30. The parent line and subpopulations were characterised by their modal chromosome numbers, in vitro and ultrastructural morphology, in vitro growth, anchorage-independant growth, transport properties, antigen expression and their behaviour as xenografts in nude mice. Finally, the possibility of in vitro interactions occurring between subpopulations which alter the growth rate of one or other of the cell lines, was investigated.

Chromosome analysis.

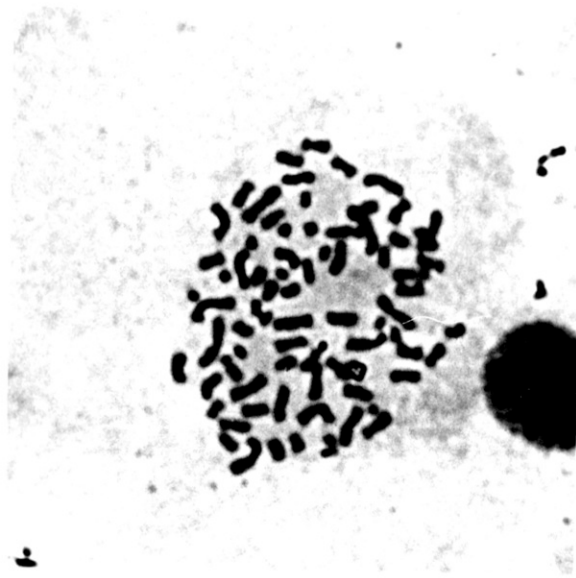
Modal chromosome numbers determined for each of the cell lines varied from 42 to 44 (Table 2). The range of chromosome numbers observed is also indicated in Table 2. The parent cell line was found to have a modal chromosome number of 43, with a range of 42-46. Fig. 8 shows a typical chromosome spread from which modal numbers were obtained. All the cell lines contained some tetraploid cells though these were generally few in number. However, approximately one third of col 3 cells were found to have chromosome numbers above 80 which were not possible to count with accuracy. These near tetraploid cells were therefore not included in the calculation of the modal chromosome number of col 3. Normal human foreskin fibroblasts were used as a control to confirm the accuracy of counting. In this case the modal chromosome number was found to be 46 with no variance.

Cell Type	Modal No.	Range
HCA-7	43	42-46
Col 1 but	44	42-46
Col 3	44	40-46
Col 6	42	38-46
Col 11	44	40-46
Col 24	43	41-46
Col 26	44	40-46
Col 27	42	40-46
Col 29	43	40-46
Col 30	44	40-46
NHFF*	46	None

Table 2. Modal chromosome numbers and ranges obtained from spreads of 50 cells from the HCA-7 cell line and subpopulations.

* NHFF= normal human foreskin fibroblasts.

Fig. 8. Photomicrograph of a representative chromosome spread of HCA-7 cells (x 1,025).



Histological staining.

Neither monolayers of the HCA-7 parent cell line or any of the subpopulations were found to show any positive staining to the Alcian blue or the Grimelius silver stains though staining was observed with the periodic acid Schiff and haematoxylin and eosin stains in all lines.

Morphology.

Phase contrast microscopy.

At early passage HCA-7 cells showed an extensive morphological heterogeneity (Fig. 9a). Many domes were observed in confluent monolayers of HCA-7. This heterogeneity and dome formation was lost between passage 25-30 and the monolayer took on a uniform appearance of tightly packed rounded cells. All the subpopulations isolated displayed different morphologies, some, but not all, with dome formation. These characteristic morphologies have remained stable for at least 21 passages in vitro and a brief description of each morphology is indicated in Table 3.

Photomicrographs which demonstrate these morphologies more clearly are shown in Figures 9 and 10. The majority of the colonies grew as smooth rounded colonies characteristic of epithelial cells (Fig. 11a), however col 29 was unusual in that it grew as spikey edged colonies (Fig. 11b). Col 24 displayed a change in morphology after passage 21 where tubule-like and doughnut-shaped structures were observed apparently sitting above the monolayer.

Ultrastructural Morphology.

HCA-7 cells also displayed their morphological heterogeneity when viewed under the electron microscope. Cells of HCA-7 differed in their shape, presence of microvilli, intercellular spacing and nuclear polarity (Fig. 12a).

Microvilli were often well developed on cells of HCA-7 (Fig. 12b and c). All the subpopulations, as well as the parent line (Fig. 12d), displayed tight junctions with desmosomes at the apical surface. However many differences were noted between the subpopulations with respect to the polarity of their nucleus, abundance of microvilli, intercellular spaces and cell shape.

Several of the cell lines displayed large intercellular spaces (Fig. 13a) whereas other subpopulations formed a very compact monolayer (Fig. 13b). Marked differences were observed in the polarity of the cells of each line. For

<u>Cell Type</u>	<u>Characteristic Morphology</u>	<u>No.</u>
HCA-7	Heterogeneous mixture of cells at early passage with domes.	122
Col 1 but	Pavement-like cells doming before confluency.	37
Col 3	Uniform cells showing areas of whorling.	38
Col 6	Defined edging to cells, dome formation when confluent.	27
Col 11	Loosely packed cells with dome formation.	26
Col 17	Regularly shaped cells with indistinct borders.	5
Col 23	Well defined edges to cells with some doming.	5
Col 24	Small cells with ill-defined borders.	24
Col 26	Mainly large uniform cells.	31
Col 27	Elongated cells with doming at confluency.	25
Col 29	Very regular, small, tightly packed cells.	35
Col 30	Loosely packed cells with abundant dome formation.	21

Table 3. Characteristics of the subpopulations of the human colonic adenocarcinoma cell line HCA-7.
No. = number of passages.

Fig. 9. Phase contrast micrographs (x 100) of monolayers of

A) HCA-7 cells.

B) Col 1 but cells.

C) Col 3 cells.

D) Col 6 cells.

E) Col 11 cells.

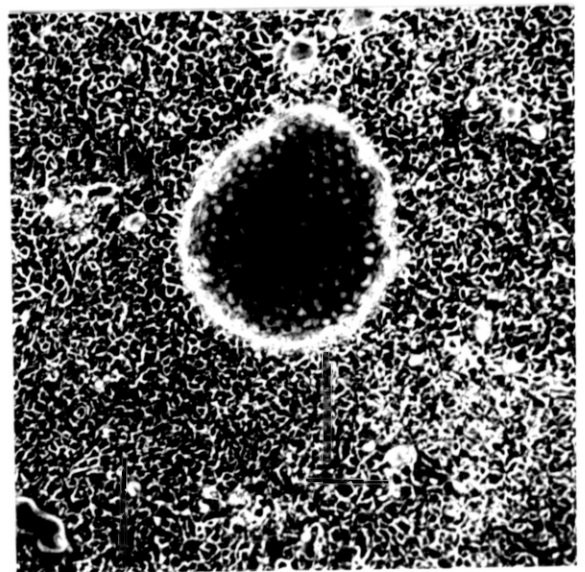
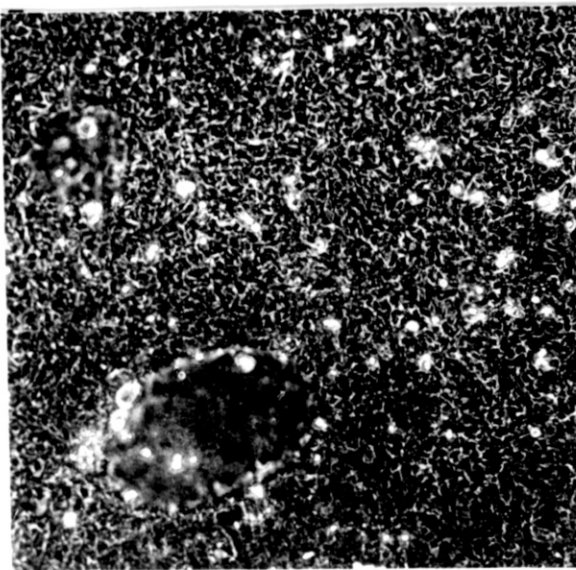
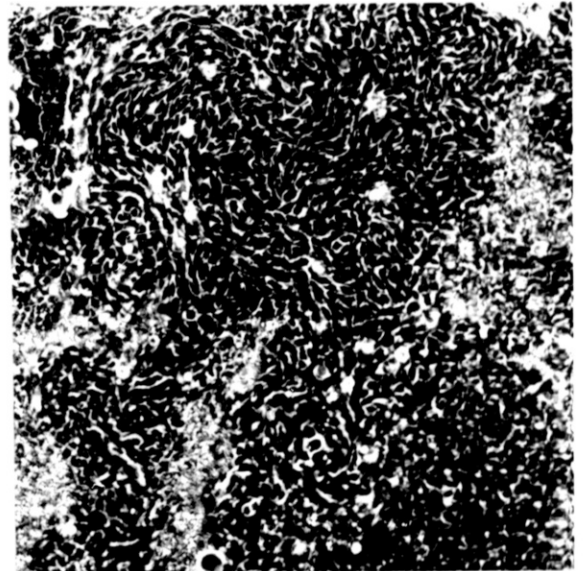
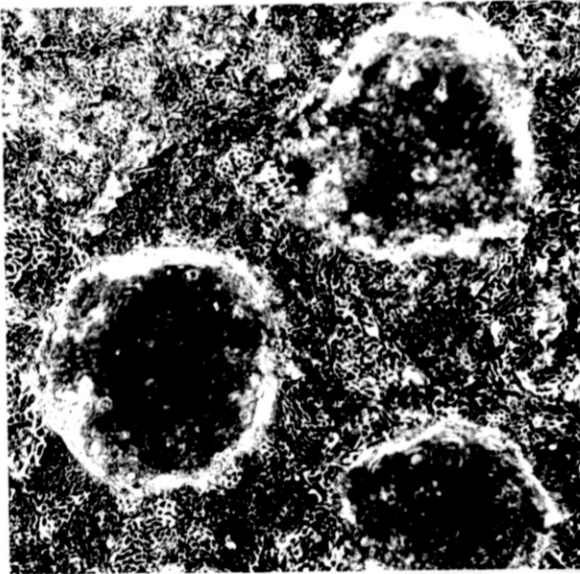
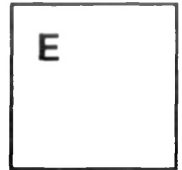
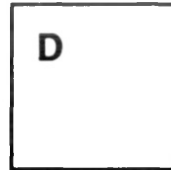
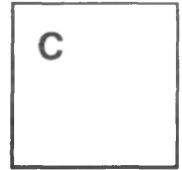
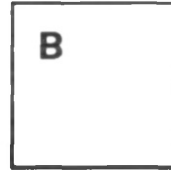
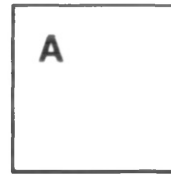
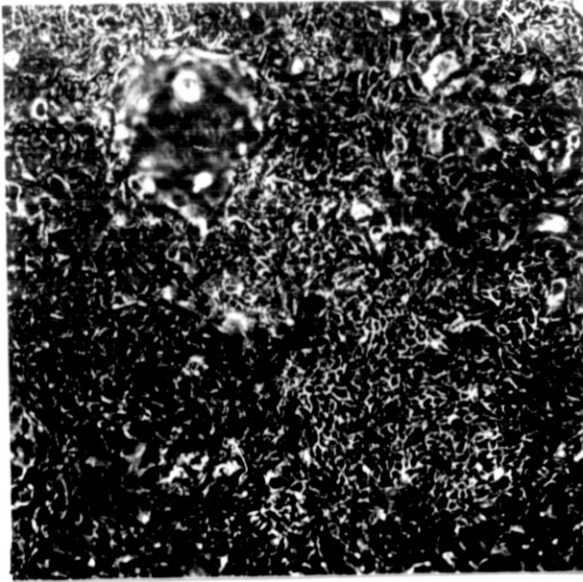


Fig. 10. Phase contrast micrographs (x 100) of monolayers of

A) Col 24 cells.

B) Col 26 cells.

C) Col 26 cells.

D) Col 27 cells.

E) Col 30 cells.

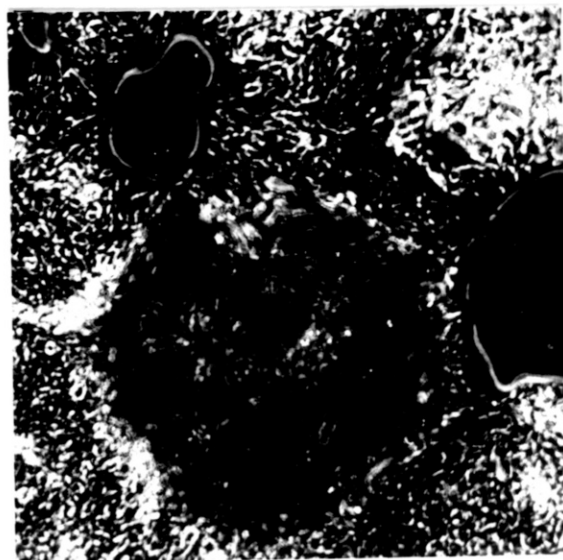
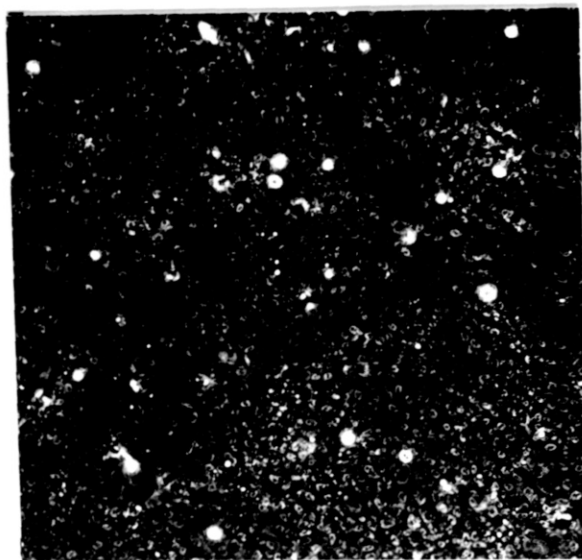
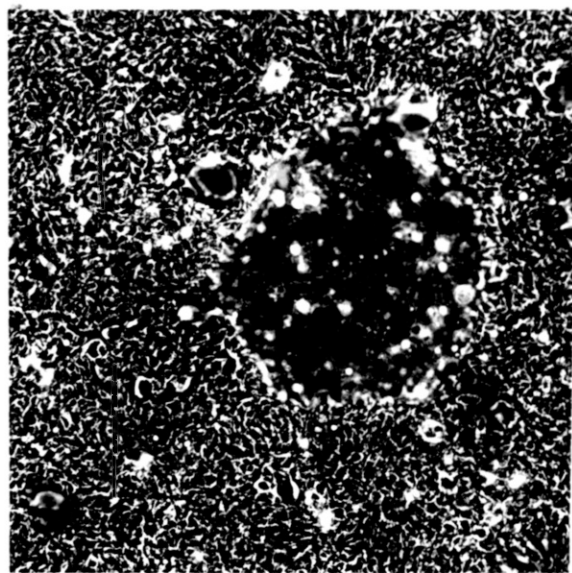
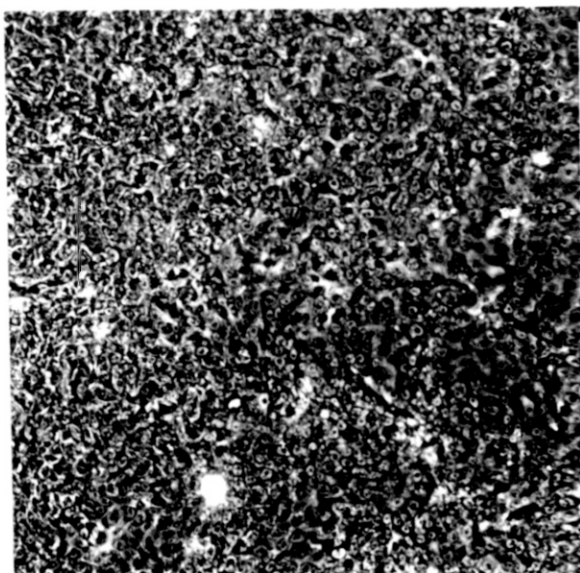
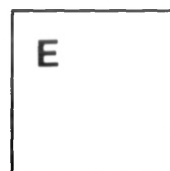
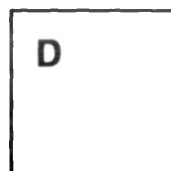
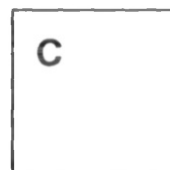
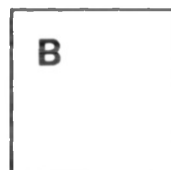
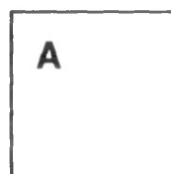
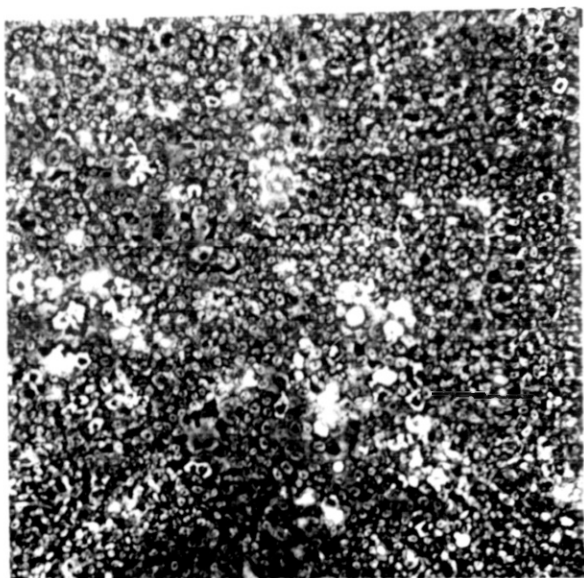


Fig. 11. Phase contrast micrographs of monolayers of

A) Col 26 (x 103)

B) Col 29 (x 93).

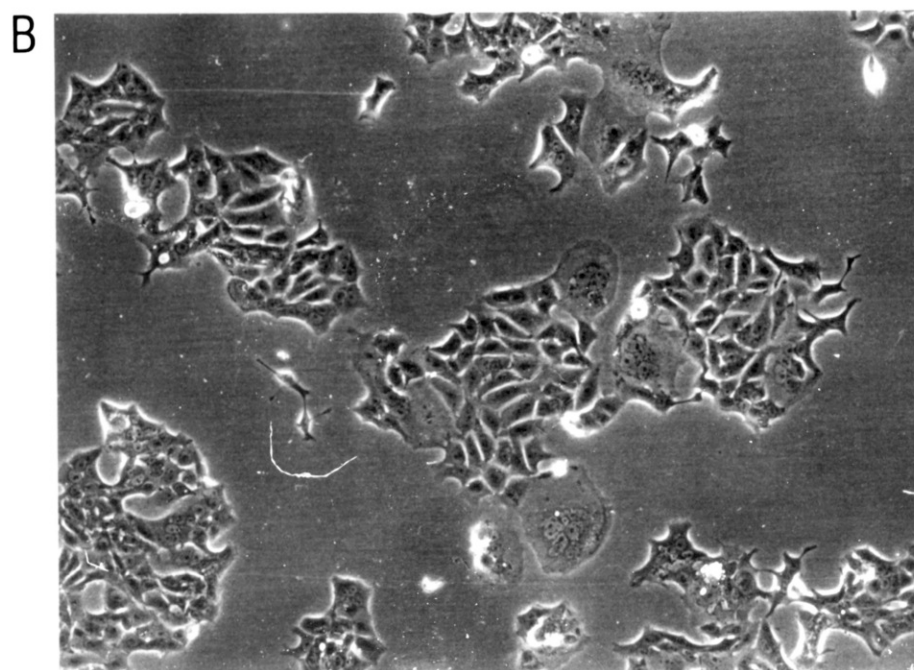
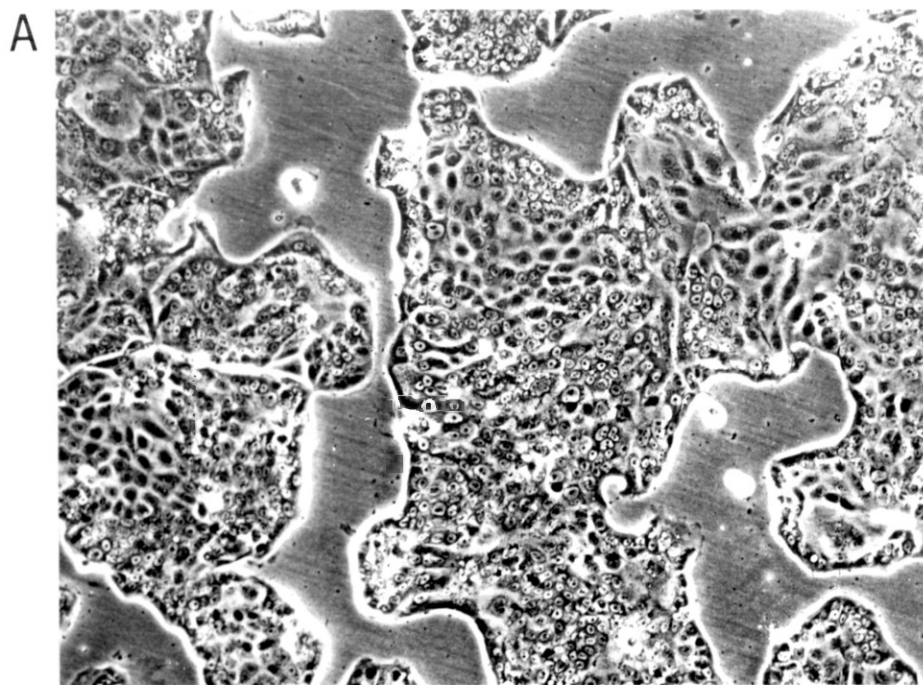


Fig. 12. Electron micrographs of monolayers demonstrating

A) morphological heterogeneity of HCA-7 cells (x 1,650).

B) well organised microvilli situated on the apical surface of HCA-7 cells (x 15,000).

C) High magnification of microvilli on HCA-7 cells (x 90,130).

D) tight junctions and desmosomes towards the apical surface of HCA-7 cells (x 28,000).

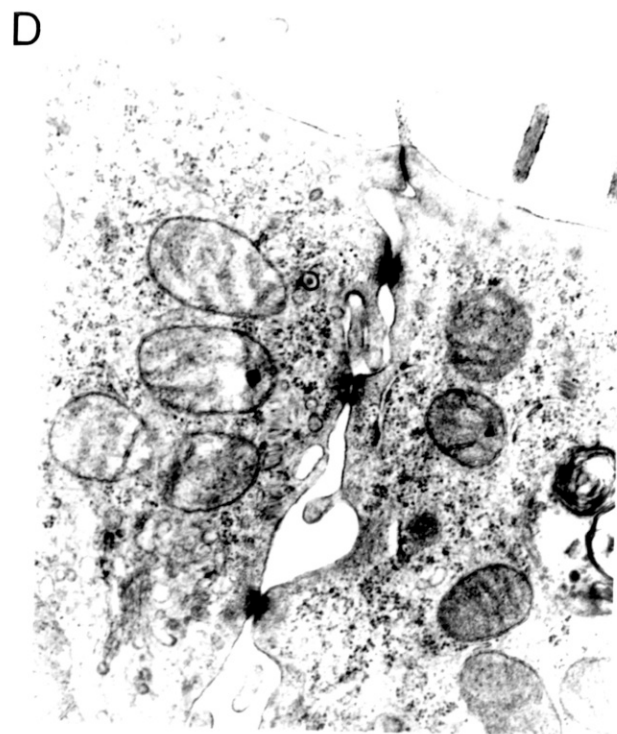
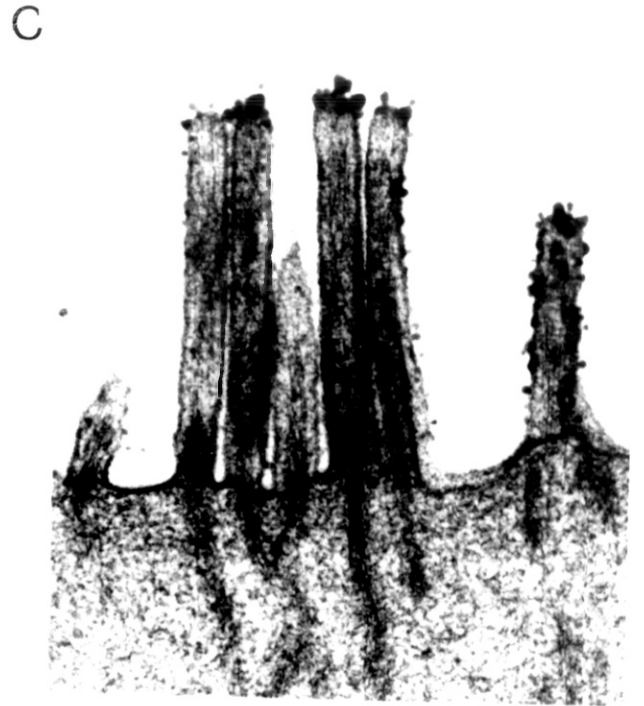
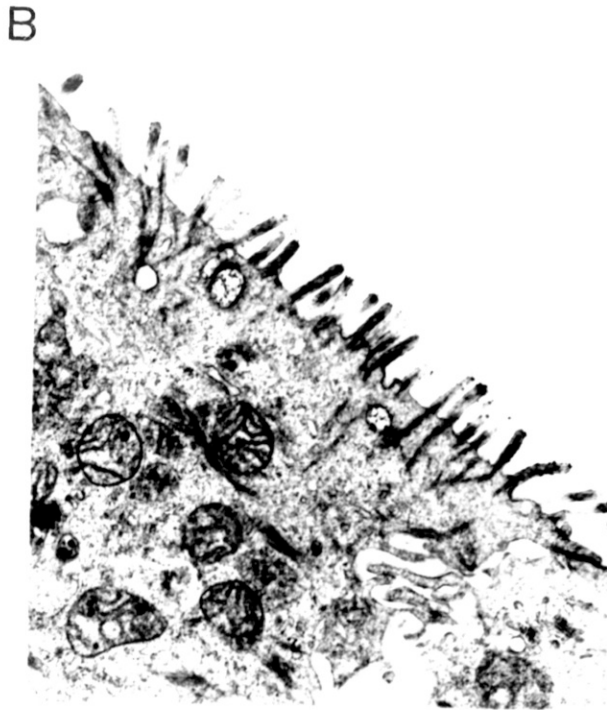
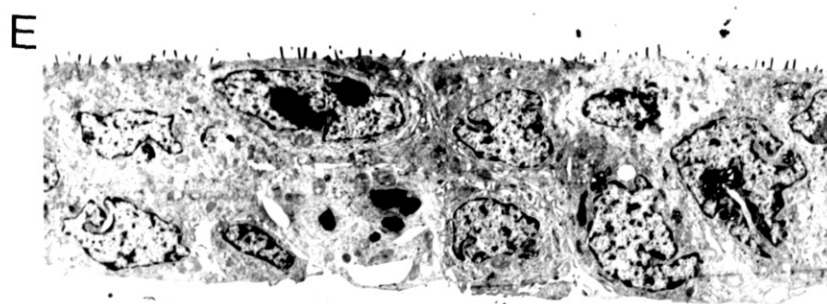
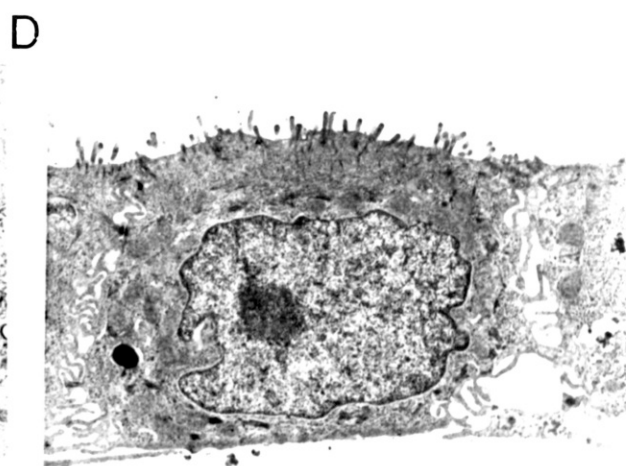
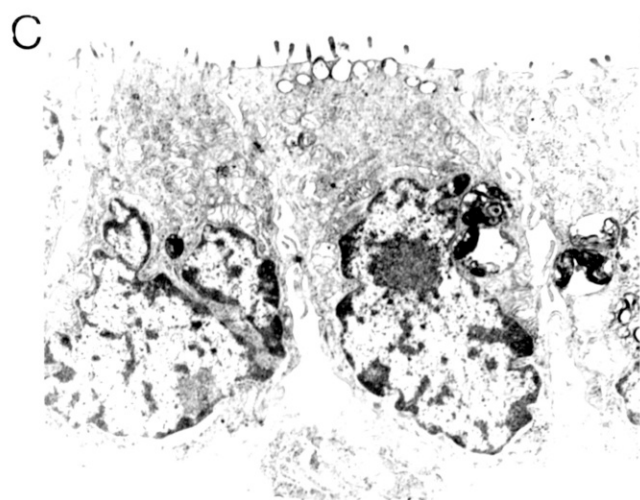
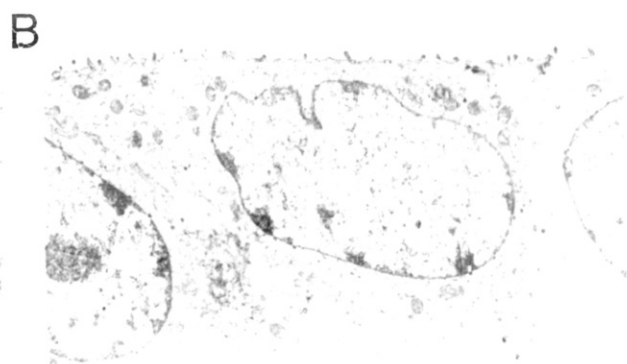
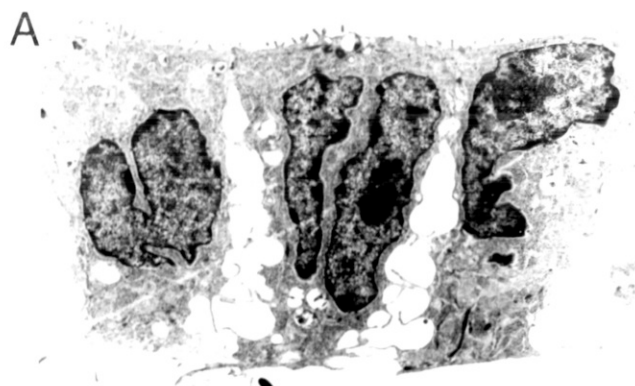


Fig. 13. Electron micrographs of monolayers demonstrating

- A) large intercellular spaces between col 27 cells (x 5,850).**
- B) compact monolayer of col 26 cells (x 4,860).**
- C) well differentiated, polarised col 1 but cells (x 3,900).**
- D) non-polarised col 30 cells (x 5,850).**
- E) cellular piling of col 1 but cells (x 2,580).**



example the cells of col 1 but were more polarised with respect to the position of the nucleus (Fig. 13c) compared to those of col 30 (Fig 13d), although in both cases microvilli were present on the apical surface of the cells only.

Interdigitations were also observed between many of the cells. These variations between the cell lines are summarised in Table 4.

Col 1 but displayed cellular piling in certain areas of the monolayer (Fig 13e) which was a feature not exhibited by any of the other cell lines.

Cell Type	Microvilli	Polarity	Cell shape	Intercellular spaces
HCA-7	++	+	Mixed	+
Col 1 but	++	+++	Columnar	++
Col 3	+	+	Cuboidal	+
Col 6	+	-	Cuboidal	+
Col 11	++	+	Columnar	+++
Col 24	+/-	-	Cuboidal	-
Col 26	+/-	-	Cuboidal	-
Col 27	++	++	Columnar	+++
Col 29	+/-	+	Cuboidal	-
Col 30	++	+	Cuboidal	++

Table 4. Summary of the ultrastructural morphologies of the HCA-7 cell line and subpopulations.
- to +++ indicates the relative occurrence of each feature.

In Vitro Growth.

Growth of the parent line and subpopulations was assessed in a single experiment by DNA quantification. As can be seen from Fig 14, all the cell lines began the experiment with the same amount of DNA in each well and appeared to grow at different rates during the time course. In order to evaluate the complex and varied individual growth rates of each cell line, growth rates were calculated for each section of the individual growth curves between two consecutive time points. Table 5 shows the results of these calculations where growth rate is expressed as the increase in DNA concentration per hour between days 2 and 6, days 6 and 10, days 10 and 14 and days 14 and 18.

These results clearly demonstrate that the growth rates of the parent line and subpopulations are very different from each other in each stage of their growth curves. For example, between day 2 and 6 the HCA-7 parent line had a lower growth rate than its derivative subpopulations. However, between days 10 and 14 the same line had the second fastest growth rate. Not only are the growth rates of each cell line different at each stage but they also demonstrate different patterns in their growth rates where some lines increase their growth rates in a certain time period where another line is slowing down.

The growth curves in Fig 14 indicate that the saturation densities of the cells would be somewhat different from each other when all had reached their stationary growth phase. Fig. 14 and Table 5 also indicate that the lag phase occurring in the growth rates, before exponential growth is attained, is different for each of the parent line and subpopulations.

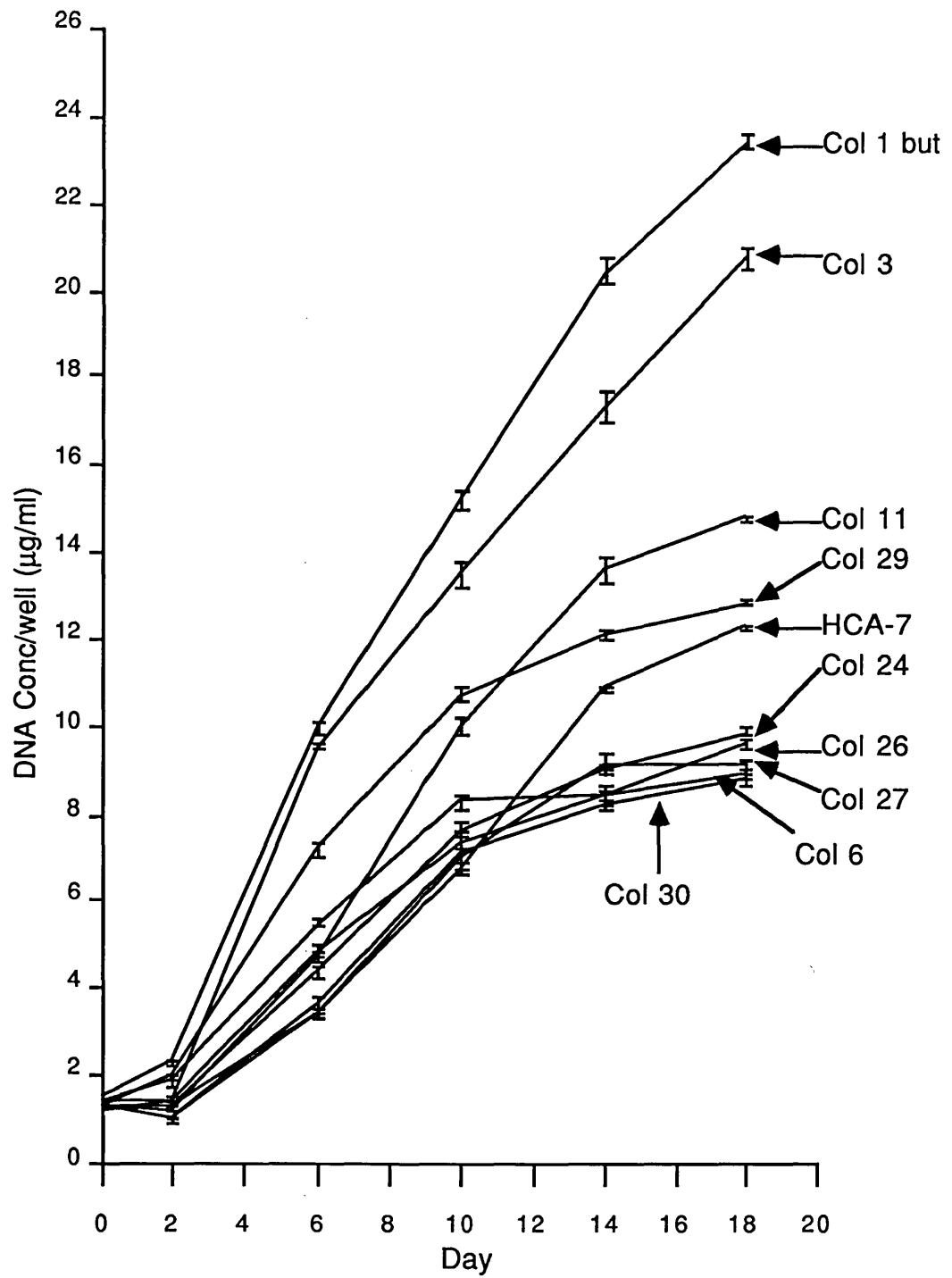


Fig. 14. Growth curves of the HCA-7 cell line and subpopulations. Growth was assessed by assaying DNA content per well for each cell line. Points, means : Bars, S.E. (n=6).

	Growth rate (ng DNA/ml/hour) Day			
Cell Type	2 - 6	6 - 10	10 - 14	14 - 18
HCA-7	22	35	43	15
Col 1 but	80	54	55	31
Col 3	84	41	40	36
Col 6	38	29	2	4
Col 11	37	55	38	12
Col 24	32	34	15	9
Col 26	37	26	11	13
Col 27	25	37	23	0
Col 29	55	37	14	8
Col 30	27	36	11	7

Table 5. Growth rates, of the HCA-7 cell line and subpopulations, calculated from the concentrations of DNA present at each time point of the individual growth curves shown in Fig. 14. Rates are expressed as the increase in DNA concentration per hour over the time period indicated.

Anchorage-Independence.

HCA-7 cells were found to grow independently of anchorage in semi-solid medium, forming organised structures (Figures 15a and b).

Col 3 formed branching structures in soft agarose (Fig 15c). Col 1 but and col 30 also formed organised structures in soft agarose (Fig 16a). Col 24 appeared to demonstrate large lumen within their structures when grown in semi solid medium (Figures 16 b and c). Col 6 and col 11 showed no growth at all and cells soon died. The remaining colonies showed very little growth in an disorganised manner.

Structures formed in suspension were similar to those formed in agarose in the majority of cases. HCA-7 cells grew in suspension forming well organised structures (Fig. 17a). Col 3 and col 30 formed large branching structures (Fig. 17b) with well defined cellular junctions (Fig. 17c). Col 29 and col 1 but formed small spherical structures (Fig 18a). It appeared that col 24 was forming lumen within the structures formed in suspension similar to those observed in agarose gels (Figures 16b and c). The other colonies did not grow in suspension. Cells of col 3 and col 30 were found to settle and grow on the non tissue culture-treated plastic of the bacteriological plates. This growth was quite extensive with col 30 cells.

Fig. 17. Phase contrast micrographs of cells grown in bacteriological dishes

A) HCA-7 (x200).

B) Col 30 (x200).

C) Col 30 (x400).

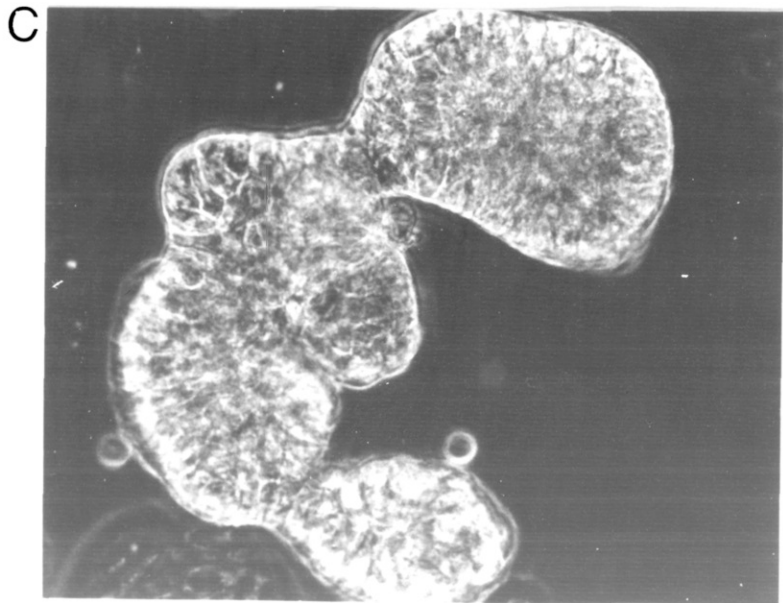
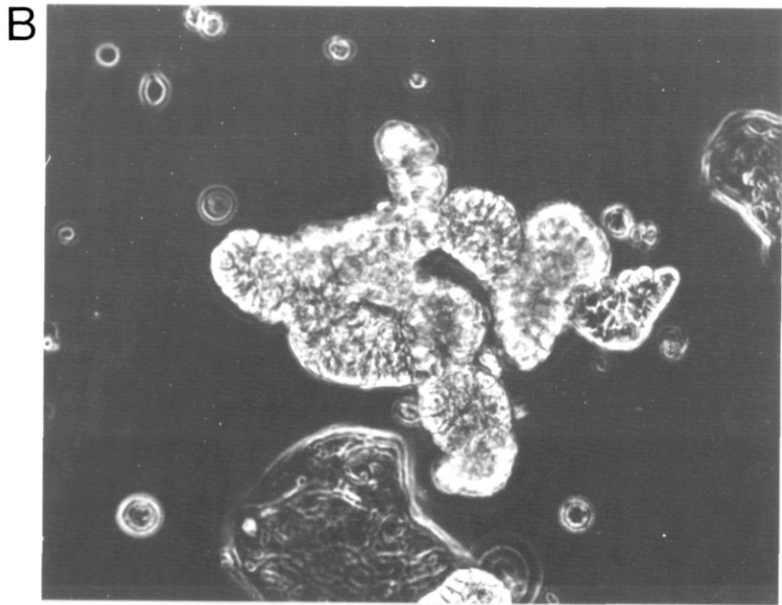
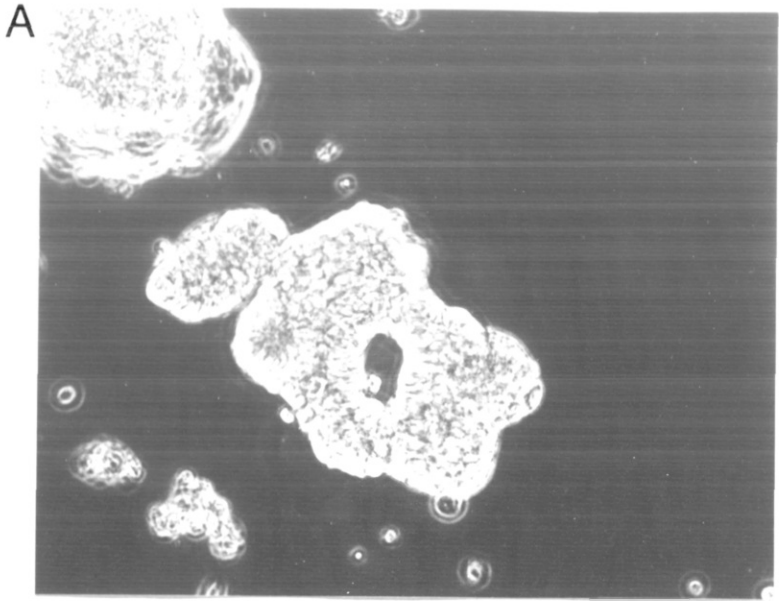


Fig. 18. Phase contrast micrographs of cells grown in bacteriological dishes

A) Col 1 but (x300).

B) Col 24 (x300).

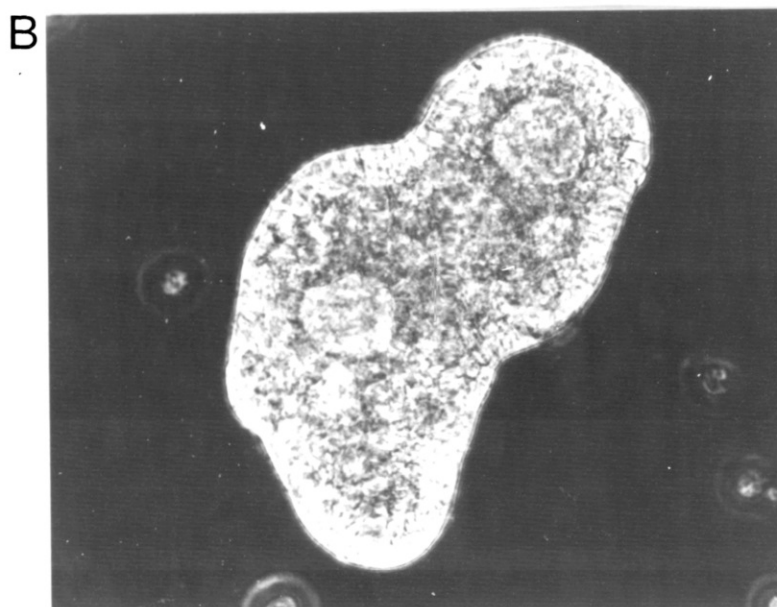
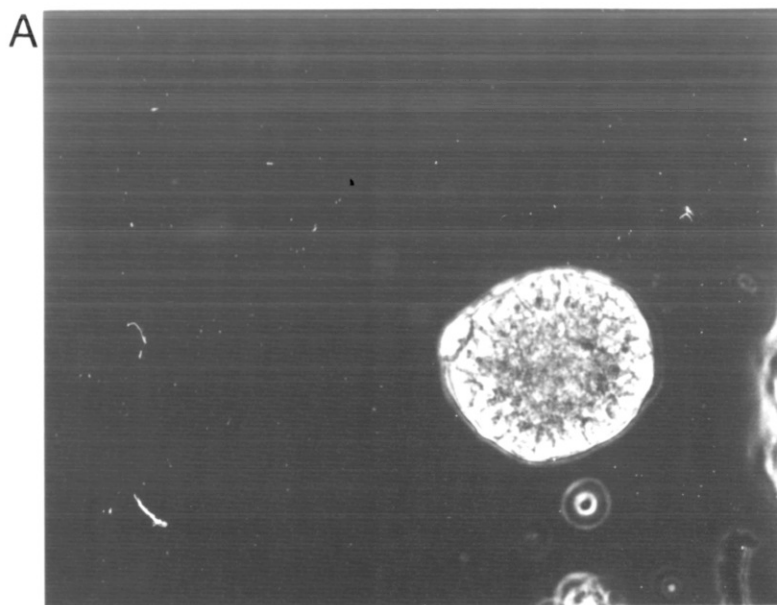


Fig. 15. Photomicrographs of cells grown in soft agarose, fixed in neutral buffered formalin and 4 μ m sections stained with haematoxylin and eosin.

A) HCA-7 (x247).

B) HCA-7 (x247).

C) Col 3 (x247).

A



B



C



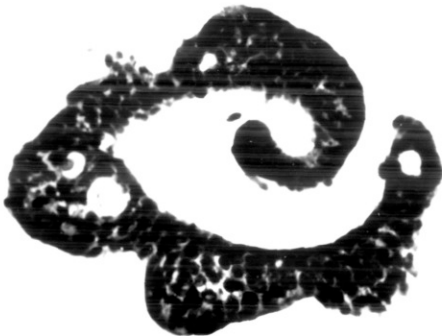
Fig. 16. Photomicrographs of cells grown in soft agarose, fixed in neutral buffered formalin and 4 μ m sections stained with haematoxylin and eosin.

A) Col 1 but (x247).

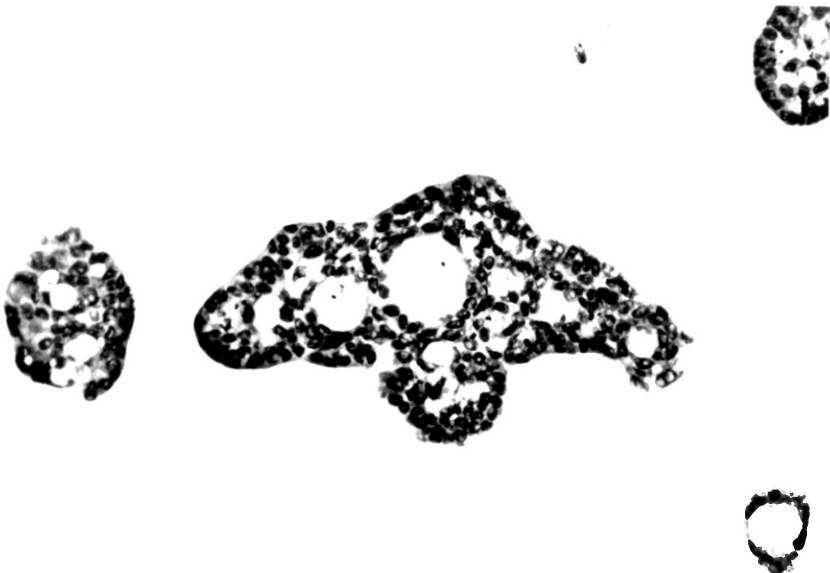
B) Col 24 (x247).

C) Col 24 (x247).

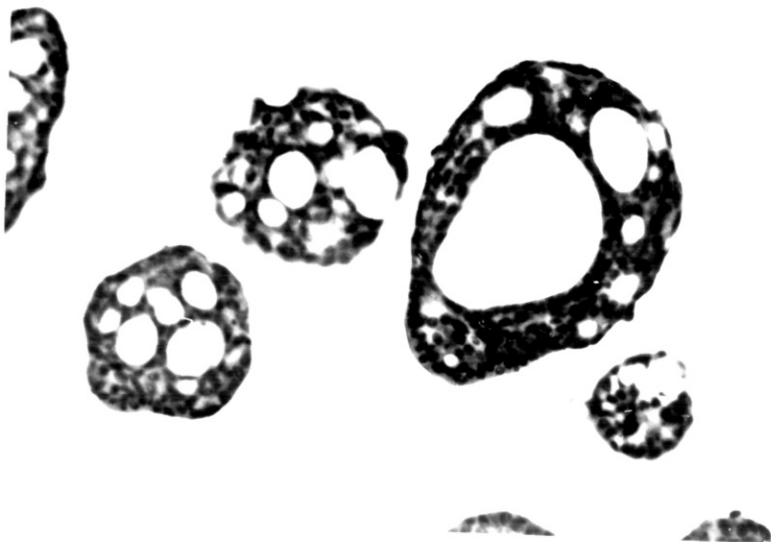
A



B



C



Transport Properties.

Dome quantification.

Monolayers of HCA-7, col 1 but, col 6, col 11, col 27 and col 30 all display dome formation at some stage in their growth. Differences were observed in the quantity and pattern of domes formed by these colonies (Fig. 19). The parent line showed a steady increase in the number of domes with respect to time, though few domes were observed before confluency. Col 6 and col 11 displayed no doming before confluency was reached and their final numbers of domes were somewhat less than that of HCA-7. Col 27 showed a general increase in dome number until shortly after confluency when all the domes disappeared leaving a flat monolayer with a pavement-like appearance. There was an increase in the number of domes formed by col 1 but until day 12 when the number then decreased due to the domes merging together. Col 30 had by far the greatest ability for dome formation where domes were often seen in small colonies of approximately 50 cells.

Ussing Chamber.

Results displaying the transport properties of HCA-7, col 1 but and col 3 have previously been reported by Cuthbert et al (1987). This group have also carried out initial investigations into the transport properties of col 29.

In this study a set protocol was followed to assess the transport profiles of colonies 6, 11, 24, 26, 27 and 30.

Col 11 had a very unstable resting current and showed no response to any agent added either apically or basolaterally. The resting currents of the remaining subpopulations varied markedly from 0.6 μA (col 27) to 3.7 μA (col 24), compared to the parent line at 1.9 μA (Cuthbert et al, 1987). Also the transepithelial resistance of the monolayers was found to differ, col 26 having 5 times the resistance of col 24 cells (Table 6).

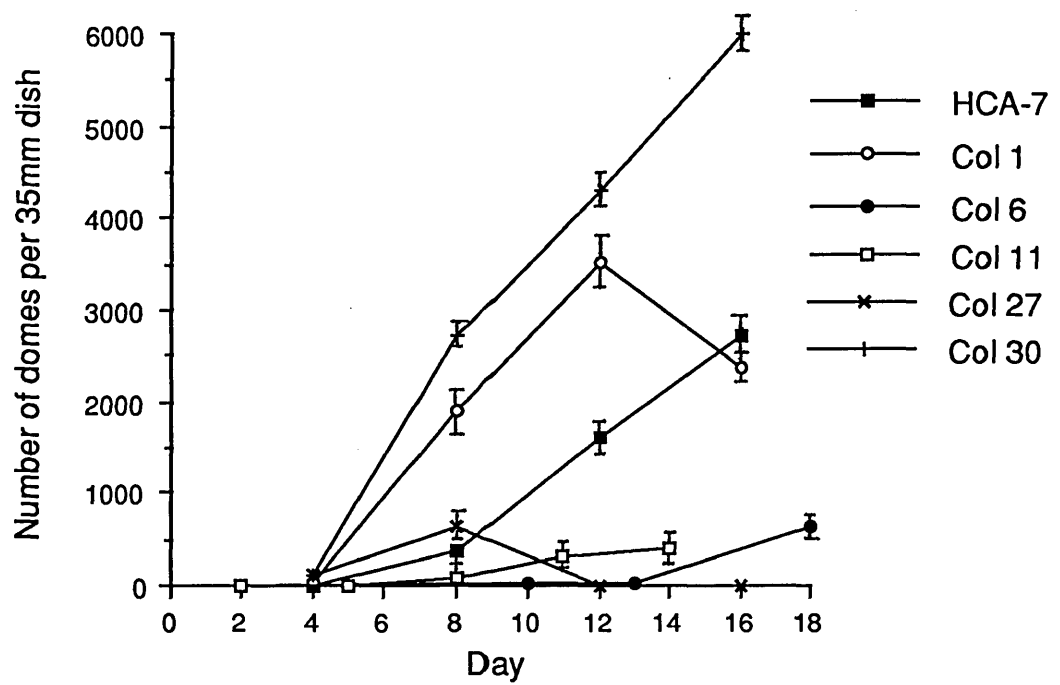


Fig. 19. Quantification of dome formation in monolayers of HCA-7 and selected subpopulations. The total number of domes per 35mm dish were counted for each cell line. Points, means ; bars, S.E. (n =3).

The resulting changes in short circuit current induced by the various agents was calculated as the increase in current above that of the resting current of each subpopulation. Agents were added to the KHS in the following order:- lysylbradykinin (LBK 100 nM apically), forskolin (10 μ M basolaterally), LBK (100 nM basolaterally), carbachol (10 μ M basolaterally), A23187 (1 μ M apically) and piretinide (200 μ M basolaterally). There was no washing of the monolayers between these applications.

Scanned images of typical traces obtained are shown in Figures 20 and 21. In general the pattern of responses obtained for each subpopulation tested was very similar though the induced increases of current varied. Col 6 (Fig. 22a), col 24 (Fig. 22b), col 26 (Fig. 22c) and col 27 (Fig 23a) all displayed an increase in SCC on addition of LBK to the apical side of the cells. Also they all produced increases in SCC, above the previous result, in response to basolaterally applied forskolin. When LBK was applied to the basolateral side a further increase in SCC occurred. Carbachol was also found to produce a further increase in the SCC when applied to the cells. All these increases in current were found to be significantly different from the previous response. Significance was considered at a level of probability less than 5% using the paired student 't' test. A decrease in SCC was observed when the calcium ionophore A23187 was applied to the basolateral side of col 24 and col 27, whereas an increase in SCC was noted in the cases of col 6 and col 26. However, only the responses to A23187, of col 24 and 26 were found to be significantly different from the carbachol response. Piretinide induced a large decrease in the SCC though in all 4 cases it did not completely abolish them. The overall responses to these agents by col 24 were much greater than those of the other 3 colonies.

Col 30 (Fig 23b) showed a marked difference in the responses to the added agents compared to the other colonies. LBK failed to induce any change in the SCC when added to either the apical or basolateral reservoir. However there was a very large increase in the current on addition of forskolin which

Cell Type	Resting Current ($\mu\text{A}/\text{cm}^2$)	Transepithelial Resistance ($\mu\Omega/\text{cm}^2$)	Resting Potential Difference (mV)	*No.
Col 6	11.5 ± 5.5	43 ± 13	-0.4 ± 0.2	6
Col 24	18.5 ± 6.5	31 ± 4	-0.3 ± 0.2	8
Col 26	5.0 ± 2.0	150 ± 29	-0.5 ± 0.3	4
Col 27	3.0 ± 1.0	109 ± 24	-0.2 ± 0.1	6
Col 30	7.5 ± 2.5	75 ± 5	-0.7 ± 0.3	6

Table 6. Electrical parameters of the HCA-7 cell line and subpopulations taken at rest in the Ussing chamber system.. * No. = number of experiments. Values given are means $\pm$ standard error.

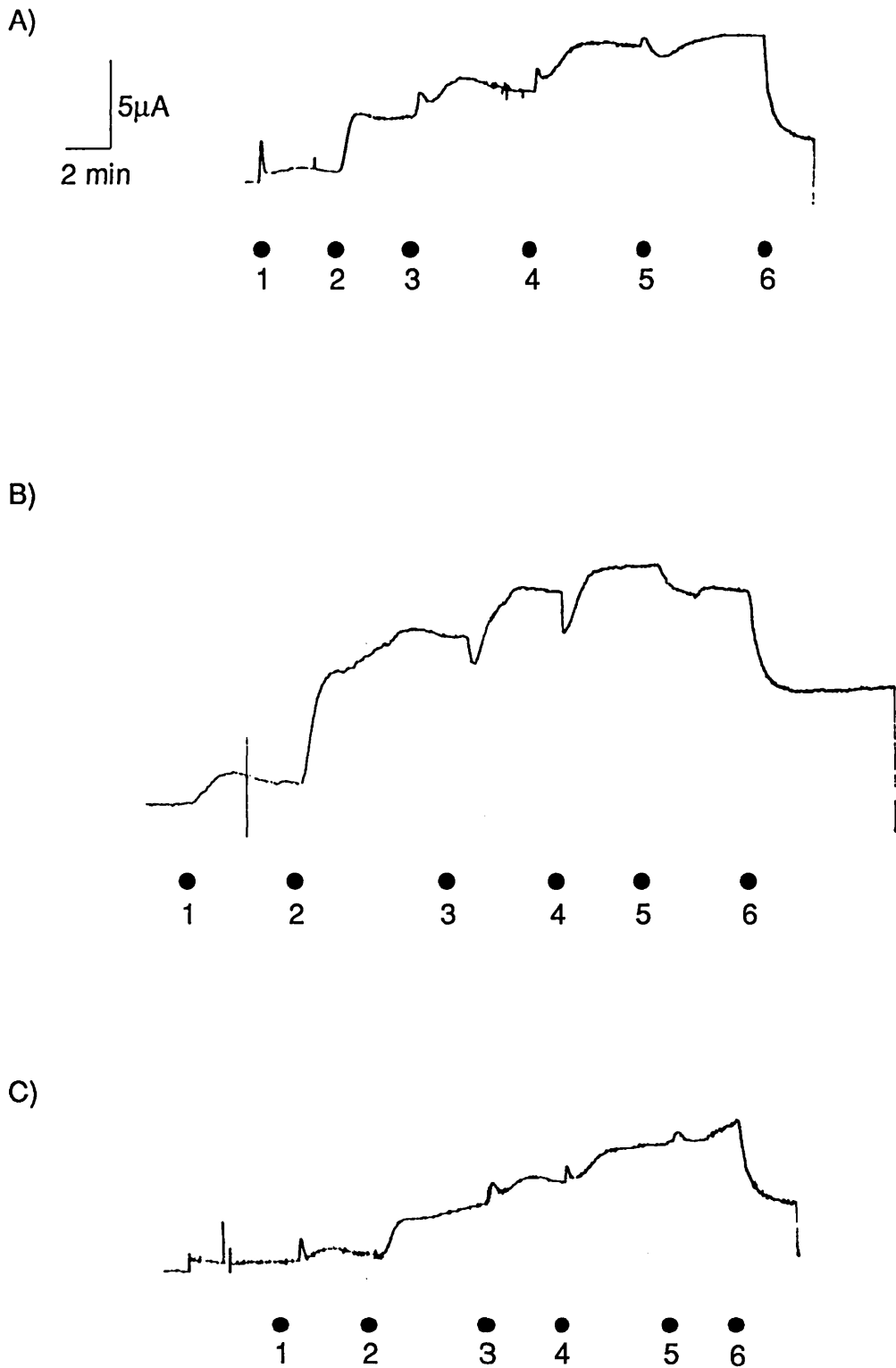


Fig. 20. Image scanned sample traces from Ussing Chamber experiments on A) Col 6, B) Col 24 and C) Col 26. Agents added as indicated:- 1 =Ly-sylbradykinin (100nM, apically); 2 = Forskolin (10 μ M, basolaterally); 3 = Lysyl bradykinin (100nM, basolaterally); 4 = Carbachol (10 μ M, basolaterally); 5 = A23187 (1 μ M, apically); 6 = Piretinide (200 μ M, basolaterally). Scale shown applies to all traces.

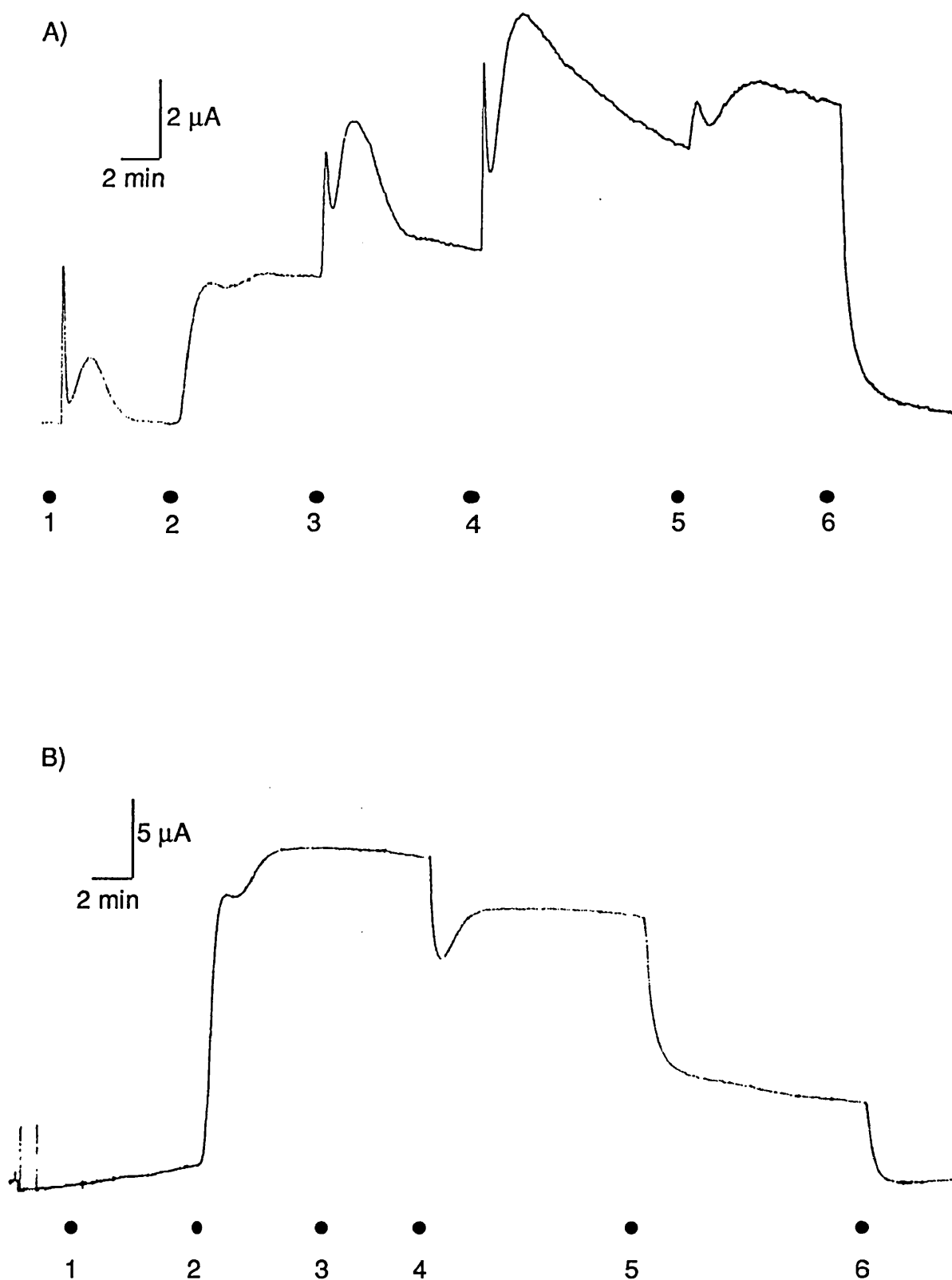
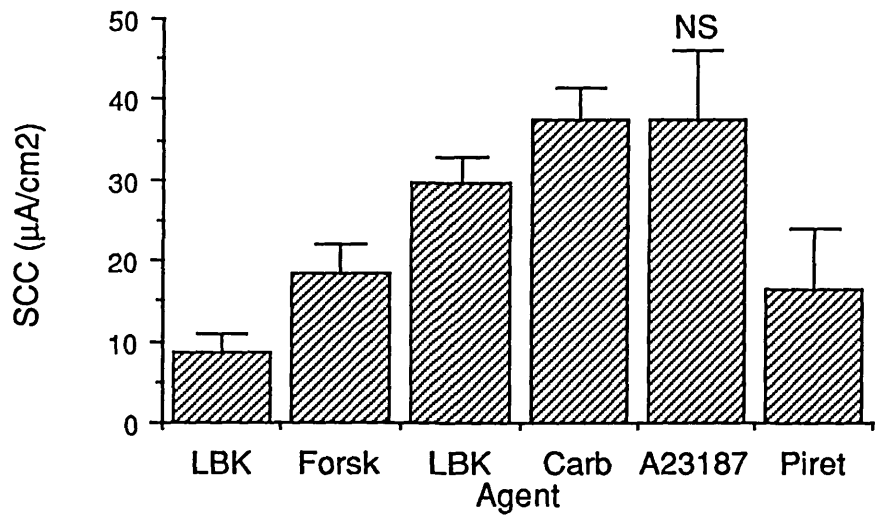


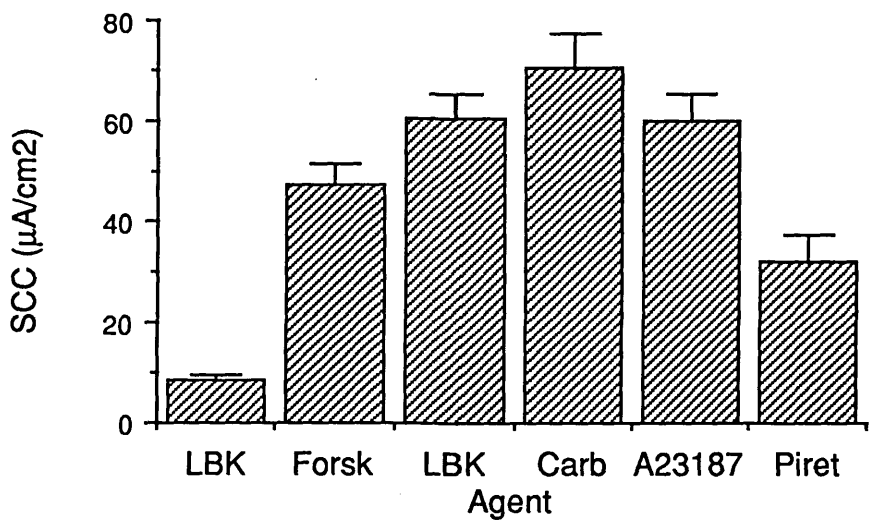
Fig. 21. Image scanned sample traces from Ussing Chamber experiments on A) Col 27 and B) Col 30. Agents added as indicated:- 1 =Lysylbradykinin (100nM, apically); 2 = Forskolin (10 μ M, basolaterally); 3 = Lysyl bradykinin (100nM, basolaterally); 4 = Carbachol (10 μ M, basolaterally); 5 = A23187 (1 μ M, apically); 6 = Piretinide (200 μ M, basolaterally).

was more than double that of any other cell line. The application of carbachol produced a slight but insignificant decrease in SCC. In contrast to the other subpopulations, A23187 produced a very large and significant decrease in the SCC of col 30 cells. Piretinide further reduced the SCC though again it was not completely abolished.

A) Col 6



B) Col 24



C) Col 26

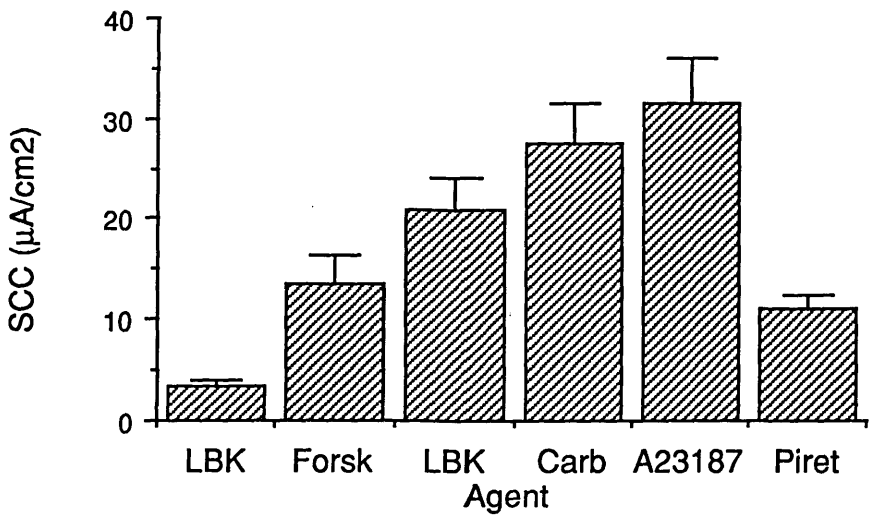
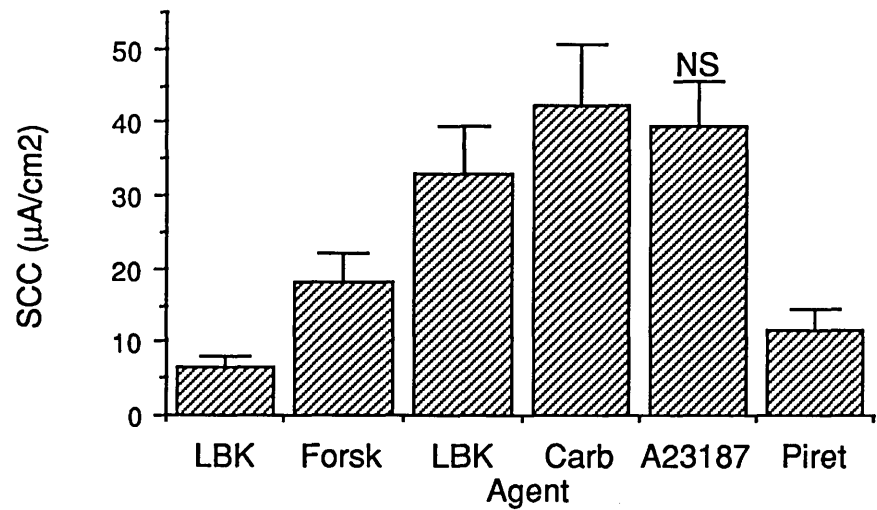


Fig. 22. Histograms showing the increases in short circuit current (SCC), above the resting currents, induced by lysylbradykinin (LBK; apical), forskolin (forsk; basolateral), LBK (basolateral), carbachol (carb; basolateral), A23187 (apical) and piretinide (piret; basolateral) in A) Col 6, B) Col 24 and C) Col 26. NS = not significantly different from previous column, $p > 0.05$.

A) Col 27



B) Col 30

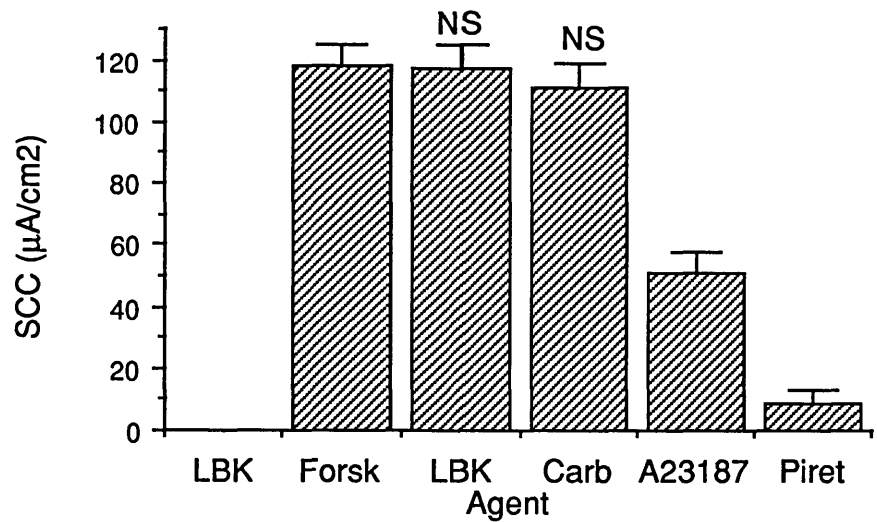


Fig. 23. Histograms showing the increases in short circuit current (SCC), above the resting current, induced by lysylbradykinin (LBK; apical), forskolin (forsk; basolateral), LBK (basolateral), carbachol (carb; basolateral), A23187 (apical) and piretinide (piret; basolateral) in A) Col 27 and B) Col 30. NS = not significantly different from previous column, $p > 0.05$.

Immunocytochemistry.

Qualitative.

The antigen recognised by the antibody AUA1 was found to be uniformly expressed in the parent line and all subpopulations (Fig. 24a). Sucrase isomaltase was also expressed by all the cell lines however the staining was patchy and uneven in all cases (Fig. 24b). The parent line and subpopulations were found to express the CEA antigen to similar extents (Fig. 24c), though col 3 did appear to express more of this antigen than the other cell lines.

All the cell lines demonstrated spontaneous expression of the HLA-DR molecule but this expression varied between the lines. Col 24 displayed the greatest amount of positive staining (Fig 25) whereas col 30 showed very little. Positively stained cells of col 3 and col 29 appeared in sparse patches of approximately 20-30 cells (Fig. 26). However only single cells of colonies 6, 11 and 27 were found to express HLA-DR with the majority of the monolayer being negative (Fig. 27). Approximately 5-10% of cells of the parent line, col 1 but and col 26 were seen to express this antigen, where positively staining cells were scattered evenly over the monolayer (Fig 28).

Only cells of HCA-7, col 1 but, col 24 and col 30 appeared to express the antigen detected by antibody 1A3 (Fig. 29). Weak staining was observed on HCA-7 cells and col 26 cells with the 4D4 antibody. The pattern of staining of these two cells lines was markedly different. Positive staining of HCA-7 cells appeared as small globules situated in the cytoplasm of the cells (Fig. 30a). However, the 4D4 antibody reacted to an antigenic determinant situated at the edge of the col 26 cells (Fig. 30b).

Strong positive staining was seen in the monolayers of HCA-7, col 1 but, col 24 and col 26 on application of the 5D5 antibody. In this case, staining evenly distributed throughout the cytoplasm of the cell (Fig. 31).

Quantitative.

Fig. 24. Representative immunoperoxidase staining of monolayers of

A) Col 3 cells with AUA1 antibody (x560).

B) Col 29 cells with anti-sucrase isomaltase antibody (x280).

C) Col 24 cells with anti-carcinoembryonic antigen antibody (x280).

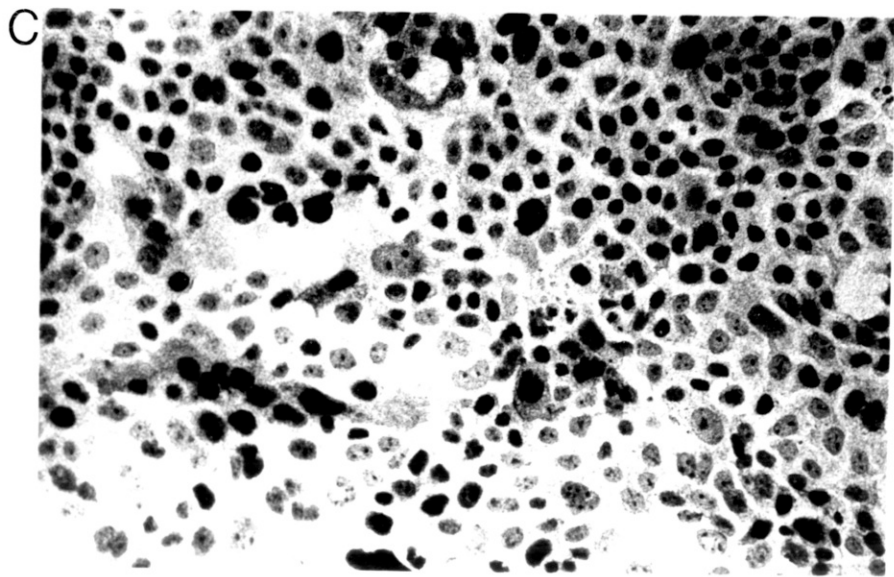
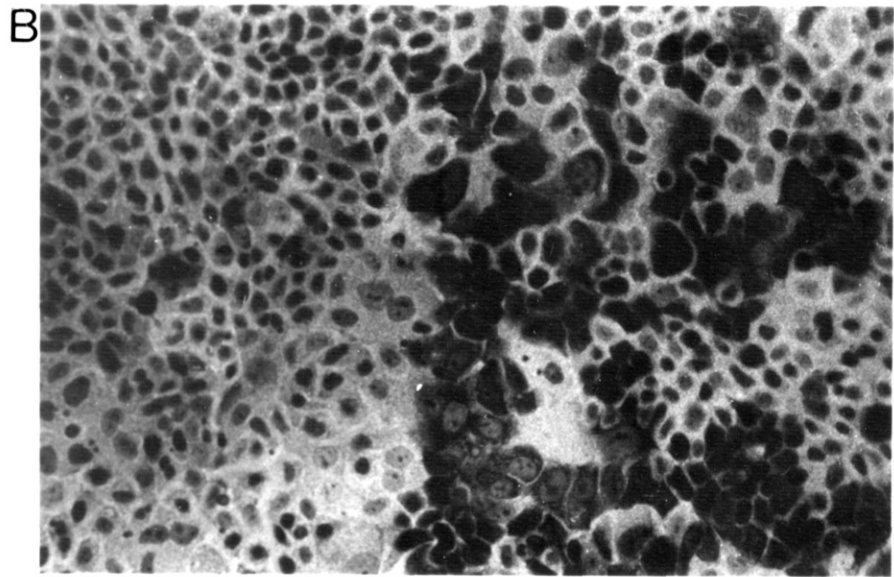
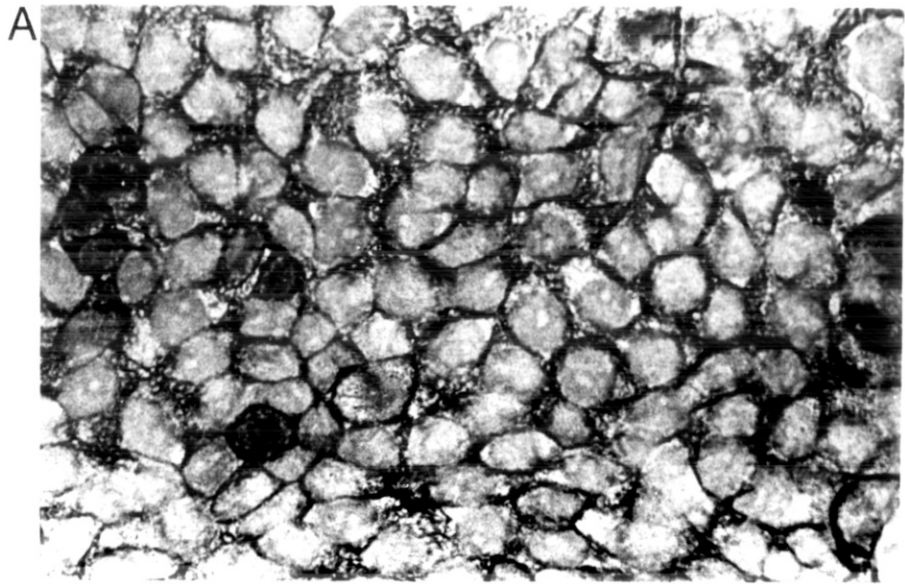


Fig. 25. Immunoperoxidase staining using the CR3/43 antibody on monolayers of

A) Col 24 cells (x140).

B) Col 24 cells (x560).

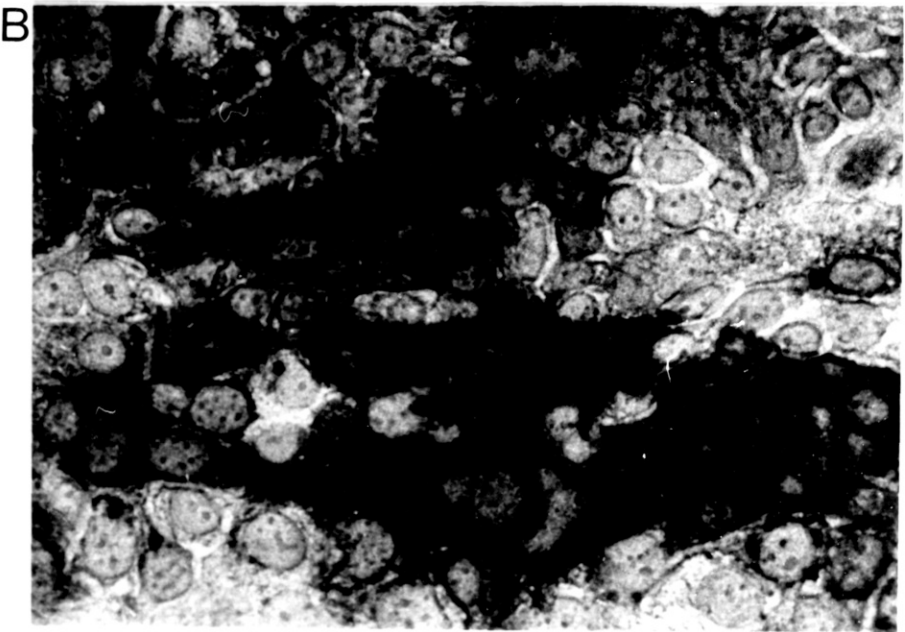
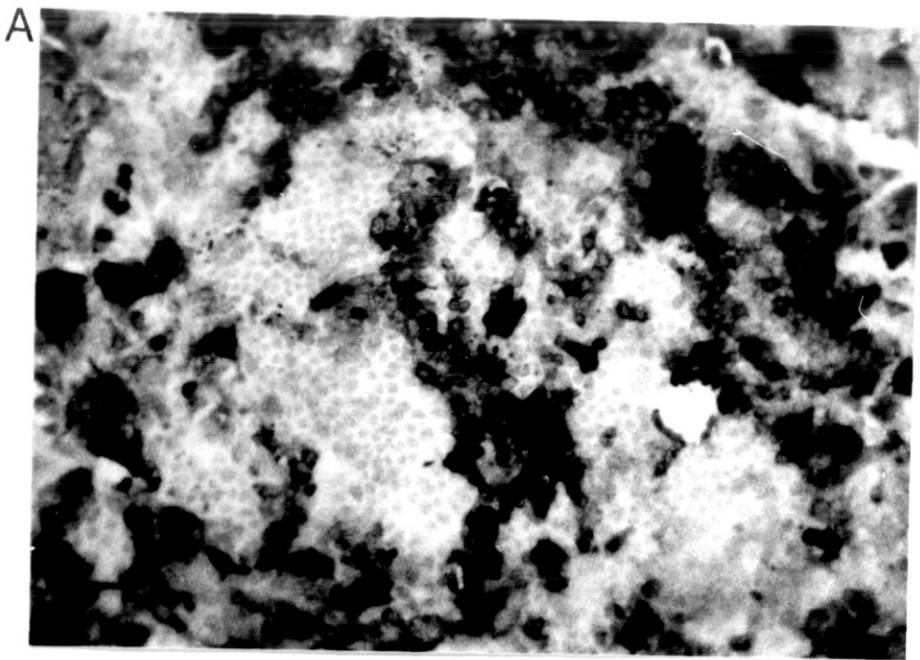


Fig. 26. Immunoperoxidase staining using the CR3/43 antibody on monolayers of

A) Col 29cells (x140).
B) Col 29 cells (x560).

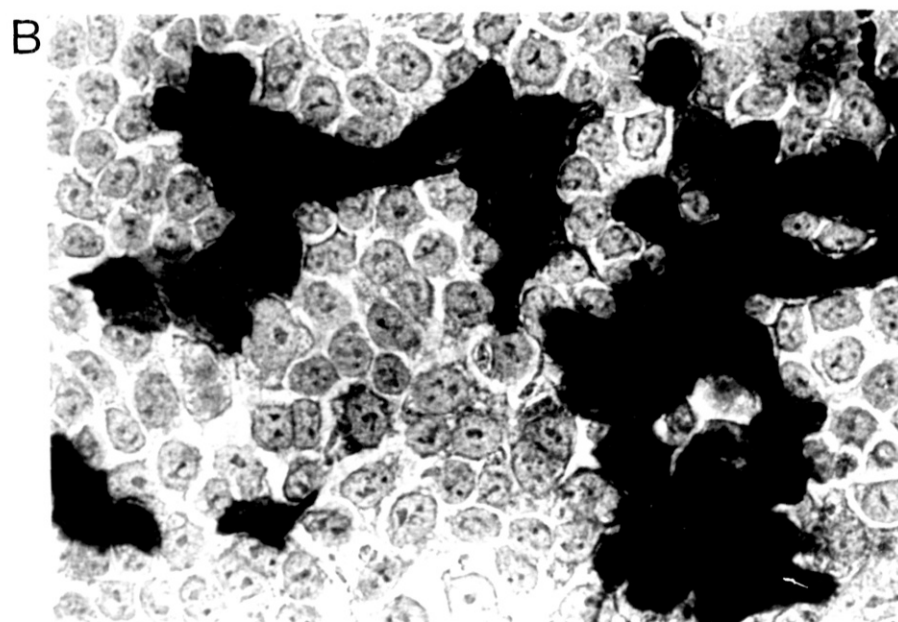
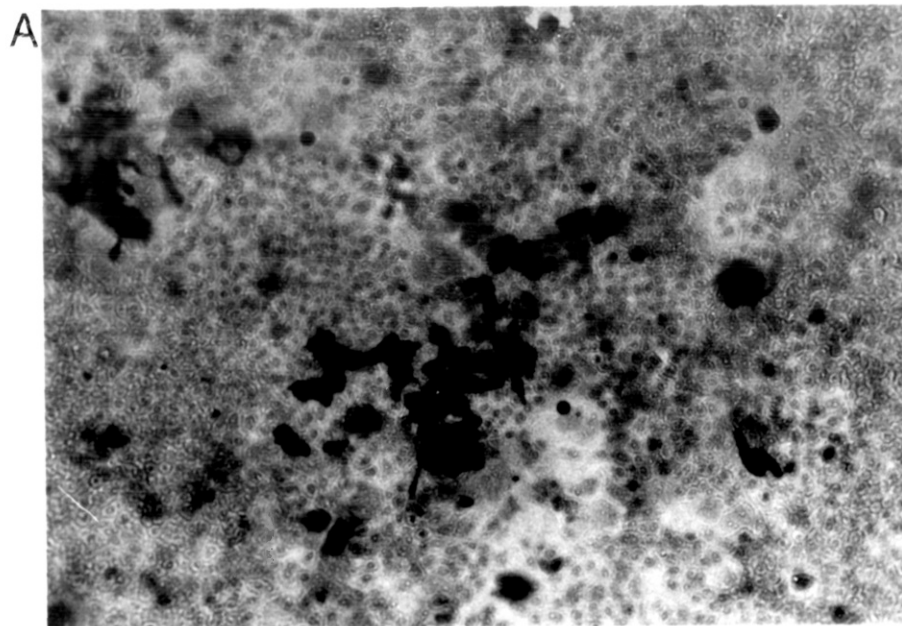
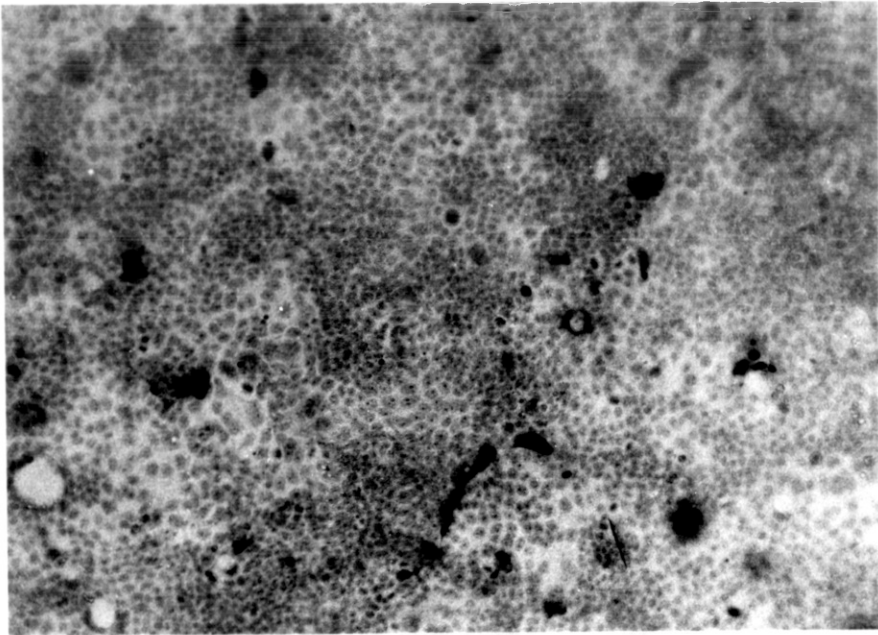


Fig. 27. Immunoperoxidase staining using the CR3/43 antibody on monolayers of

A) Col 11 cells (x140).
B) Col 11 cells (x560).

A



B

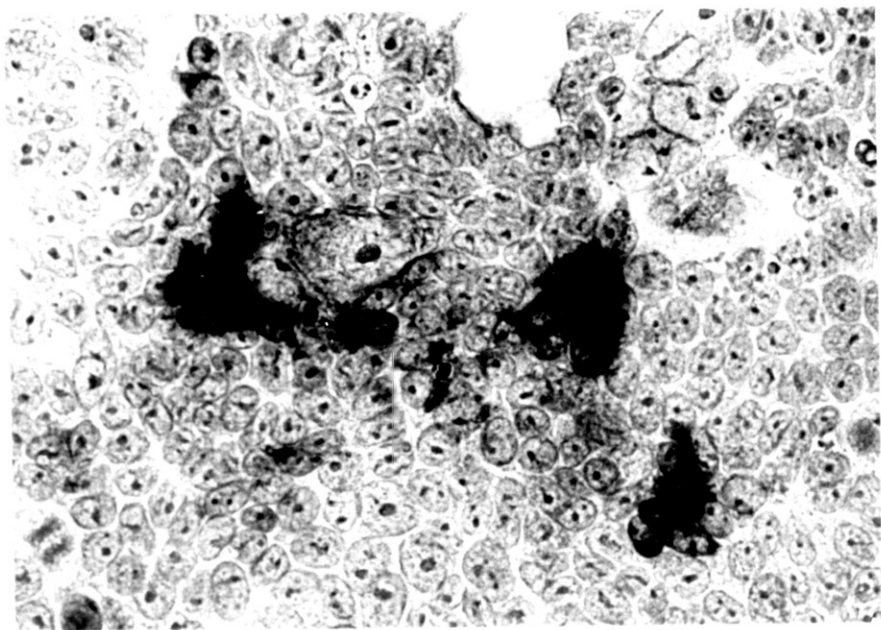


Fig. 28. Immunoperoxidase staining using the CR3/43 antibody on monolayers of

- A) HCA-7 cells (x140).
- B) Col 26 cells (x140).
- C) Col 26 cells (x560).

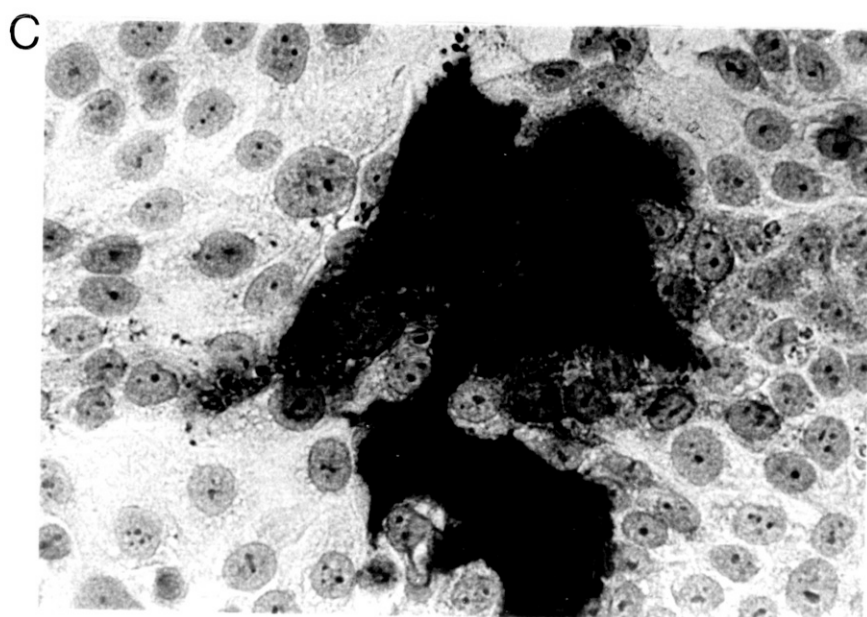
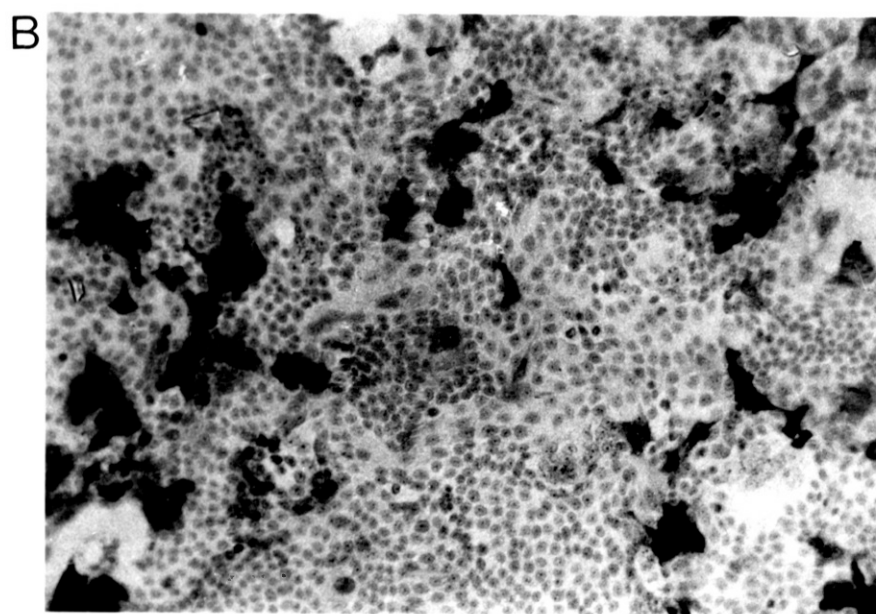
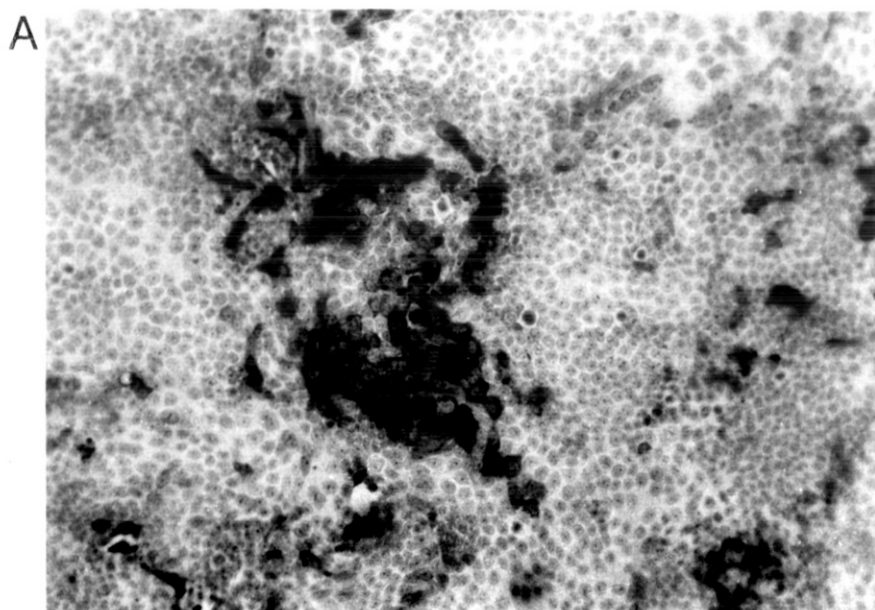


Fig. 29. Immunoperoxidase staining using the 1A3 antibody on monolayers
of
A) HCA-7 cells (x280).
B) Col 30 cells (x280).

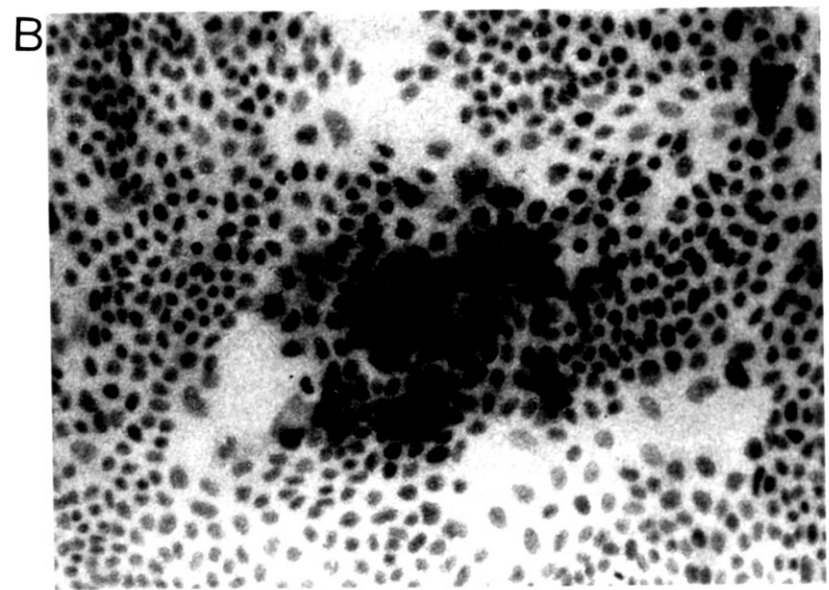
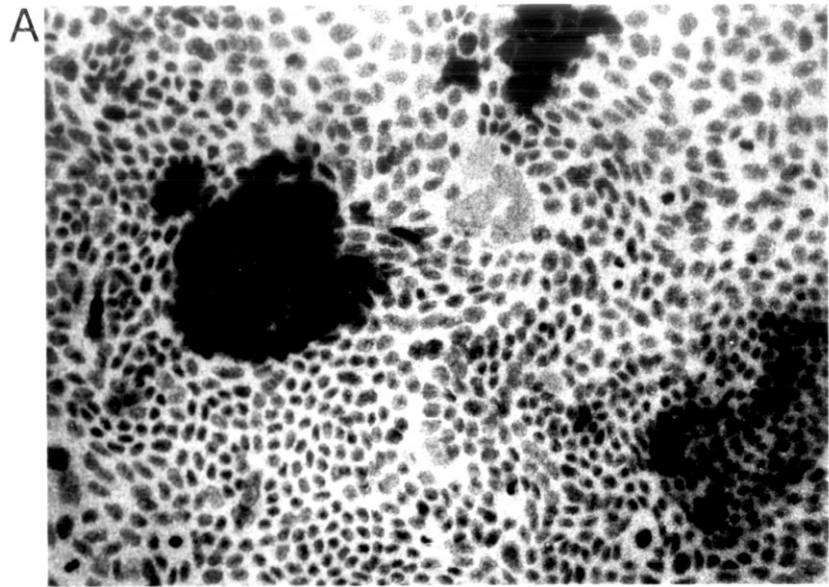


Fig. 30. Immunoperoxidase staining using the 4D4 antibody on monolayers of

A) HCA-7 cells (x880).

B) Col 26 cells (x880).

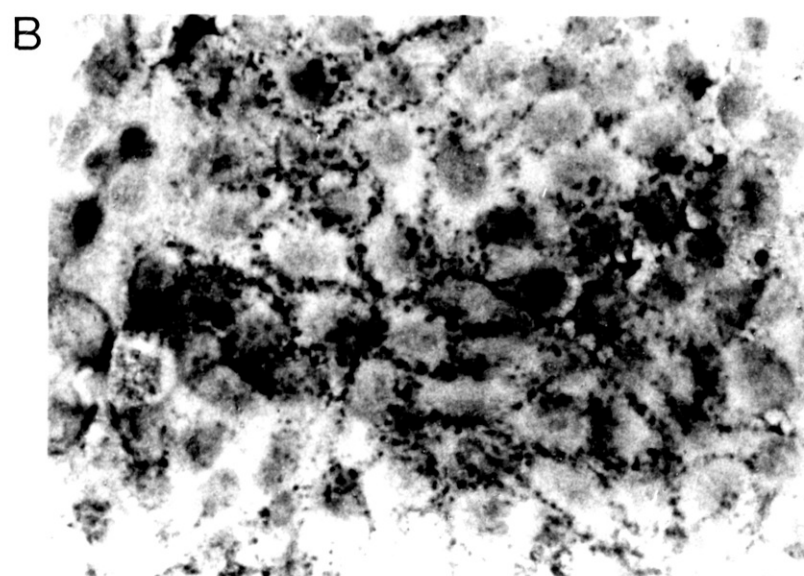
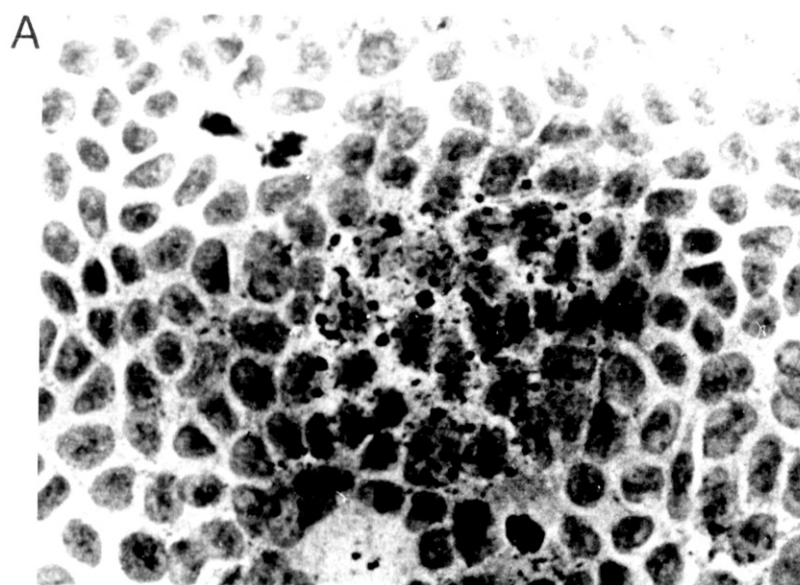
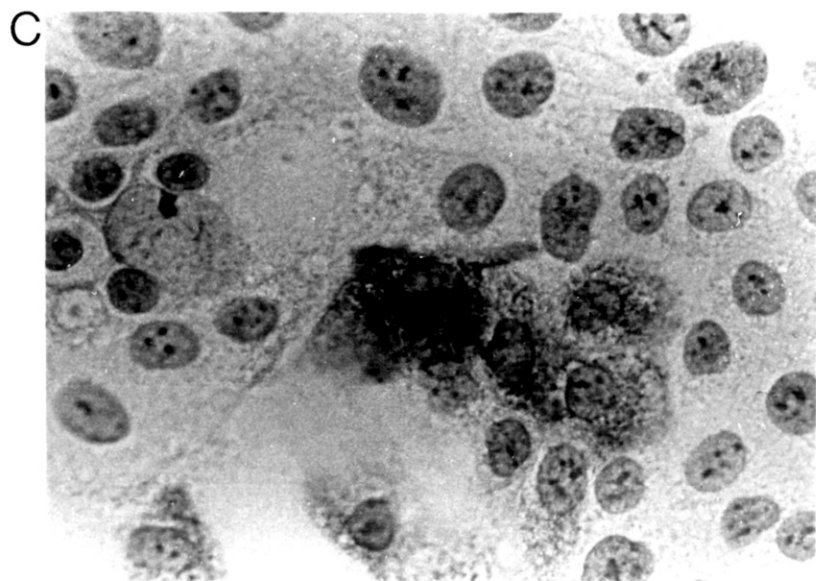
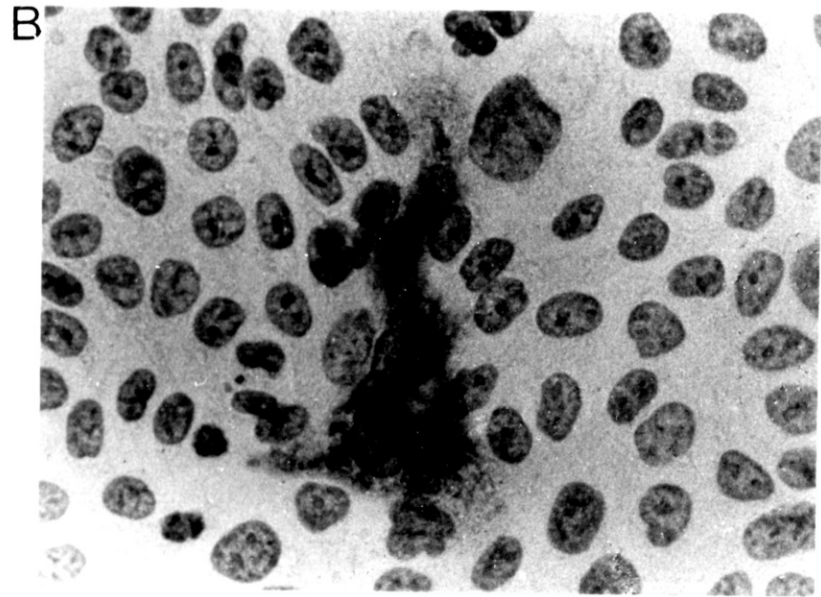
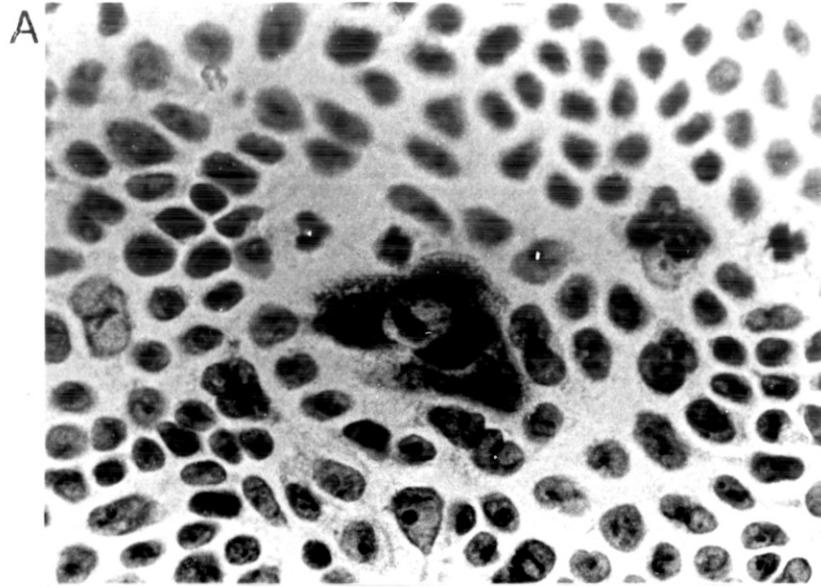


Fig. 31. Immunoperoxidase staining using the 5D5 antibody on monolayers of

- A) HCA-7 cells (x840).
- B) Col 26 cells (x840).
- C) Col 24 cells (x840).



The quantitative analysis of the expression of the various antigens by the subpopulations correlated fairly closely to that observed using the indirect immunoperoxidase method. Histograms showing the amount of antibody bound to the cells, indicated by the number of counts per min / μg of DNA, are shown in Figures 32 and 33. The cpm / μg of DNA calculated for each set of control cells was deducted from the test values obtained for each antibody. In an attempt to evaluate the differences observed between the cell lines in the expression of the various antigens, statistical analyses were carried out. The expression of antigen by each subpopulation was compared to the expression of the same antigen by the parent line, using the unpaired student 't' test. Statistically significant differences were considered at a level of probability less than 5% ($p < 0.05$).

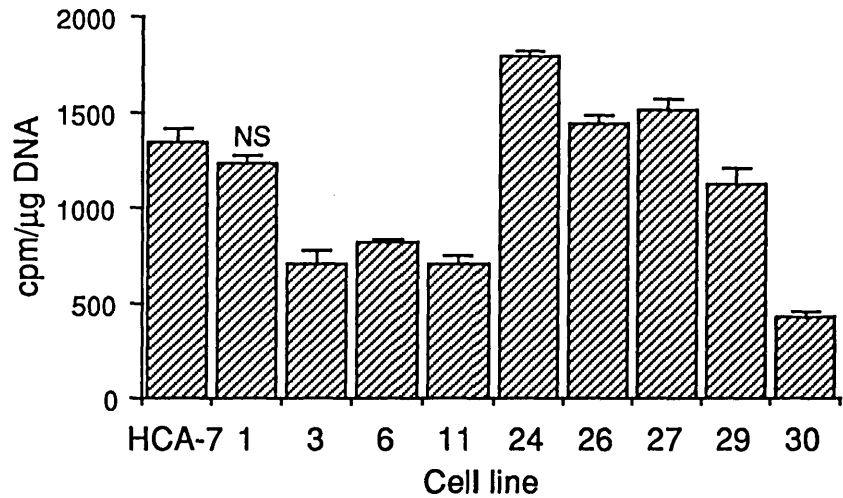
In general, the total number of counts bound was much higher in each cell lines when the antibodies CR3/43, AUA1 or anti-CEAm were applied. The differences between the cell lines in the binding of antibodies were often small but many were significantly different from the parent line. Col 24 was found to have the highest expression of HLA-DR and col 30 the lowest, confirming the qualitative results. Only col 1 but was found to have an expression of this antigen which was not significantly different from HCA-7.

The expression of the antigen recognised by AUA1 was similar for all the cell lines tested and most were not significantly different from the parent line.

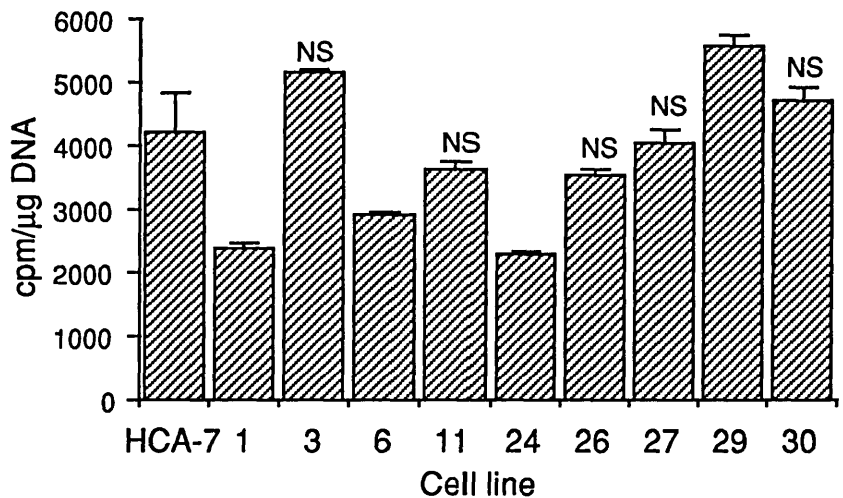
Again the expression of CEA was similar throughout the range of subpopulations, however col 3 had an increased expression of this antigen compared with the other cell lines.

Antibodies 1A3, 4D4 and 5D5 showed a much reduced level of binding compared to the other antibodies. All the cells expressed the antigen recognised by 1A3 to varying degrees though the expression by col 3 was much reduced. Col 3 was found not to express the antigens recognised by either 4D4 or 5D5, however large and significant differences were observed in the expression of these antigens by the remaining cell lines (Fig. 33). Col 27

A) CR3/43



B) AUA1



C) CEA

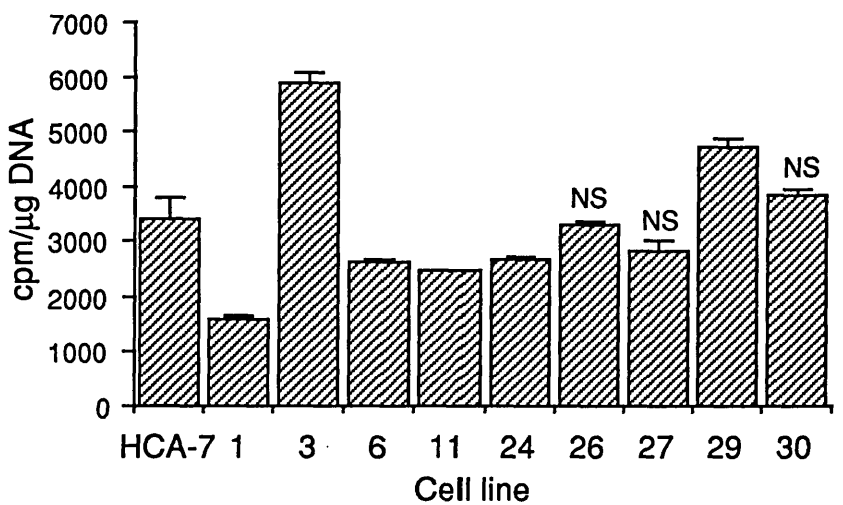


Fig. 32. Histograms showing the relative amounts of A) CR3/43, B) AUA1 and C) CEA antibodies bound to the HCA-7 cell line and each subpopulations. Binding of the antibodies is indicated by the number of counts per minute per μg DNA. See text for detail. NS = value not significantly different from HCA-7 value, $p > 0.05$.

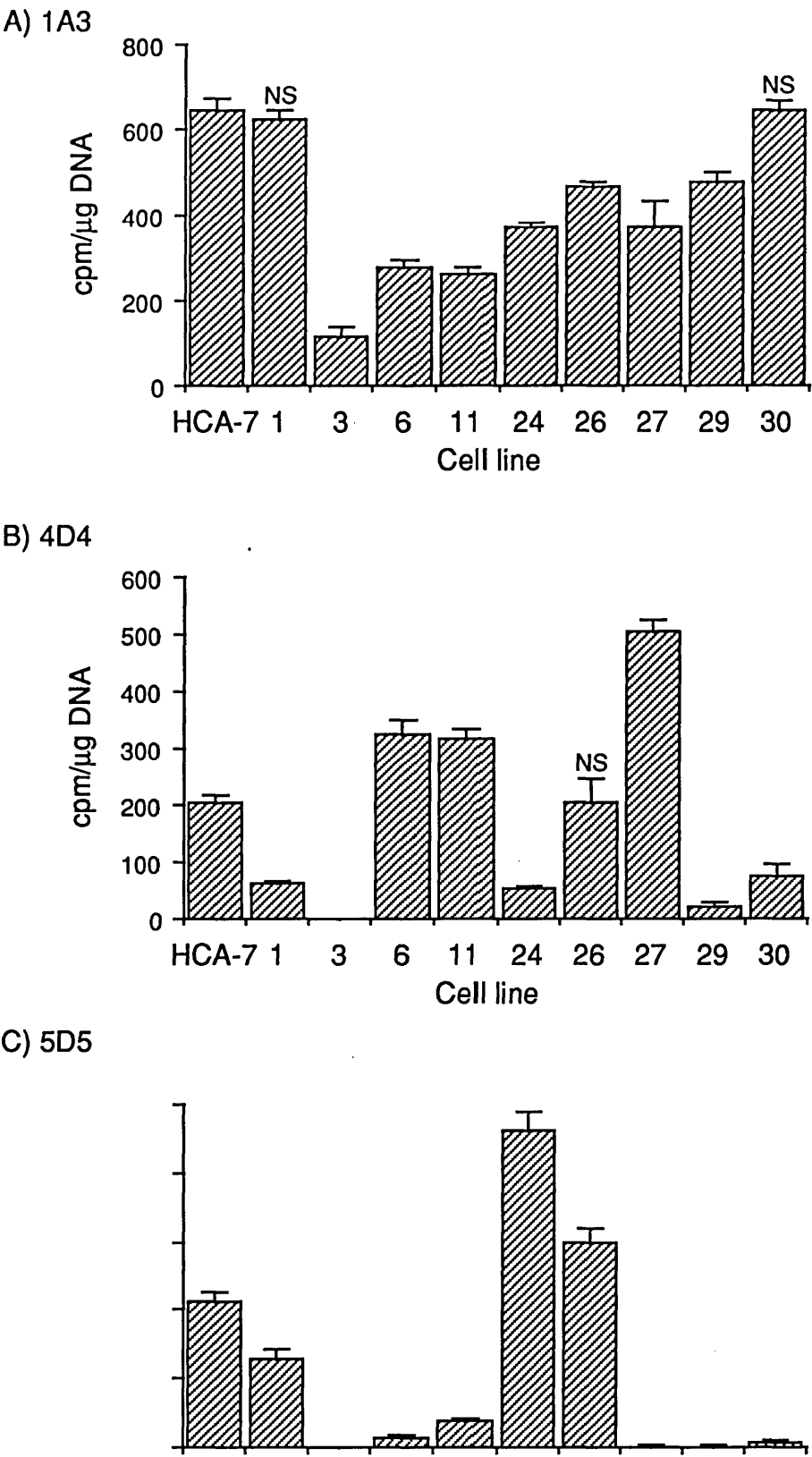


Fig. 33. Histograms showing the relative amounts of A) 1A3, B) 4D4 and C) 5D5 antibodies bound to the HCA-7 cell line and each subpopulation. Binding of the antibodies is indicated by the number of counts per minute per μg of DNA. See text for details. NS = value not significantly different from HCA-7 value, $p > 0.05$.

displayed the most binding of the 4D4 antibody which was not apparent with the indirect immunoperoxidase method. Similarly, binding of the 4D4 antibody by col 6 and col 11, which was not detected by the qualitative method, was higher than the binding by HCA-7 and col 26 cells which was detected by the previous method. Little binding was observed in the remaining subpopulations

Colonies 6, 11, 27, 29 and 30 all showed very little binding of the 5D5 antibody. HCA-7, col 1 but and col 26 displayed an intermediate amount of binding and col 24 demonstrated the greatest affinity to this antibody.

Xenografts.

Xenograft tumours were produced in nude mice after injection of cells of the parent line or subpopulations. Several mice were lost, at random, due to infection or illness before tumours had time to develop and were not included in the results. The time taken for xenografts to form at the injection sites varied greatly between the cell lines (Fig. 34). For example all col 1 but tumours were removed after 14 days in the mice whereas col 6 cells took on average 194 days to form xenograft tumours. The number of tumours produced by each cell line and the percentage take of tumours are shown in Table 7. Half the cell lines had a 100% success rate in forming tumours. Histological examination of the tumours revealed large differences in the structural organisation of the tumours and also in the degree of differentiation of the cells in different areas.

More than one histological pattern was often observed in different tumours arising from one subpopulation and often several distinct patterns were seen within a single tumour (Fig. 35a). Table 8 gives the general description assigned to all the patterns discovered in the xenografts, which were based on architectural organisation and cytological differentiation. Patterns observed in the subpopulations, which were sufficiently similar to a pattern already seen in the parent line or another subpopulation, were given the same pattern number although subtle differences in differentiation were sometimes present. Subpopulations which displayed a pattern not previously seen were assigned a new number. A total of eleven different patterns, which could be readily and reproducibly recognised were present in the 247 tumours examined (Figures 36, 37 and 38). Descriptive terms were formulated for these patterns which were predominantly based on their architecture, hence there was some overlap between different groups but which could be resolved at the cytological level. Table 8 also indicates the invasion characteristics of each of the tumour types observed. The less differentiated tumours tended to have an irregular destructive pattern of

Cell Type	Time xenografts removed. Mean $\pm$ SE	Total number of tumours.	Percentage take of tumours.
HCA-7	67.8 $\pm$ 4.5	50	83%
Col 1 but	14	36	100%
Col 3	48.6 $\pm$ 2.7	30	100%
Col 6	194.3 $\pm$ 22.6	9	38%
Col 11	162.5*	6	50%
Col 24	24.8 $\pm$ 8.2	30	100%
Col 26	106.3 $\pm$ 50.5	10	42%
Col 27	53 $\pm$ 27	22	73%
Col 29	29.6 $\pm$ 3.4	30	100%
Col 30	27.5 $\pm$ 10	24	100%

Table 7. Tumourigenicities of the HCA-7 cell line and subpopulations when grown as xenografts in nude mice. Time of xenografts removed indicates the mean of the times at which the number of tumours indicated were removed from the mice involved. Percentage take of tumours is the percentage of successful tumours arising from the inoculation sites.

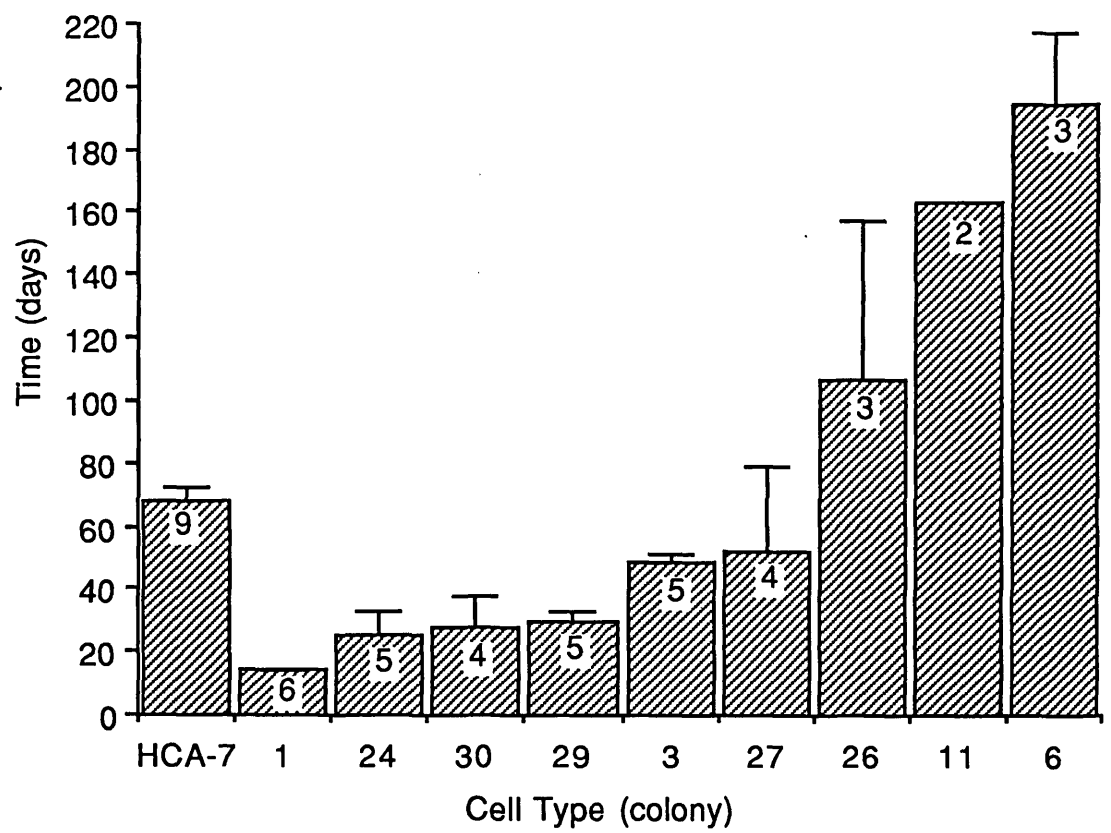


Fig 34. Histogram showing the mean number of days taken to form xenograft tumours in nude mice. Mean calculated from the number of mice indicated in the boxes $\pm$ SE (bars).

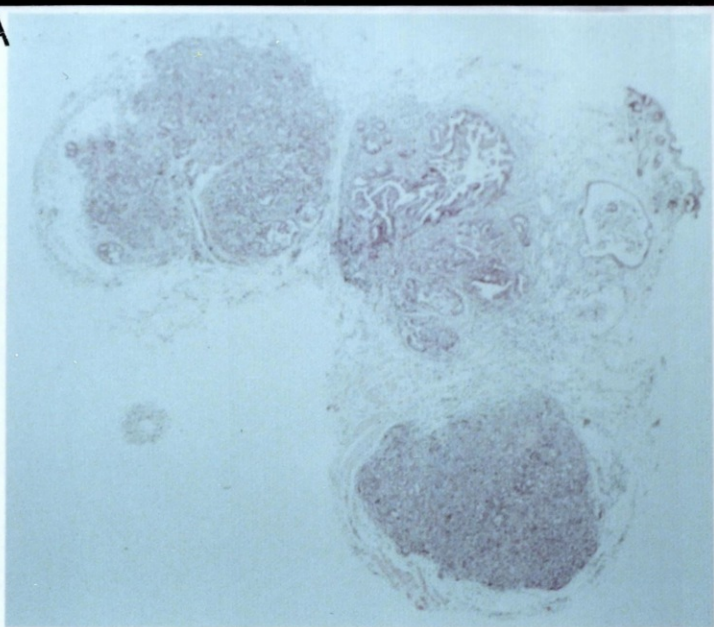
Fig. 35. Photomicrographs of sections of xenograft tumours demonstrating

A) H&E of whole HCA-7 tumour (x40).

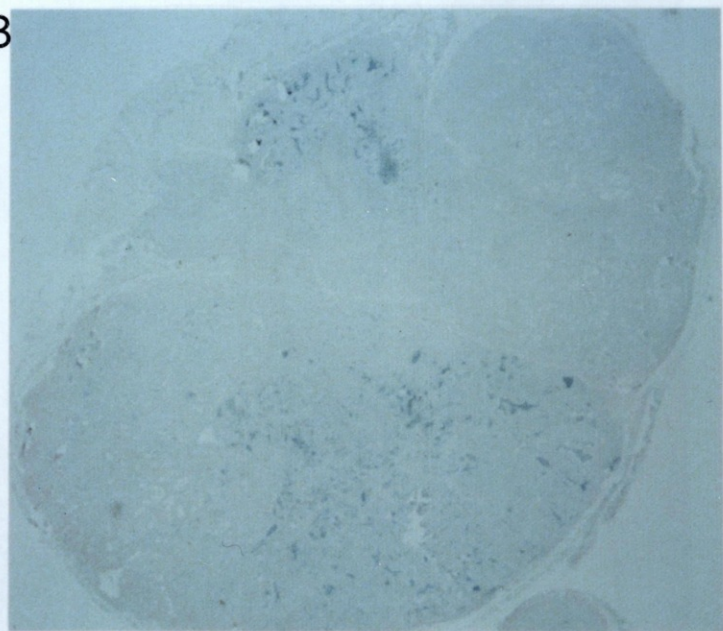
B) Alcian blue stain of whole HCA-7 tumour (x40).

C) Alcian blue stain of col 30 tumour (x310).

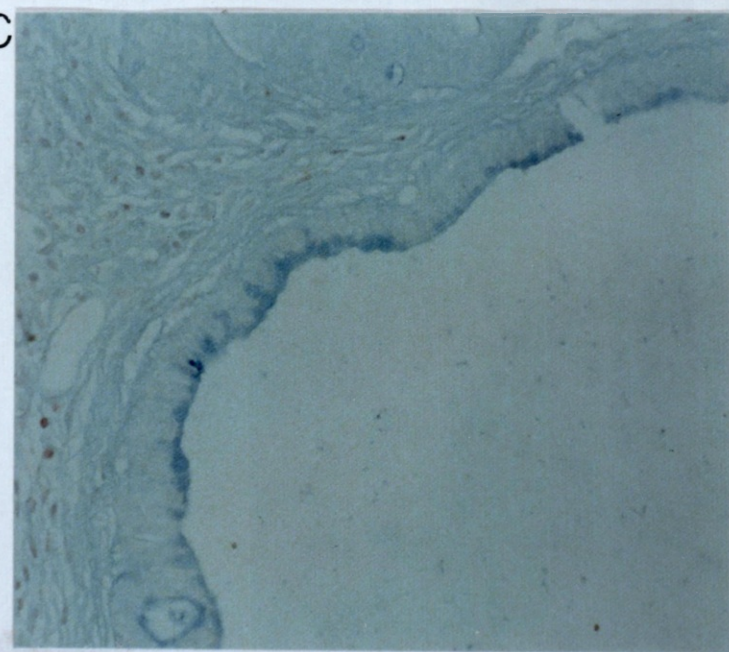
A



B



C



<u>Cell Type</u> <u>Pattern No.</u>	<u>Xenograft Organisation</u>	<u>Pattern of</u> <u>Invasion</u>	<u>Mesenchyme</u>
HCA-7			
1	Well differentiated tubulocystic	E	+ (F/C)
2	Well differentiated villotubular	B	+ (F/C)
3	Cystic glandular/papillary	E	± (C/F)
4	Simple tubular fibromatous	B	++(F/C)
5	Poorly differentiated complex glandular villous	B	+ / ++ (F/C)
6	Solid alveolar/tubular	I	+ (F)
Col 1 but			
7	Glandulovillous/cribriform	FI	+ (C)
Col 3			
8	Poorly differentiated villous	I	+ (F)
Col 6			
9	Simple cystic	E	± (C)
10	Poorly differentiated complex tubular	I	+ (C)
Col 11			
3	Cystic glandular/papillary	E	+ (C)
6	Solid alveolar/tubular	B	++(C/F)
Col 24			
3	Cystic glandular/papillary	E	± (F)
6	Solid alveolar/tubular	E	++(C)
Col 26			
3	Cystic glandular papillary	E	± (F)
4	Dilated tubular fibromatous	E	+++ (F/C)
5	Complex glandular	I	+ (C/F)
Col 27			
3	Well differentiated cystic glandular	E	+ / ++ (F/C)
10	Poorly differentiated complex tubular	FI	++(C)
Col 29			
3	Well differentiated cystic glandular/papillary	B	++(F)
3	Cystic glandular/papillary	FI	++(F/C)
4	Simple tubular fibromatous	B	
6	Solid alveolar	I	+ (F)
Col 30			
11	Well differentiated villotubular/cystic	B	++(C)

Table 8. Patterns of organisation, presence of mesenchyme and invasion characteristics of the xenografts established from HCA-7 and subpopulations. B - broad ; E - expansile ; I infiltrative ; FI - focally infiltrative ; F fibrous ; C cellular.

Fig. 36. H&E photomicrographs of sections of xenograft tumours demonstrating

A) Pattern 1 (x324).

B) Pattern 2 (x88).

C) Pattern 3 (x324).

D) Pattern 4 (x208).

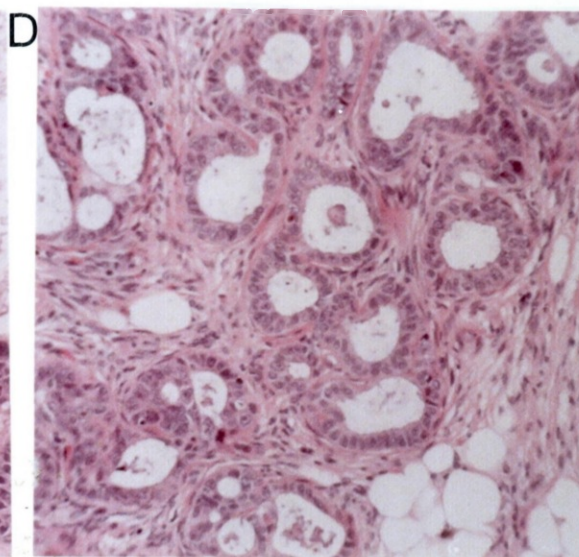
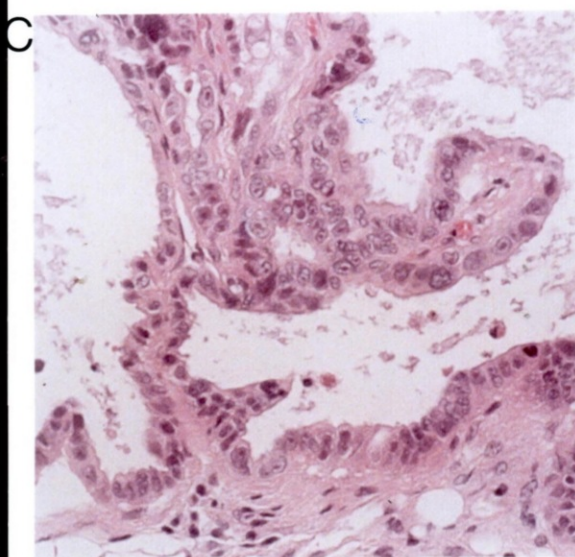
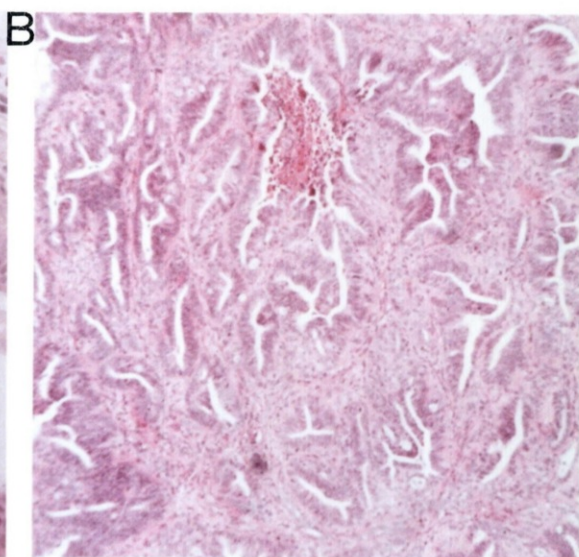
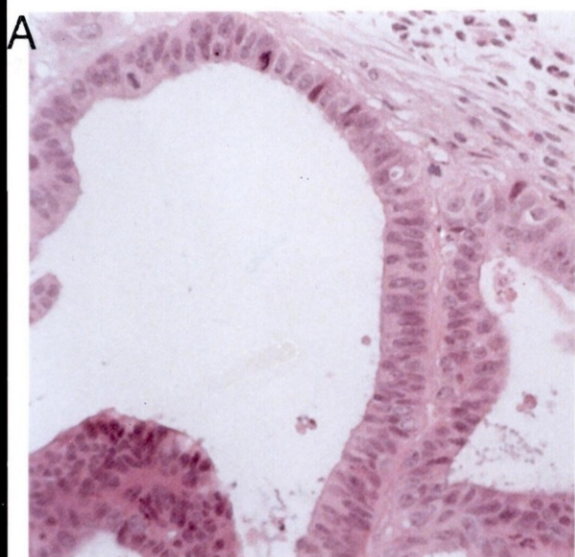


Fig. 37. H&E photomicrographs of sections of xenograft tumours demonstrating

A) Pattern 5 (x520).

B) Pattern 6 (x324).

C) Pattern 7 (x324).

D) Pattern 8 (x324).

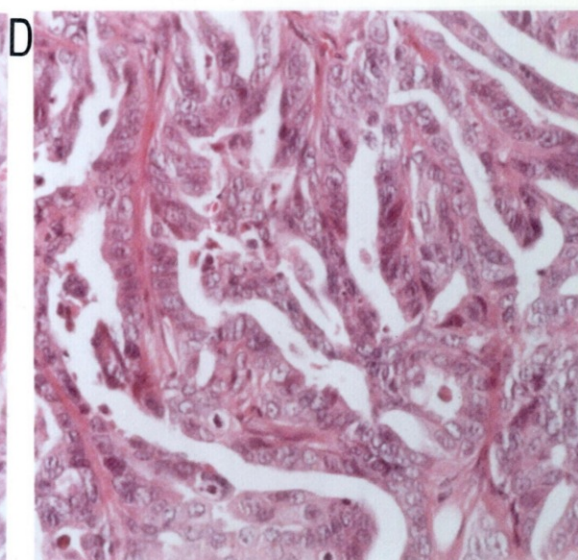
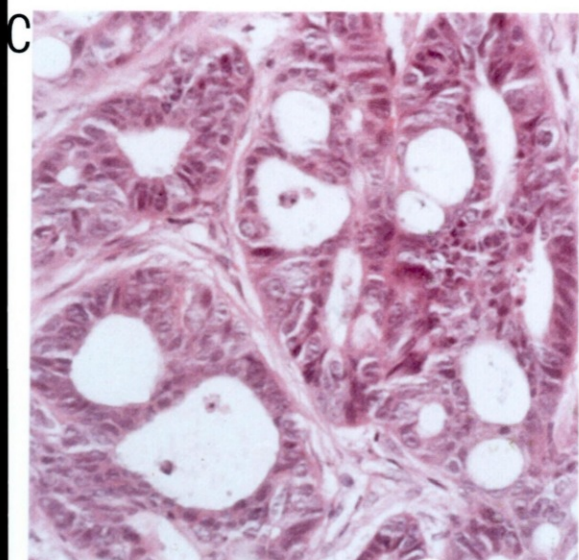
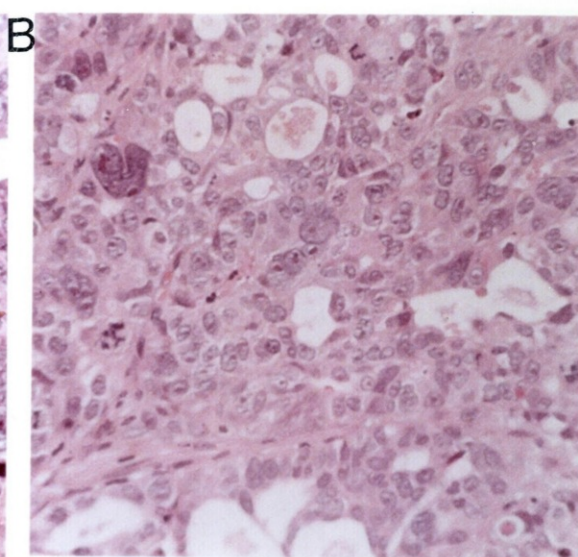
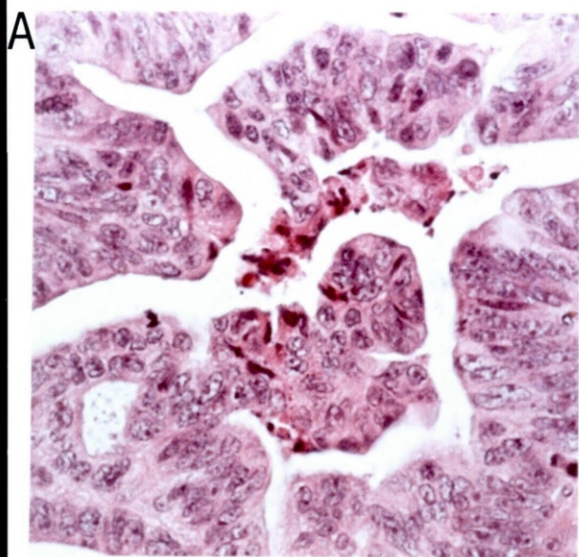
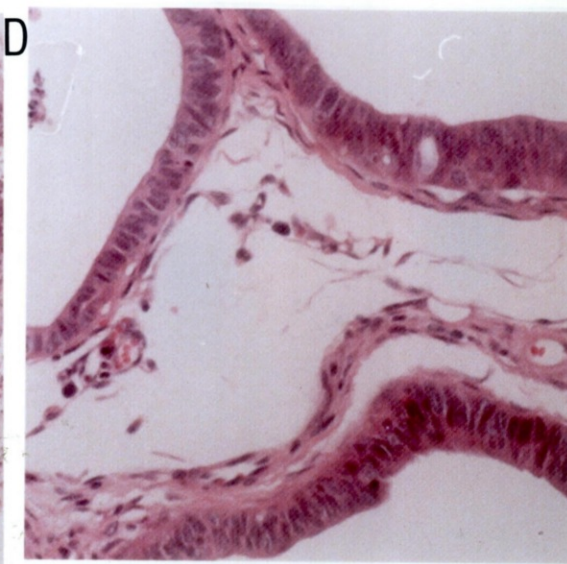
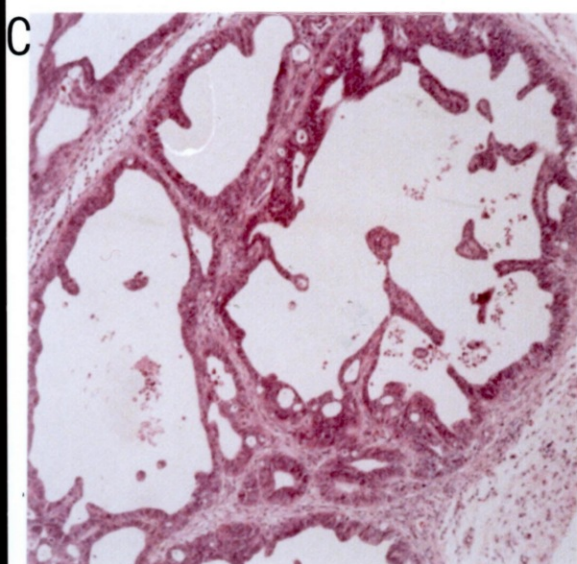
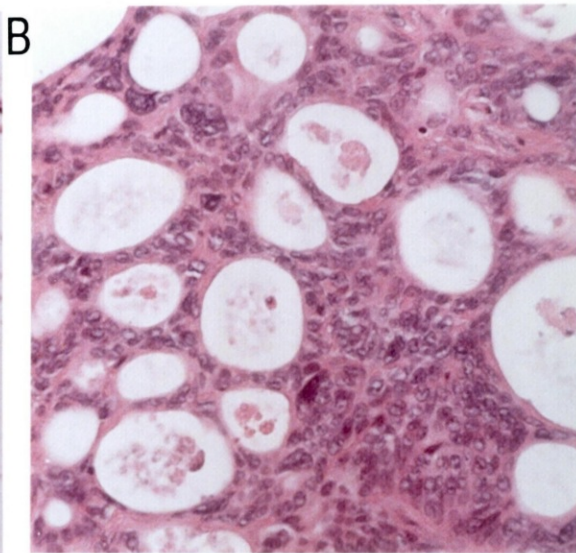
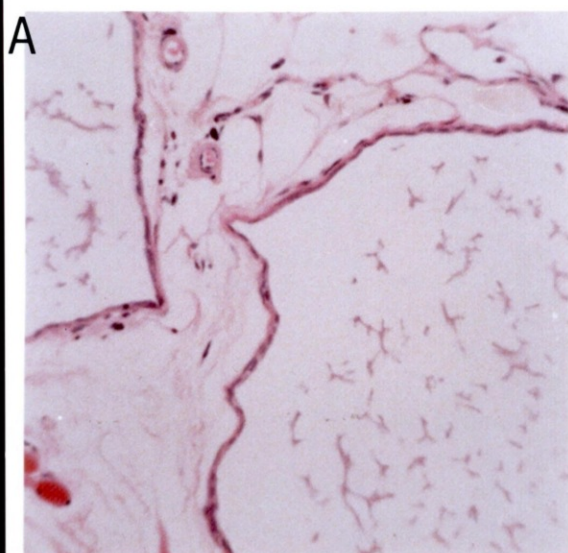


Fig. 38. H&E photomicrographs of sections of xenograft tumours demonstrating

- A) Pattern 9 (x208).**
- B) Pattern 10 (x324).**
- C) Pattern 11 (x208).**
- D) Pattern 11 (x520).**



invasion, often into muscle and occasionally into bone. Tumours having a widely invasive periphery were designated a broad fronted pattern of invasion. Some patterns, especially cystic varieties, showed marginal compression rather than destructive invasion, classified as an expansile pattern of growth. The abundance and type, either fibrous or cellular, of mesenchyme also differed between the xenografts (Table 8), but this appeared to have little relation to the organisation of the tumour cells. Several cytological features were also taken into account (Table 9). Tumour cells were often cuboidal though columnar cells predominated in the more differentiated patterns. These columnar cells were usually more polarised though a few cuboidal cells did attain some degree of nuclear polarity. Non of the tumours from any cell line showed endocrine cell differentiation that was detectable by the Grimelius silver stain.

Cytoplasmic mucin (detected by Alcian blue staining) was found to be present in some tumours. Single tumours established from HCA-7 cells were noted to contain foci of goblet cells adjacent to cells void of any cytoplasmic mucin (Fig. 36b.). Xenografts from colonies 24, 29, 3 and 11 showed no goblet cells or cytoplasmic mucin at all, however pattern 1 from the HCA-7 xenografts showed abundant mucin and a few goblet cells (Fig. 36c). Brush border staining varied in intensity though its presence could be detected in most instances. Nuclear size and morphology were also noted, along with features such as mitoses, apoptotic bodies, pleomorphism and other minor criteria conventionally used in making an assessment of cytological differentiation. Taking into consideration the structural organisation and invasiveness of the tumours and their cellular features, an arbitrary histological grade, from 1 - 6, was assigned to each of the patterns observed, grade 6 being assigned to the least differentiated pattern (Table 9).

The histological characteristics of the resected primary carcinoma, from which the HCA-7 cell line was established, were then evaluated in the same way as the xenografts (Table 10). Only two of the patterns observed in the xenograft

<u>Cell Type</u> <u>Pattern No.</u>	<u>Cell</u> <u>Shape</u>	<u>Cell</u> <u>Polarity</u>	<u>Goblet</u> <u>Cells</u>	<u>Brush</u> <u>Border</u>	<u>Nuclei</u> <u>Size</u>	<u>Xenograft</u> <u>Grade</u>
HCA-7						
1	Co	+++	+++	+ / ++	S	2
2	Co	++	++	++	M	3
3	Cu	-	±	+	M	4
4	Cu	-	-	++	M	4
5	Cu	+	++	++	L	5
6	Cu	-	-	±	L	6
Col 1 but.						
7	Cu	+	+	++	L	4/5
Col 3						
8	Cu	-	-	+	L	6
Col 6						
9	Cu	-	±	+	S	2/3
10	Cu	-	-	+	L	5
Col 11						
3	Cu	+	-	+	M	4
6	Cu	++	-	++	M	5
Col 24						
3	Cu	+	-	±	M	3
6	Cu	-	-	±	L	5
Col 26						
3	Cu	+ / ++	-	+	S	2/3
4	Cu	+	-	++	S	3/4
5	Cu/Co	±	±	+ / ++	M	5
Col 27						
3	Co	+ / ++	++	++	M	3
10	Cu	-	-	+	M	4(6)
Col 29						
3	Cu	-	-	±	S	3
3	Cu	-	-	±	M	4
4						
6	Ro	-	-	-	L	6
Col 30						
11	Co	+ / ++	++	+ / ++	L	3

Table 9. Characteristics of cells present within xenografts established from injection of cells of HCA-7 or subpopulations into nude mice.
Cu - cuboidal ; Co - columnar ; Ro - rounded ; S - small ; M - moderate ; L - large.

tumours were recognised in the original carcinoma. However three new patterns were present (Fig. 39) and in pattern 13 signet cells were observed. Ultrastructural Morphology.

Many of the organisational patterns and cellular features of the tumours were confirmed when the xenografts were observed under the electron microscope. Fig. 40a shows the more cystic pattern of HCA-7 xenografts, whereas Fig. 40b displays a lumen in an HCA-7 xenograft with a well developed brush border. A more solid, compact arrangement of cells of col 3 was well illustrated (Fig. 40c) in comparison to col 24 tumours which displayed large intercellular spaces (Fig. 40d).

The majority of cells in all xenografts were found to have tight junctions, many with desmosomes (Fig. 41a). An abundance of well formed microvilli were seen on many col 30 cells (Fig. 41b). Also present in col 30 tumours were many goblet cells surrounding the lumina (Fig. 41c). The majority of the cells were found to have a cuboidal shape however columnar cells were observed, for example in col 26 cells, with some degree of polarity (Fig. 41d).

<u>Pattern No.</u>	<u>Xenograft Organisation</u>	<u>Pattern of Invasion</u>
2	Well differentiated villotubular	B
5	Moderately differentiated complex glandular villous	B
12	Mucin secreting complex glandular villous	I
13	Mucoid carcinoma	B
14	Undifferentiated carcinoma	I

<u>Cell Type</u> <u>Pattern No.</u>	<u>Cell</u> <u>Shape</u>	<u>Cell</u> <u>Polarity</u>	<u>Goblet</u> <u>Cells</u>	<u>Brush</u> <u>Border</u>	<u>Nuclei</u> <u>Size</u>	<u>Xenograft</u> <u>Grade</u>
2	Co	++	++	++	S	3
5	Co	++	-	++	S	4
12	Co/Cu	+	++	+	M	4/5
13	Ro	-	++++	-	S	6
14	Ro	-	-	-	S	6

Table 10. Characteristics of the organisational patterns and cellular features observed in the original colonic adenocarcinoma removed from a 54 year old female.
Cu - cuboidal ; Co - columnar ; Ro - rounded ; S - small ; M - moderate ; B - broad ; I - infiltrative.

Fig. 39. H&E photomicrographs of sections of the original primary colonic carcinoma from which the HCA-7 cell line was derived.

- A) Pattern 2 (x460)
- B) Pattern 5 (x460)
- C) Pattern 12 (x460).
- D) Pattern 13 (x460)
- E) Pattern 14 (x460).

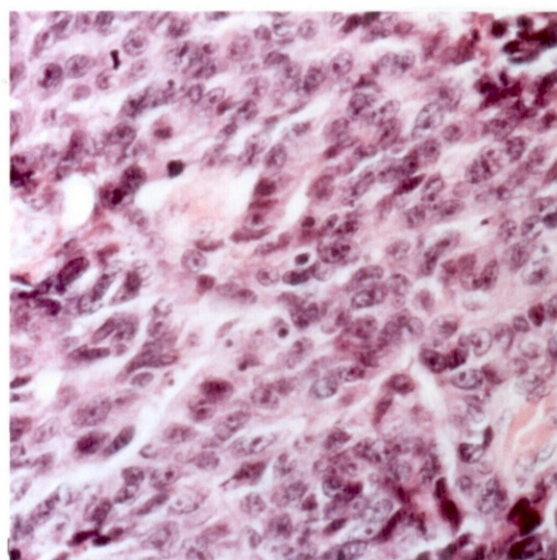
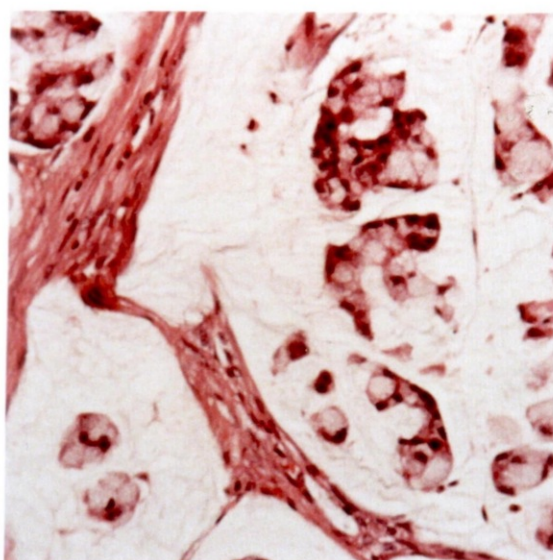
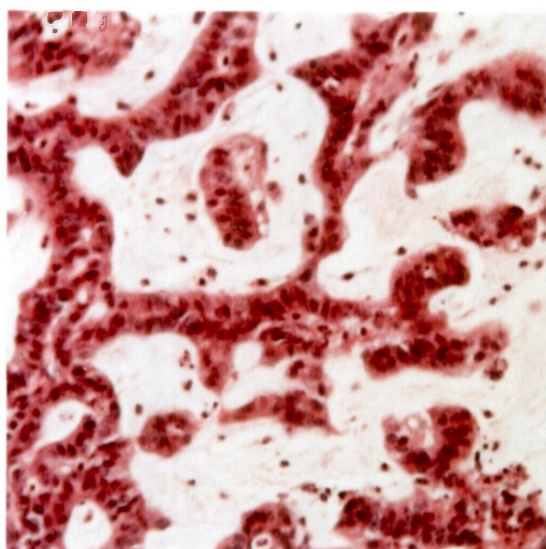
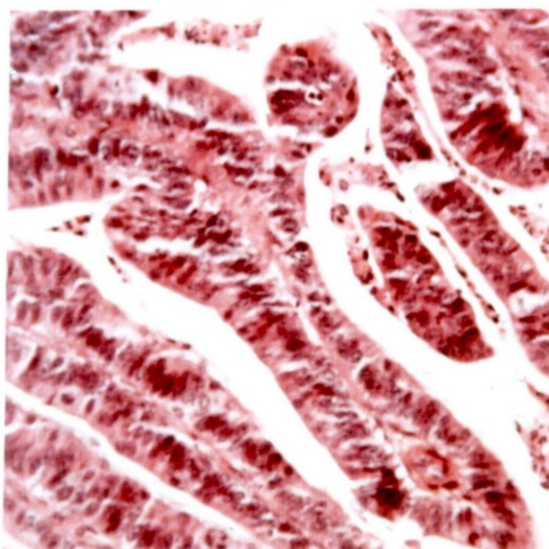
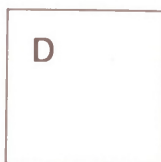
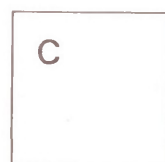
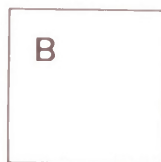
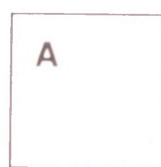
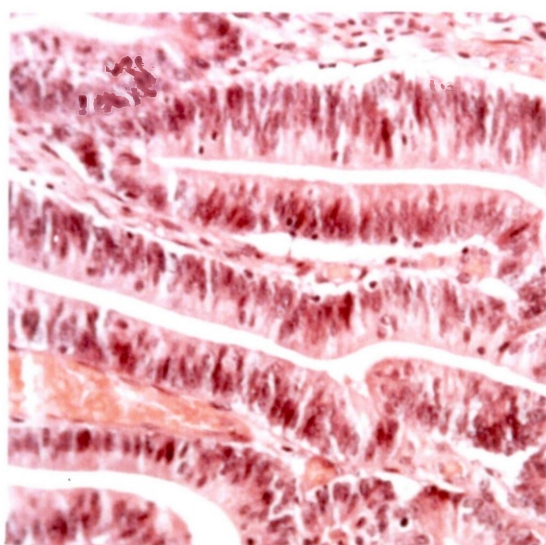


Fig 40. Electron micrographs of xenograft tumour sections demonstrating

A) Cystic pattern of HCA-7 (x 5,906)

B) Well developed luminal brush border in HCA-7 (x 3,300)

C) Compact arrangement of Col 3 cells (x 1,850)

D) Large intercellular spaces between Col 24 cells (x 1,768).

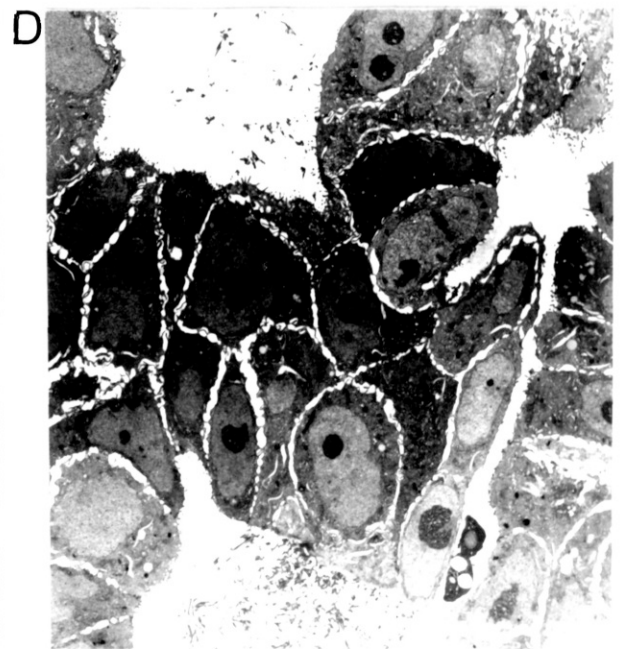
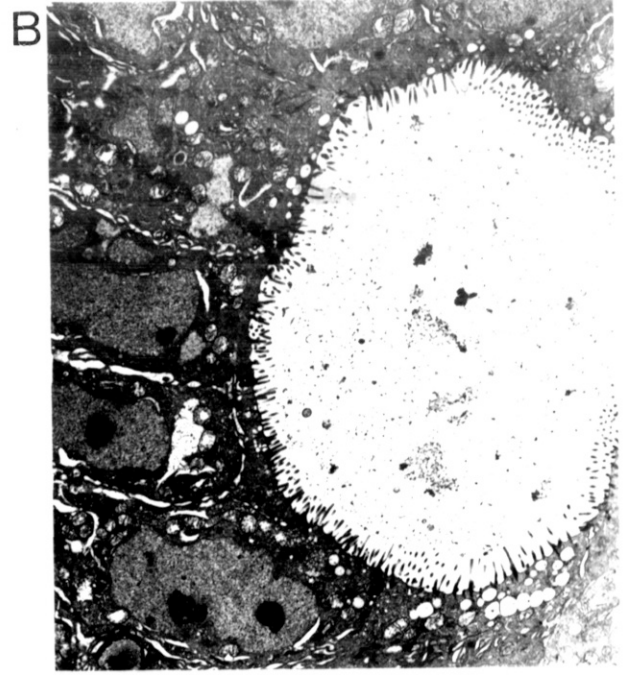


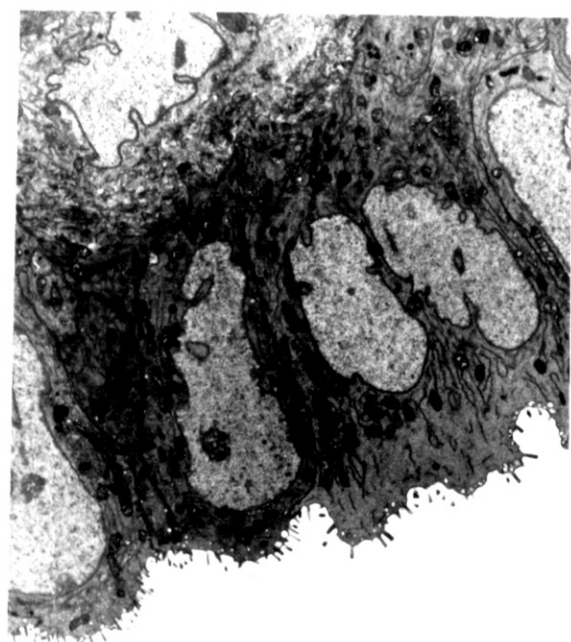
Fig 41. Electron micrographs of xenograft tumour sections demonstrating

**A) Desmosomes towards the apical surface of Col 30 cells
(x 59,429)**

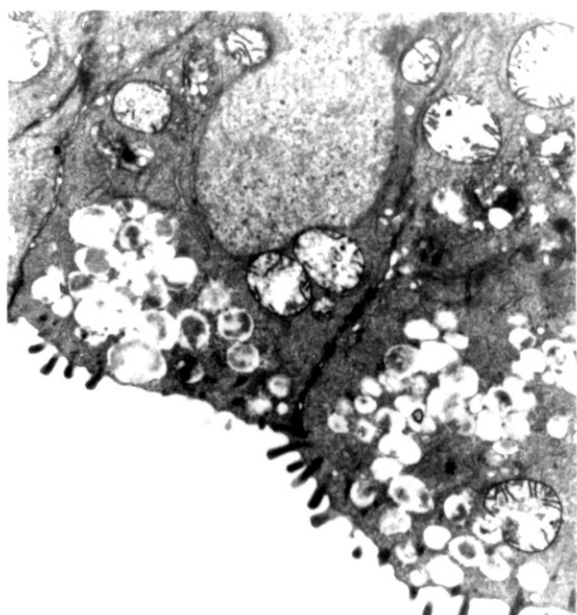
B) Well formed microvilli of Col 30 cells (x 14,375)

C) Col 30 goblet cells (x 8,516)

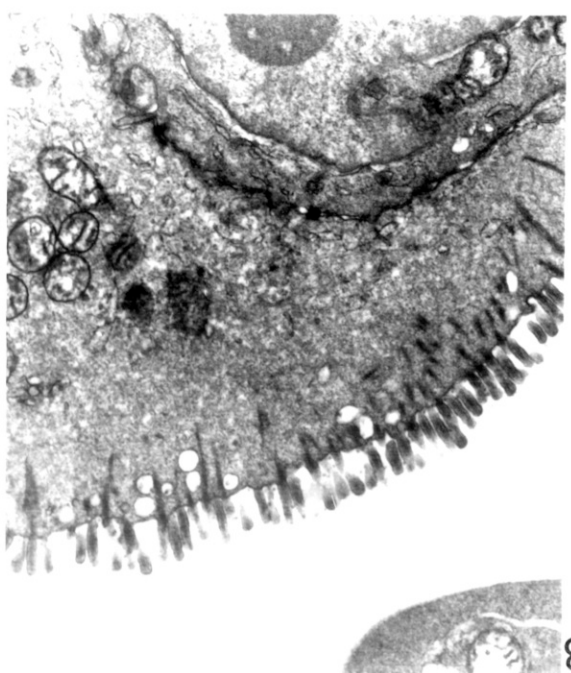
D) Polarised columnar Col 26 cells (x 3,540).



D



C



B



A

Interactions.

Co-cultures.

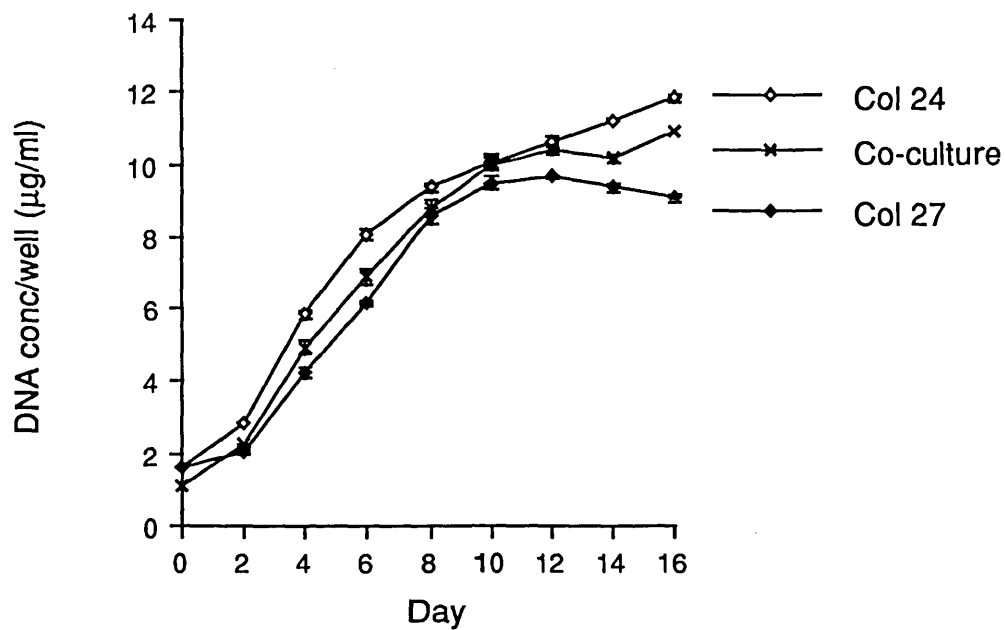
The co-culture of several cell types was carried out in order to establish whether or not any contact mediated interactions occurred between the cell lines. Cells of each of two cell types were grown individually and together in a 1:1 ratio. Cells investigated in this way were combinations of HCA-7 / col 1 but, HCA-7 / col 3, col 1 but / col 3, col 24 / col 27 and col 29 / col 30. The growth of the majority of these co-cultures did not diverge to any large degree from the growth rate predicted from their individual growth rates, for example see col 24 / col 27 and col 29 / col 30 in Fig. 42. However, the growth rate of the HCA-7 / col 3 co-culture was found to be much higher than that predicted from the growth rates of HCA-7 and col 3 growing separately (Fig. 43). The co-culture in this case tended towards the higher growth rate of col 3. This experiment was repeated three times with similar results.

Conditioned media.

In this set of experiments only the possible interactions occurring between HCA-7 cells and col 3 cells were investigated. The conditioned media appeared to alter the growth rates of both the HCA-7 cells and the col 3 cells compared to their growth in fresh media (Figures 44 and 45). These differences were small compared to the overall number of cells present. To evaluate these small changes in growth, the number of cells present at the last time point were looked at more closely. Figures 44b and 45b show histograms of the DNA content present in the wells of each cell type at day 14. To assess the statistical significance of these results, unpaired 't' tests were performed comparing the amount of DNA present in wells fed with media conditioned by HCA-7 cells or col 3 cells, to that present in wells fed with fresh media. Again significance was considered at a level of probability less than 5%.

After 14 days in culture, HCA-7 cells fed with HCA-7 conditioned or col 3 conditioned media, had a lower amount of DNA present compared to cells fed

A)



B)

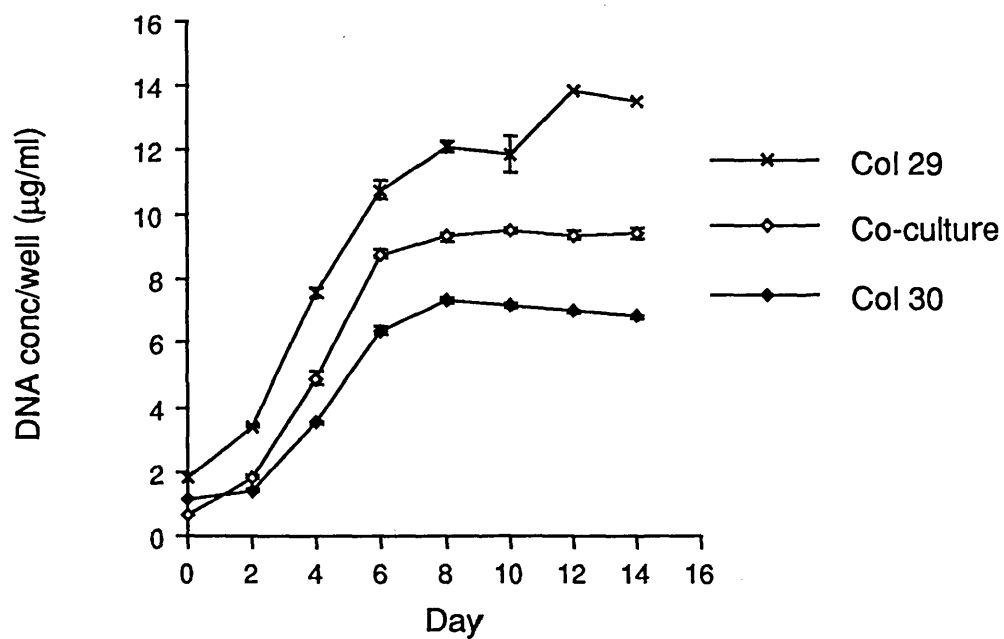


Fig. 42. Growth curves of individual cell lines and 50:50 mixtures of the two cell types grown in 24-well plates, A) Col 24, Col 27 and co-culture and B) Col 29, Col 30 and co-culture. DNA concentration in each well was assessed using a fluorimetric assay. Points, means; bars, S.E.(n=4).

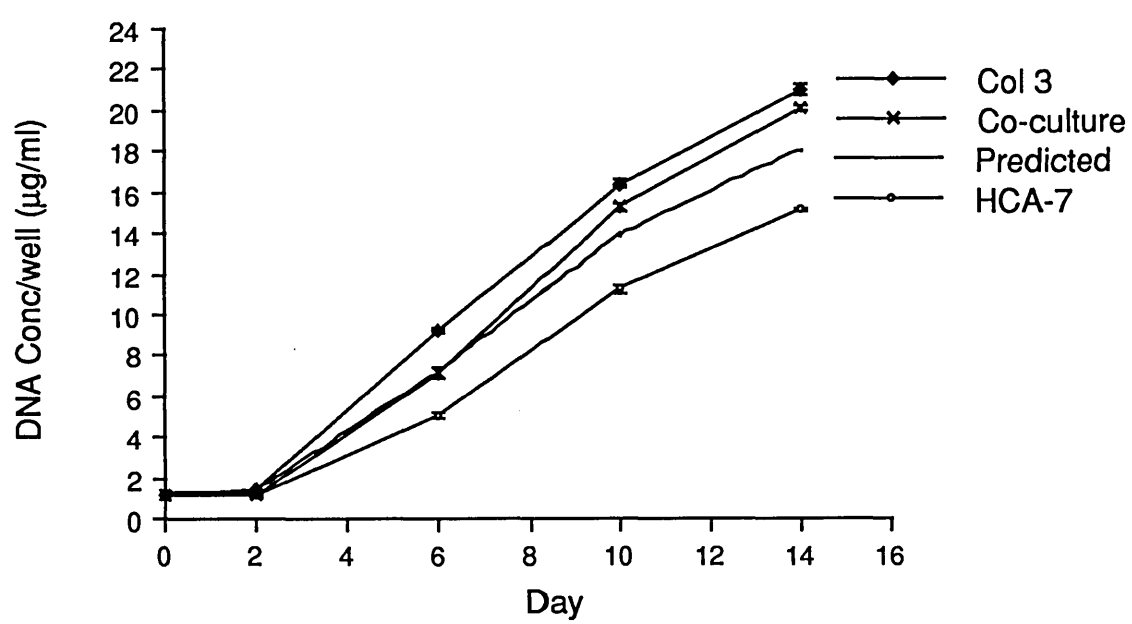


Fig. 43. Growth curves of HCA-7, Col 3 and a 50:50 mixture of both cell types. DNA concentration was assessed by a fluorimetric assay. The arbitrary predicted growth curve of the co-culture was constructed from the mean of the DNA values from the two individual cell lines. Points, mean ; bars, S.E. (n=8).

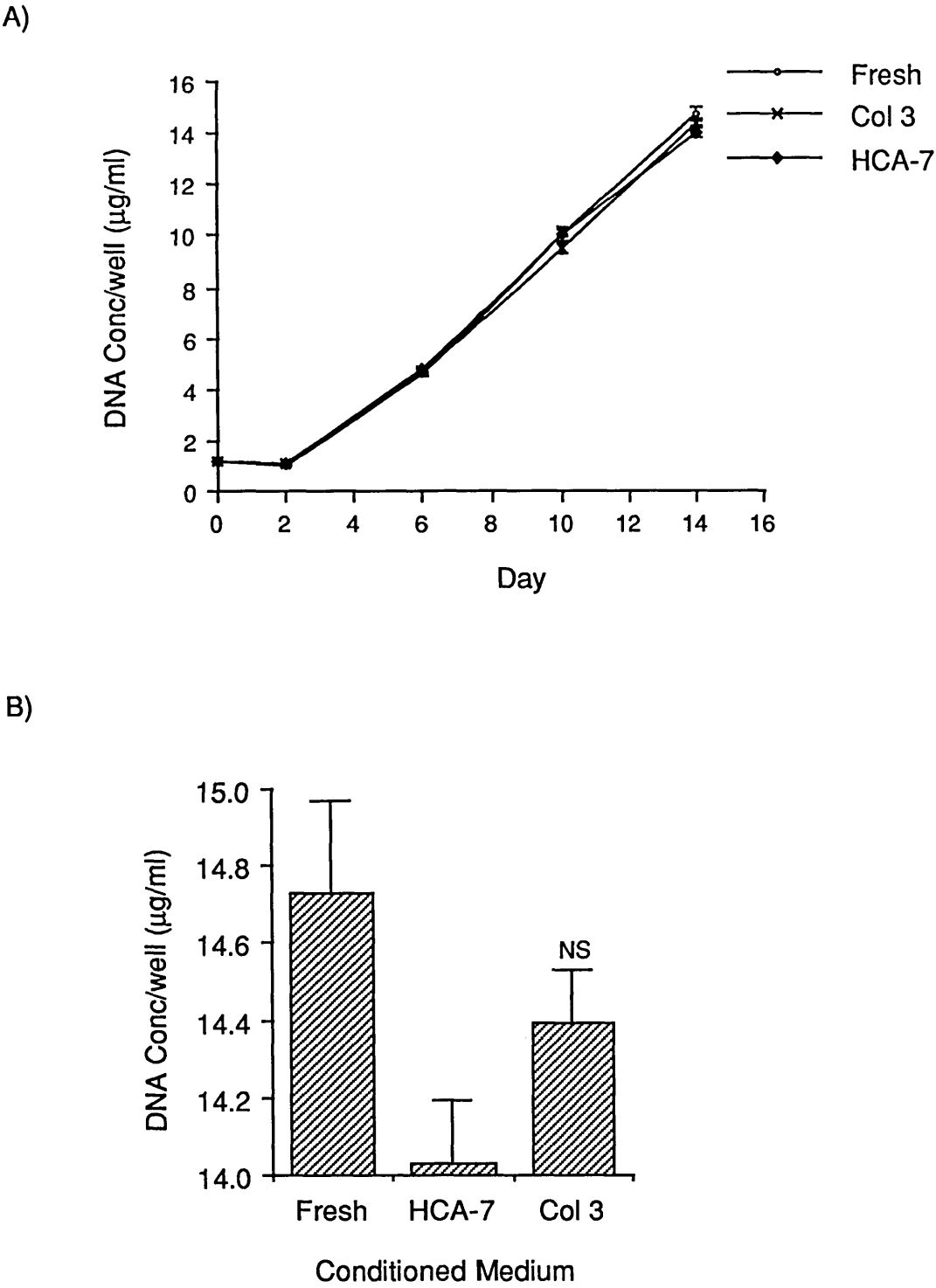


Fig. 44. HCA-7 cells grown in the presence of fresh media, media conditioned by HCA-7 cells or media conditioned by Col 3 cells. A) Growth curve of cells from day 0 to day 14. Points, means ; bars, S.E. (n =8). B) Histogram showing concentration of DNA present at day 14 taken from A). Concentrations of DNA were assessed using a fluorimetric assay. NS = value not significantly different from fresh media value, $p > 0.05$

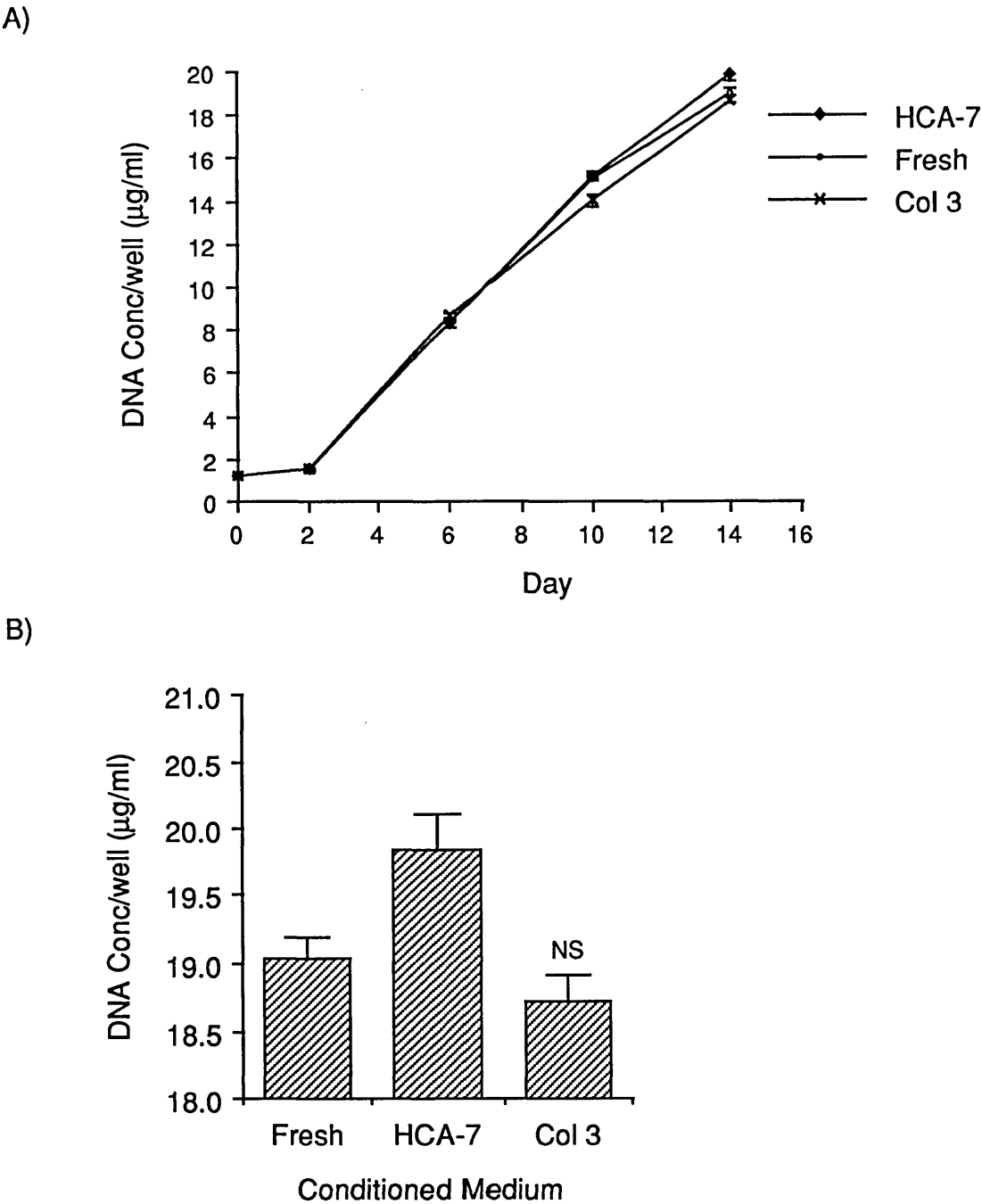


Fig. 45. Col 3 cells grown in the presence of fresh media, media conditioned by HCA-7 cells or media conditioned by Col 3 cells. A) Growth curve of cells from day 0 to day 14. Points, means ; bars S.E. (n =8). B) Histogram showing concentrations of DNA present at day 14 taken from A). Concentrations of DNA were assessed by a fluorimetric assay. NS = value not significantly different from fresh media value, $p > 0.05$.

with fresh media. The reduction was statistically significant in the case of HCA-7 conditioned media but not in that of col 3 conditioned media.

The growth of col 3 cells was also altered by the conditioned media. A significant increase in the number of col 3 cells was observed with HCA-7 conditioned media. However an apparent decrease in the number of col 3 cells occurred when supplied with media conditioned by col 3 cells, though this change was found not to be statistically significant.

Transwells.

The growth of HCA-7 cells and col 3 cells, in the presence of transwells containing either HCA-7 or col 3 cells, was compared to the growth in the absence of any other cell type. In addition, the effect of HRA-19 cells on growth was also examined. Again a decrease in DNA content of HCA-7 wells in the presence of HCA-7 or col 3, was observed (Fig. 46). Although comparatively small these decreases were found to be statistically significant. In this experiment, a slight, but significant, decrease was observed in the growth of col 3 cells in the presence of transwells containing HCA-7 cells (Fig. 47). A much larger decrease in growth of col 3 cells was demonstrated when col 3 cells occupied the transwell inserts. Non-contact co-culture with HRA-19 cells produced a reduction in growth of both HCA-7 cells and of col 3 cells.

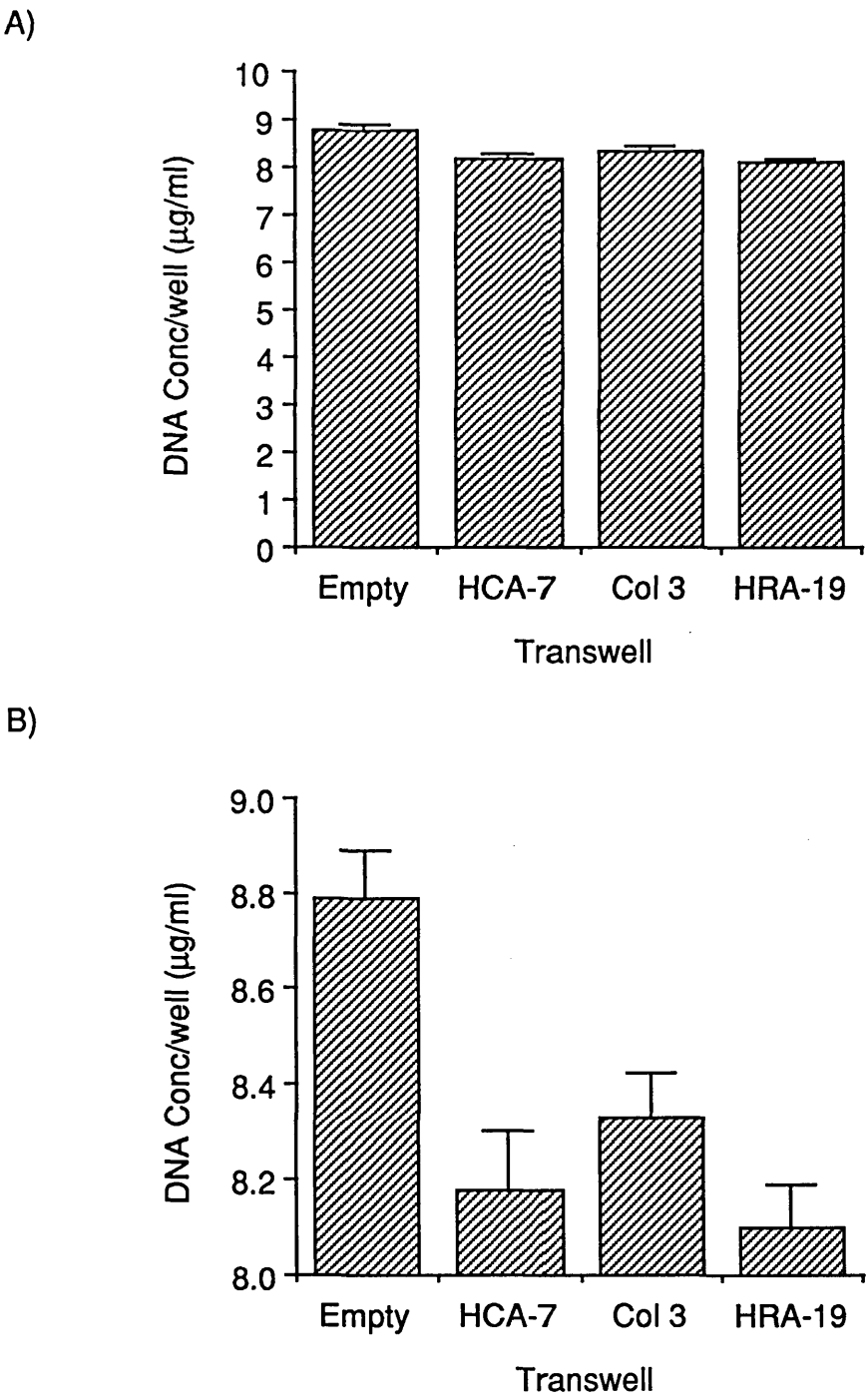
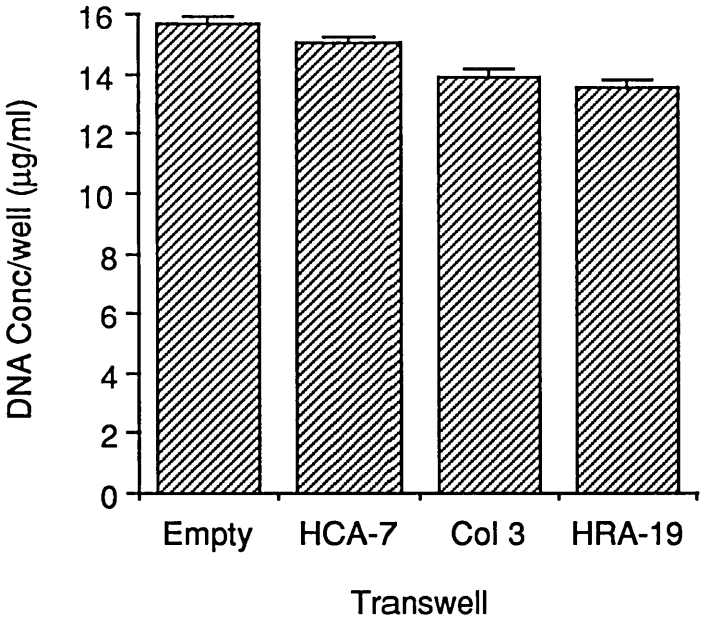


Fig. 46. HCA-7 cells grown for 14 days in the presence of transwell inserts containing fresh media, HCA-7 cells, Col 3 cells or HRA-19 cells. A) Histogram showing the concentration of DNA present in each well at day 14. Columns, means ; bars, S.E. (n =8). B) Enlargement of the upper portion of A). DNA concentrations assessed by fluorimetric assay.

A)



B)

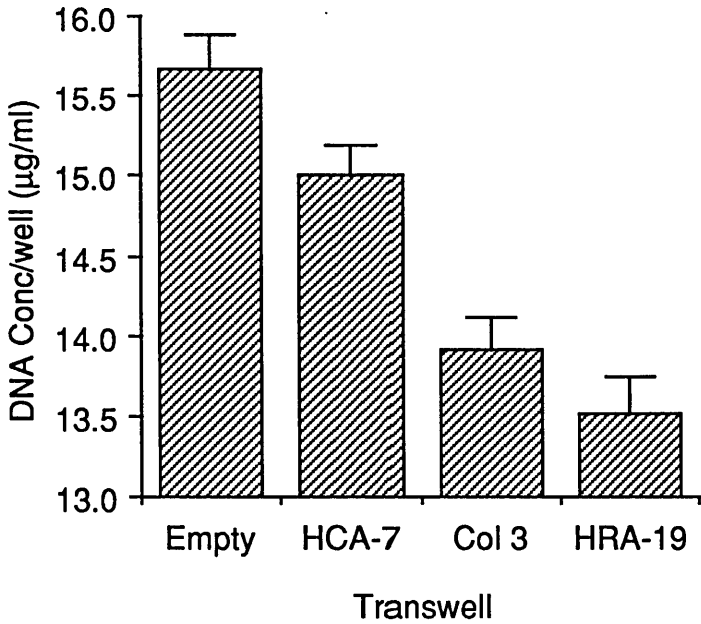


Fig. 47. Col 3 cells grown for 14 days in the presence of transwell inserts containing fresh media, HCA-7 cells, Col 3 cells or HRA-19 cells. A) Histogram showing concentrations of DNA present in each well at day 14. Points, means ; bars, S.E. (n =8). B) Enlargement of upper portion of A). DNA concentrations assessed by fluorimetric assay.

DISCUSSION.

Modal Chromosome Numbers.

Transport Properties.

Mucous Production.

Antigenic Heterogeneity.

In Vitro Growth Characteristics.

Anchorage Independent Growth.

Xenografts.

Tumourigenicity.

Histology.

Subpopulation Interactions.

Colorectal Carcinoma Cell Heterogeneity.

In this study nine subpopulations have been isolated from a cell line established from a single primary human colonic adenocarcinoma (HCA-7). It had previously been attempted to clone the HCA-7 cell line (Kirkland, personal communication) however this proved to be unsuccessful even with the use of feeder layers. In the light of this knowledge it was not attempted to clone the subpopulations isolated in this study but it is assumed that these subpopulations arose from a very few number of cells if not one cell and are therefore likely to be clonal though this is not confirmed.

The subpopulations isolated have been shown to differ, from each other and the parent cell line, in many of their characteristics. All the subpopulations are morphologically distinct and have retained these characteristic morphologies for at least 21 passages in vitro. The modal chromosome numbers of the cell line were all found to be hypodiploid though these differed between the cell lines. The in vitro growth characteristics of the cell lines were also observed to be markedly different.

The parent line and subpopulations differed in several properties which are characteristic of malignant cells. The cell lines all demonstrated different expression of carcinoembryonic antigen and also differed in their ability to grow in an anchorage-independant environment. When the parent line and subpopulations were grown in vivo as xenografts in nude mice they demonstrated different tumourigenicities and the tumours produced from the individual cell lines differed in their structural organisation and degree of differentiation.

Many of the subpopulations were also shown to retain some of the differentiated properties exhibited by normal colorectal epithelial cells. The parent line and all subpopulations were shown to transport electrolytes and fluid across the cell monolayer demonstrated either by the formation of domes or by short circuit current measurements. The cell lines also differed in their expression of several antigenic determinants, such as sucrase isomaltase, mucous cell determinants, the HLA-DR molecule and an absorptive cell

determinant, which are suggested to indicate a certain level of differentiation. Interactions between the HCA-7 parent line and col 3 have been shown to occur in vitro. It has been demonstrated that these interactions are at least in part due to an unknown soluble factor.

Characterisation of the HCA-7 Cell Lines.

All the characterisations of the subpopulations performed in this study were performed using cells between passage 10 and 20 minimising any possible variation in results due to differences in passage number.

Modal Chromosome Numbers.

All the subpopulations and the parent line demonstrated a modal chromosome number lower than the normal human diploid 46 chromosomes. Half of the cell lines, namely colonies 1 but, 3, 11, 26 and 30, had a modal chromosome number of 44, a loss of only two chromosomes. However col 24, col 29 and HCA-7 showed a loss of three chromosomes and the remaining lines demonstrated a loss of four. Without a detailed karyotypic analysis of the cell lines it is impossible to fully interpret the implications of these findings with respect to other phenotypic differences displayed by the cell lines. Whether or not the same chromosomes are missing from each cell type, would be an interesting avenue to explore.

However, it is of interest to note that each cell line is hypodiploid. This feature appears to be a rare event in primary colorectal neoplasms. A study by Reichmann *et al.* (1981) showed that only 2 out of 56 patients with colorectal neoplasms had tumours demonstrating a hypodiploid karyotype. Similarly, in two other studies, 1 of 14 cases (Sonta and Sandberg, 1978) and 2 of 16 cases (Martin *et al.*, 1979) displayed overall chromosome number reductions. Chromosome counts have also been performed on the majority of cell lines listed in Table 1 (see Introduction). Most of these cell lines were found to be hyperdiploid to near tetraploid with modal chromosome numbers ranging from 46 to 92. Exceptions include the S-7512 cell line with a modal chromosome number of 115 (Machida *et al.*, 1977). Few of the cell lines showed a hypodiploid count. The cell lines LS-174T, SW 837, SW 1083, HT29, 5 of 17 of the VACO series and S/RG had modal chromosome numbers in the range

of 41 to 46 (Tom et al., 1976 ; Leibovitz et al., 1976 ; Kimball and Brattain, 1980 ; McBain et al., 1984 ; Willson et al., 1987 ; Paraskeva et al., 1989).

It may be suggested that with increasing time in culture the cells acquire an increased genetic instability giving rise to an altered modal chromosome number. It was demonstrated using the DLD-1 parent cell line that although the modal chromosome number remained constant from early to late passage cells, the range of numbers did increase (Dexter et al., 1981).

However there seemed to be little relation, in the afore mentioned studies, between the modal chromosome numbers and the passage at which they were calculated. For example, the hypodiploid S/RG cell line (Paraskeva et al., 1989) and the hypertetraploid S7512 cell line (Machida et al., 1977) were both tested at early passage, whereas the hyperdiploid Co115 cell line which demonstrated very little divergence from the mode (Carrell et al., 1976) was tested at passage 50, where one might have expected a more unstable karyotype. In this study the modal chromosome numbers were calculated when the subpopulations were all at a similar passage.

In cell lines which have yielded subpopulations with differing phenotypic characteristics, as is the case with the HCA-7 cell lines, modal chromosome numbers have been investigated. The HCT-116, HCT-116a and the HCT-116b subpopulations all have the same modal chromosome number of 46 (Brattain et al., 1981). However, from the DLD-1 cell line, clone A cells were hyperdiploid and clone D cells had a mode of 46 chromosomes, the parent line also having a mode of 46 (Dexter et al., 1981). In addition, the HCT-C and HCT-C col subpopulations had modal chromosome numbers of 46 and 47 respectively (Brattain et al., 1983). These differences may have some implications in the mechanisms involved in the generation of tumour cell diversity, possibly supporting the role of increased genetic instability proposed by Nowell (1976).

Differentiated Features of the HCA-7 Cell Lines.

Transport Properties.

Differences in dome formation were observed between the cell lines both in the quantity and size of domes present. Dome formation has been suggested to be a result of electrolyte and fluid transport from the apical to the basolateral side of the cells. However, from the evidence obtained of the transport characteristics of the HCA-7 cell line and subpopulations using the Ussing chamber system, it has been shown that fluid transport by cells does not necessarily result in the formation of domes. Indeed, the parent line and subpopulations, with the exception of the dome forming col 11, all demonstrate a short circuit current (SCC) when unstimulated, indicating that active transport of electrolytes and fluid is occurring, however not all produce domes in monolayer culture. The resting currents of the cell lines were found to vary quite considerably. Col 30 which has by far the greatest capacity for dome formation has a resting current of $7.5 \mu\text{A}/\text{cm}^2$, whereas col 24 which does not show any dome formation displays a resting current of $18.5 \mu\text{A}/\text{cm}^2$. These results demonstrate that other factors are involved in the formation of domes in monolayer culture in addition to the ability of the cells to vectorially transport electrolytes and fluid. From their investigations of the effect of ouabain (an inhibitor of Na^+ , K^+ -ATPase) on Caco-2 cells, Ramond *et al.* (1985) also concluded that dome formation was not simply due to electrolyte and water transport and may be due to different processes in different cell lines. It is possible that the presence of tight junctions and desmosomes influence the ability of the cells to retain fluid under the monolayer in order to form a dome. The transepithelial electrical resistance measurements, taken when the monolayers were at rest, indicate the 'tightness' of the cell monolayer, that is its resistance to fluid movement due to the presence of tight junctions. However, no direct correlation was observed to suggest that the more resistant monolayers have an increased capacity to form domes.

The opportunity was taken in this study to investigate the responses of the subpopulations to various agents known to affect transepithelial transport in the mammalian colon. Prof. Cuthbert and his group in Cambridge have previously performed experiments which indicated different responses to secretagogues by the HCA-7 parent line, col 1 but and col 3 (Cuthbert *et al.*, 1987) and have also carried out initial studies on col 29. The results of these four lines shall be included in this discussion where possible. Absolute comparisons cannot be made between all the cell lines due to the differences in protocol carried out in separate studies, however the responses of the above four cell lines are clear.

Lysylbradykinin (LBK) and other kinins have been shown to induce electrogenic chloride secretion in intestinal epithelia when applied to the basolateral side of the tissue (Cuthbert and Margolius, 1982). However Cuthbert *et al.* (1985) demonstrated that the HCA-7 cell line responded to LBK when applied to either the basolateral or apical side of the cells suggesting that kinin receptors are present in both domains in this cell line. They later went on to show that col 1 but also had functional kinin receptors located in both domains however these were absent in col 3 cells (Cuthbert *et al.*, 1987). The responses of the remaining subpopulations investigated here also varied quite considerably. As was the case with HCA-7 and col 1 but, LBK was shown to induce an increase in SCC in colonies 6, 24, 26 and 27 when applied to either the apical or the basolateral side of the cells, again indicating functional kinin receptors. Initial studies indicate that col 29 demonstrates approximately twice the increase in SCC in response to apically applied LBK than any of the other cell lines (Cuthbert, unpublished observations). However more striking is the absence of any response in col 30 cells to LBK applied either apically or basolaterally, as was the case with col 3 cells. The fact that these two subpopulations did not respond to LBK does not mean that kinin receptors are not present in the cells. It is possible

that the processes which couple the receptor to the chloride secretory response are absent or malfunctioning.

The mechanisms by which kinins increase chloride secretion are unclear. However it was concluded by Cuthbert *et al.* (1984a, 1984b) that one of the primary actions of kinins was to increase calcium influx through the basolateral aspect of epithelial cells. They postulated that this increased calcium influx was needed for eicosanoid formation in order to increase apical chloride permeability via cyclic AMP (Cuthbert *et al.*, 1984b). However they later showed that chloride secretion, induced by kinins, does not require eicosanoid synthesis (Cuthbert *et al.*, 1984c). The reasons why the HCA-7 cell line and subpopulations differ in their responses to LBK is unknown. It may simply be due to a difference in the number of functional kinin receptors present in each cell line or that different mediators are involved in their secretory responses.

Forskolin is a known activator of adenylate cyclase (Seamon *et al.*, 1981), thus increasing levels of intracellular cyclic AMP, which has been shown to stimulate chloride secretion in the mammalian colon (Frizzell and Heintz, 1979). The SCC of the parent line and all subpopulations were increased by forskolin but to differing extents. Of interest were col 3 and col 30 and their responses to increased intracellular cyclic AMP, as neither responded to LBK and therefore not considered to be influenced by raised intracellular calcium levels. On addition of forskolin to the basolateral bathing fluid, there was a ten fold greater response elicited in col 30 compared to that in col 3.

From these results it is indicated that the mechanisms and mediators involved in chloride secretion by cells of the HCA-7 cell line and the subpopulations differ between the cell lines and require either cyclic AMP or calcium ions or indeed a combination of the two mediators.

In order to investigate this further, other agents were used that are known to utilise calcium ions in their signals. Carbachol, a muscarinic receptor agonist, is thought to use calcium as a second messenger and A23187, a calcium

ionophore, increases intracellular calcium directly. On examination of the five subpopulations investigated in this study, carbachol was found to increase the SCC of colonies 6, 24, 26 and 27 above that induced by LBK and forskolin. Therefore, taking into account the responses to the secretagogues of these cell lines, the results would indicate that chloride secretion is stimulated by calcium ions in col 6, col 24, col 26 and col 27. However on addition of A23187 to the bathing solution a further increase in SCC was only observed in col 26 cells. It may be the case that in the other cell lines a threshold in the chloride secretory response has been reached after addition of the other agents and A23187 was unable to induce any more chloride secretion. Col 30, on the other hand, showed no increase in SCC by these two agents and in fact the response elicited by forskolin was reduced slightly by carbachol and quite dramatically by A23187. It seems to be indicated that calcium ions are not necessary for chloride secretion by col 30 cells and indeed may have an inhibitory involvement in this subpopulation.

Piretanide is a frusemide-like loop diuretic which inhibits active chloride transport (Zeuthen *et al.*, 1978). The ability of this agent to reduce the SCC displayed by the subpopulations confirms that the short circuit currents are at least in part due to electrogenic chloride secretion. However, the fact that the SCC of each cell line are not completely reduced to resting rate, may imply that part of the SCC induced in the cell lines is due to transport of other ions. Initial investigation indicated that sodium transport was not involved in the short circuit responses of the subpopulations as these responses were not inhibited by amiloride which blocks sodium channels in the apical surface of epithelial cells of the colon (Schiller *et al.*, 1988 ; Wills *et al.*, 1984).

The mechanisms by which the HCA-7 cell line and its derivative subpopulations transport chloride across the cells are complex and possibly manyfold. The results obtained here do however clearly indicate that there are differences in the transport properties of the cell lines and in their response to various secretagogues. Many more investigations, such as binding studies,

are needed to establish the presence or absence of receptors for these secretagogues. Also evaluations of the requirement of calcium ions and cyclic AMP are needed to establish the mechanisms by which the different cell lines respond to the secretagogues and whether or not these mediators are coupled to both the receptors and the secretory response. Unfortunately it has only been possible in this study to see the tip of a potentially enormous iceberg.

Mucous Production.

Neither monolayers of the parent line or any of the subpopulations demonstrated demonstrated mucous production by positive staining with Alcian blue. However using the monoclonal antibodies 4D4 and 5D5, which are directed against mucous cell components of normal human colorectal epithelium, positive results were obtained for the parent line and some subpopulations. Large differences were observed between the amount of antibody bound to the different cell types and also between the two antibodies. When tested for specificity, the 4D4 antibody was found to be more specific to colonic epithelium cross reacting with only ileal and tracheal epithelium. However the 5D5 antibody was found to react to components in epithelial cells of the stomach, ileum, gall bladder, trachea, cervix, placenta and skin (Richman and Bodmer, 1987).

It is of interest to note the differences in staining patterns of the two antibodies. The 5D5 antibody demonstrated an even stain over each cell whereas the 4D4 antibody stained small globules which were situated either cytoplasmically (HCA-7) or at the cell membrane (col 26). These differences may be due to the relative specificities of the two antibodies with the 5D5 antibody reacting with more determinants than the 4D4 antibody. However the results do suggest that mucous cell differentiation is occurring in monolayers of the parent cell line and several of the subpopulations.

Antigenic Heterogeneity.

The immunocytochemical results demonstrate antigenic heterogeneity within the HCA-7 cell line and its derivative subpopulations. One possible explanation of this heterogeneity in the parent line is that of different expression of an antigen by the different subpopulations. If this was the case then one might have expected the isolated subpopulations to be either homogeneous in their expression or demonstrate no expression of a particular antigen. However the explanation of antigenic heterogeneity has proved to be not so simple and the question then poses itself as to what factors control antigen expression in carcinoma cells.

As the subpopulations are not irrefutably clonal in origin then it is feasible that they are themselves progeny of more than one cell type with differential expression of these antigens resulting in the heterogeneous expression of several antigenic determinants. However, the possibility of polyclonal origin seems unlikely. Assuming that a particular subpopulation is of monoclonal origin, this antigenic heterogeneity must have arisen in the progeny from that one cell although we expect a homogeneous expression of an antigen in clonal cells. Should we in fact expect such homogeneity in clones when, as noted by Heppner (1984), we are, ourselves, of clonal origin?

It has previously been shown that primary colonic carcinomas also heterogeneously express the HLA-DR molecule and the carcinoembryonic antigen (Rognum *et al.*, 1983).

Antigen expression has been shown to be a function of the cell cycle of murine lymphoma cells (Cikes and Klein, 1972) and human lymphoid cells (Everson *et al.*, 1974). It was later shown by Drewinko and Yang (1976) that CEA synthesis is restricted to the stationary growth phase of the LoVo cell line, derived from a subclavicular metastasis of a patient with colonic adenocarcinoma (Drewinko *et al.*, 1974). This cell cycle-dependant expression of antigens is one possible explanation of the results obtained in this study. However if antigen expression is dependent upon the cell cycle

one might expect that in slower growing cultures there would be a higher proportion of cells in the stationary growth phase and therefore a greater expression of the antigen would be observed in these cultures. No correlation between growth rate and antigen expression was observed in the HCA-7 cell lines. The HCA-7 cell line and subpopulations were all examined for antigen expression at confluency. This was used as a standard with which to compare the antigen expression by these cell lines assuming that each cell line was at the same stage of growth. However, antigenic heterogeneity was present under these conditions.

On studying the differentiation of Caco-2 cells, Levy *et al.* (1988) demonstrated that morphological and functional differentiation of the cells occurred at confluency. However enzymatic differentiation occurred much later in the cells. The HCA-7 cell line and subpopulations were immunocytochemically tested at confluency and it is possible that a similar latency in differentiation occurred in some cells resulting in the heterogeneous expression of the differentiation markers used.

It may however be the case that, even if all the cells of each cell line appear to demonstrate homogeneous expression of these antigens when at the same stage of growth, differences are still observed in the actual amount of antibody binding to the cells. Only at this point would it be correct to say that the intrinsic capacity for expression of a certain antigen by each cell line was different.

If a heterogeneity continues to exist within a cell line when all the cells are in the stationary growth phase, what then controls the expression of antigens? Although it has been shown here that the cells don't all express certain antigens, as detected by immunoperoxidase staining and radio immunoassay, it is possible that they do in fact synthesise the particular molecule which fails to be detected by these methods. Trugnan *et al.* (1987) utilised the ability to control the differentiation of HT-29 cells by glucose replacement, to investigate this possibility. They demonstrated that the sucrase isomaltase

enzyme is easily detectable and active in the differentiated HT-29 cells. However, in the undifferentiated cells the protein cannot be detected by immunofluorescence or immunodetection in whole cells or membrane enriched fractions. Using L- [³⁵S] -methionine pulse chase experiments, they demonstrated that the enzyme was in fact synthesised by the undifferentiated cells. They concluded that the absence of sucrase isomaltase expression was a result of an impaired glycosylation and a rapid degradation of the enzyme. Similar experiments would be required to establish whether or not such mechanisms are in operation in the HCA-7 cell line and subpopulations. It would then be the case of determining why a default in the expression of a certain antigen occurs in one cell of a cell line and not another.

Antigenic heterogeneity may of course may be simply due to the possibility that some cells in a subpopulation are 'programmed' to express a certain antigen whereas others are not.

Antigenic heterogeneity of tumour cells may be an important factor in certain therapies such as antibody targeting, the implications of which have been discussed by Edwards (1985). Unless an antigen is expressed by all the cells of a tumour, the therapy is likely not to be entirely successful due to the survival of subpopulations not expressing the antigen, although this may be overcome by using a combination of antibodies. It must be noted here that antigenic heterogeneity is not a property solely confined to tumour cells. In the colon certain antibodies have been shown to predominantly stain either the crypt cells or the luminal cells (Finan *et al.*, 1982 ; Daar and Fabre, 1983). Antigenic heterogeneity may also be important in the survival of certain subpopulations of a tumour. Expression of a certain antigen by a particular subpopulation may result in that subpopulation being more immunogenic in the host, exposing the cells to attack from the host defences. However, if all the cells of one subpopulation do not express the antigen then this could contribute to the survival of that subpopulation in the tumour environment thus aiding the maintenance of heterogeneity. That antigenic heterogeneity may

be a possible basis for progression of tumours has been discussed at length by Prehn (1982).

In Vitro Growth Characteristics.

To obtain a direct comparison of the growth rates it would have been preferable to count cell numbers in a time course study. As it is impossible to obtain a single cell suspension of the HCA-7 cell line and subpopulations without damaging the majority of cells, cell numbers were not counted. In order to overcome this problem, quantitation of DNA content of the cells present was employed as a standard to assess growth rates. As the modal chromosome numbers of the parent line and subpopulations did not vary to any great extent, this method of growth assessment was deemed to be the most accurate available with which to compare the growth rates of the cell lines.

When growth assessment of the cell lines was performed, the subpopulations were all at a similar passage number. It is possible that at different passage numbers the subpopulations grow at different rates to those elucidated here and this possibility would have been examined if there had been time to do so.

In addition, growth rates may be altered if the cells had been seeded at different densities. For example, a more sparse seeding may have induced a greater growth rate in some subpopulations, yet decreased that in others. At this stage it can only be speculated as to what controls the growth rates of these carcinoma cells and whether the same control is effective in all the subpopulations or whether different mechanisms and mediators are needed to control the growth of each cell line.

Several mediators in the control of cultured human colonic carcinoma cells have been suggested. Cyclic AMP analogues have been shown to have an inhibitory effect on the WiDr, LS174T and HT29 cell lines (Tagliaferri et al., 1988). Both cyclic AMP and calcium have previously been postulated, by many groups of workers, to be intracyclic regulators in cell proliferation (see MacManus et al., 1978).

Glycogen storage has been suggested to have a role in cell proliferation mechanisms. These stores have been demonstrated to be much higher in colorectal carcinomas than in normal mucosa, implying that glycogen metabolism could be specifically modified by the neoplastic process (Rousset *et al.*, 1979). It was shown that the colorectal carcinoma-derived cell lines, Caco-2, HRT-18, HT29, HCT-8R, Co-115 and SW480, had widely differing levels of glycogen both in culture and *in vivo* when grown as xenografts in nude mice (Rousset *et al.*, 1980). They found that in cultured cells glycogen levels increased three to four fold in the stationary growth phase (Rousset *et al.*, 1979). A similar approach would be required to examine the possibility of glycogen involvement in the control of proliferation of the HCA-7 cell line and subpopulations. However it is unclear whether the storage of glycogen by a cell is actually a method of controlling growth or is indeed an effect of growth. Differences in growth rate have also been noted between subpopulations of a mouse mammary tumour cell line where doubling times *in vitro* range from 12 to 40 hours (Calabresi *et al.*, 1979). However, the clone A and clone D subpopulations isolated from the DLD-1 colonic carcinoma cell line, were shown to have similar doubling times of 22 and 20 hours respectively (Dexter *et al.*, 1981). This also proved to be the case with the HCT-116, HCT-116a and HCT-116b subpopulations from a human colonic carcinoma, where their doubling times were found to be in the range of 21 to 24 hours (Brattain *et al.*, 1981). Unfortunately, as cell numbers of the HCA-7 cell line and subpopulations were not counted here, a direct comparison in growth rate could not be made to these other systems. It would appear though, that the model developed in this study will provide an ideal and extensive system with which to investigate the different controls of growth in human colorectal carcinoma cells which may result in the differences in growth rates of the subpopulations observed.

Recently, the HCT-116 and HCT-116a subpopulations have been adapted to grow in serum-free medium. This has enabled the growth requirements of

these two subpopulations to be evaluated and differential sensitivities to transferrin, insulin and EGF by the subpopulations has been observed, suggesting separate growth controls (Boyd *et al.*, 1988b). This would indicate that although growth rates may be similar, growth requirements may greatly differ between given subpopulations.

Anchorage-Independent Growth.

Not all the subpopulations of the HCA-7 cell line possess the ability to grow in suspension or semi-solid medium which has been suggested to be a marker of malignant carcinoma cells (Marshall *et al.*, 1977). Although this has proved to be true in some cases (Brattain *et al.*, 1981 ; Trainer *et al.*, 1988), it has been indicated that anchorage-independent growth is not a good indicator of tumourigenic potential in colorectal carcinoma cells (Paraskeva *et al.*, 1984). In this study there did appear to be a good correlation between growth in soft agarose and tumourigenicity in nude mice. Col 6 and col 11 demonstrated no growth in soft agarose or suspension and also were least tumourigenic, whereas the more tumourigenic col 1 and col 30 produced large organised structures in both systems. Ideally the numbers of colonies, formed by each cell line under these conditions, should have been scored to enable a more complete correlation between anchorage-independent growth and tumourigenicity to be assessed.

At the present time it is not clear what mechanisms control the ability of a cell to grow in the absence of a solid substrate. However, transferrin has been indicated to have an important role in maintaining the transformed state of colonic carcinoma cells, as assessed by anchorage-independent growth of the HCT-116 and HCT-116a subpopulations (Boyd *et al.*, 1988c).

It would be of interest to investigate the possible presence of extracellular matrix components produced by the different cell lines. It may be that the anchorage-independent cell lines have the ability to lay down their own

substrata which enables them to continue growth in suspension. Of interest was col 30 and its growth in bacteriological dishes. This subpopulation was found to attach and grow extremely well on the non tissue culture-treated plastic of these dishes, a property not displayed by any of the other cell lines. Whether or not col 30 has an increased capacity to produce its own substrata remains to be seen. Substrata, shown to contain laminin and fibronectin, derived from one colonic carcinoma cell line has previously been shown to alter the behaviour of a different cell line (Boyd et al., 1988a ; Boyd et al., 1988c). Further investigation into the production of extracellular matrix components such as laminin, fibronectin and collagens by HCA-7 and its subpopulations are required to evaluate any possible involvement of such constituents.

Xenografts.

Tumourigenicity.

The HCA-7 parent line and subpopulations have been shown in this study to express different tumourigenicities when grown as xenografts in nude mice and also different histologies of the resulting tumours.

The value of athymic nude mice in the investigations of human neoplasms was first appreciated when it was discovered that such tumours can be successfully heterotransplanted and grown in these recipients (Rygaard and Povlsen, 1969). Since then the nude mouse has become a widely used tool in the investigations of the biology and growth kinetics of human colorectal carcinomas, by implantation of primary tumour fragments (Jessup *et al.*, 1988) and many colorectal carcinoma cell lines (for examples see Noguchi *et al.*, 1979 ; Brattain *et al.*, 1983 ; Kirkland and Bailey, 1986). The nude mouse has also been used as an intermediate host in the transfer of primary colorectal carcinoma cells into culture (Carrel *et al.*, 1976).

In the search for markers of malignant potential of carcinoma cells, several attempts have been made to correlate certain *in vitro* characteristics of colorectal carcinoma cell lines to their tumourigenicities as xenografts *in vivo*. Such traits include growth potential in monolayer culture, growth in semi-solid medium as discussed earlier and carcinoembryonic antigen expression (Brattain *et al.*, 1984 ; Drewinko *et al.*, 1984). Although trends can be seen correlating such *in vitro* characteristics with tumourigenicity, no absolute *in vitro* markers have been demonstrated to consistently predict the behaviour of the same cells *in vivo*.

Leibovitz *et al.* (1976) suggested that human colorectal carcinoma cell lines could be divided into three distinct groups based on their morphological differentiation, chromosome number and CEA production. Since this first classification several other groups have also performed similar segregations of cell lines (Brattain *et al.*, 1981 ; Drewinko *et al.*, 1984 ; Taylor *et al.*, 1984 ; Trainer *et al.*, 1988).

Brattain *et al.* (1981) separated fourteen colorectal carcinoma cell lines into three groups based on their tumourigenicity in nude mice. They found that this separation correlated well with the degree of differentiation of the tumours CEA production *in vitro* and growth in semi-solid medium. The highly tumourigenic group 1 produced poorly differentiated tumours, small amounts of CEA and grew well in soft agarose. Group 2 cell lines were weakly tumourigenic but yielded differentiated tumours. Similar amounts of CEA were produced compared to group 1 though were less anchorage-independent. In contrast, group 3 cells were non-tumourigenic, anchorage-dependant and produced on average ten fold more CEA *in vitro*.

A report published by Drewinko *et al.* (1984) supporting this proposal presented a more detailed examination of proposed markers of the three groups including chromatin distribution, *in vitro* cytokinetics, plating efficiencies and xenograft studies at different sites of inoculation in nude rats. The hypothesis that human colorectal carcinoma cell lines can be separated into three groups, which demonstrate reliable markers for their behaviour *in vivo*, was also put to the test by Trainer *et al.* (1988). As in the previous studies they based their group separations on tumourigenicity in nude mice. However they found no relationship between tumourigenicity, generation time in monolayer culture and CEA secretion. Nor did they find any correlation between the histology of the xenografts and their tumourigenicity. In addition, they also examined and found no correlation between tumourigenicity and the expression of certain oncogenes.

The great interest in correlating *in vitro* characteristics of cell lines to their behaviour *in vivo* stems from the desire to find a phenotypic or genotypic marker which will help predict the behaviour of primary tumours in the host, thus giving an improved indication of the prognosis for the patient. It remains to be established whether or not such an infallible marker exists and what value it would contribute to the clinical treatment and management of the disease.

Although these investigations may go a long way to aiding our understanding of the diversity which exists between individual colorectal tumours, the problem still remains regarding the different phenotypic variants within a single neoplasm and their malignant potential. As the availability of colorectal carcinoma cell lines with derivative subpopulations is severely limited, the possible differential tumourigenicities and correlating *in vitro* characteristics of such subpopulations has not been a major area of research.

Brattain *et al.* (1981) found that the HCT-116, HCT-116a and HCT-116b subpopulations, isolated from a single primary colorectal tumour, demonstrated different tumourigenicities with respect to the percentage of mice producing tumours and the latency period of tumour development. All three subpopulations resulted in tumours which also displayed different histological patterns and degree of differentiation. These tumourigenicities were also found to correlate well with their ability to grow in semi-solid medium.

Extensive research has gone into elucidating the characteristics of the DLD-1 cell line and its derivative subpopulations, clone A and clone D *in vivo*. Initial investigations demonstrated that all three lines had similar latency periods. However the parent line produced moderately to poorly differentiated tumours, clone A poorly differentiated tumours and clone D moderately differentiated tumours (Dexter *et al.*, 1981). Further examination of the behaviour of this cell line has demonstrated that the xenografts, resulting from the inoculation of these cells, differ in their sensitivities to photon irradiation (Leith *et al.*, 1984). These kind of results tend to provide an incentive to find *in vitro* markers to predict the response of subpopulations to therapeutic regimes. The need for a more extensive system, with which to study the different behaviours of tumour cell subpopulations, in order to realise the extent of biological diversity within tumours and ultimately to effect more successful treatments and prognostic markers, is quite apparent.

In comparison to the other colorectal carcinoma cell lines mentioned above, the HCA-7 parent line and subpopulations are all relatively tumourigenic in that they all form tumours in nude mice. However there are large differences, in both the percentage of inoculum sites yielding tumours in the mice and in the time taken for the tumours to form, between the individual subpopulations. There is a good correlation between the time taken to form tumours and the percentage yield, that is the faster the tumours are formed, the greater the percentage yield. Whether or not this indicates that the faster forming tumours have a greater inherent capacity to form tumours is not clear. The decrease in the percentage yield of tumours as xenografts take longer to form may be due to host factors coming into play as the mouse matures. However what is abundantly clear is that the behaviour of the parent line and subpopulations in nude mice are markedly different. In agreement with Trainer et al. (1988), no absolute correlation was found between the tumourigenicities of the parent line and subpopulations and their in vitro characteristics, other than growth in semi-solid medium as discussed earlier.

The expression of carcinoembryonic antigen, which has been debated as a marker for prognosis, by the cells in vitro bore no relation to their tumourigenicity. For example col 3 cells demonstrated over three fold more expression of CEA than col 1 but cells, however both exhibited a 100% tumour yield.

The only possible indication of a correlation between an in vitro characteristic and tumourigenicity was the expression of the antigenic determinant recognised by the antibody 4D4. Tumours from HCA-7, col 6, col 11, col 26 and col 27, all demonstrating 38-83% yield, bound on average seven times more antibody than the remaining subpopulations with 100% yield. Whether or not this antibody recognises an antigenic determinant which has some influence on the ability of tumour subpopulations to survive in vivo can only be speculated at this stage. It may be that the 4D4 antibody is recognising a marker of differentiation of a tumour. Further investigations should include the

expression of the antigen recognised by the 4D4 antibody by the xenograft tumours. Specific identification of this antigenic determinant, assumed to be of mucous cell origin, may also shed some light onto its role, if any, in the tumourigenic potential of the cells expressing it. It was, however, noted that the expression of this antigen by the cells *in vitro* bore no relation to the relative number of mucous cells present in the xenografts.

Histology.

On removal and examination of the tumours arising from the parent line and subpopulation, it became increasingly evident that different histological patterns were being exhibited by the different cell lines. It was at this stage that the tumours were initially graded according to their organisational pattern and degree of differentiation. This initial classification was performed 'blind' by a pathologist with no knowledge of the origin of the xenograft tumours other than that they were established from cultured human colonic carcinoma cells. A more detailed examination followed, resulting in the classification and grading of the xenograft tumours.

As might have been expected, the HCA-7 parent line tumours yielded several organisational patterns, two of which were observed in the original primary carcinoma. The majority of tumours raised by the subpopulations demonstrated histological patterns sufficiently similar to those observed in the tumours of the parent line to warrant the same classification of pattern number. One might have predicted, due to the fact that the subpopulations were all isolated from the HCA-7 parent line, that they would have all have exhibited patterns which were present in the HCA-7 tumours. However this did not prove to be the case as col 1 but, col 3, col 6, col 27 and col 30 all demonstrated patterns not observed in any tumours arising from the parent line cells. It is possible that these patterns were present in the HCA-7 xenografts but remained unobserved. Another possibility is that these

organisational patterns did not appear due to influences exerted by the other cell types present in the tumours.

In addition to this observation, tumours arising from individual subpopulations often demonstrated more than one histological pattern and grade. As the subpopulations are not confirmed to be clonal in origin, again we cannot rule out the possibility that they have arisen from more than one cell type, providing a simple explanation of the resulting heterogeneity of their xenograft tumours. Assuming that the subpopulations are of clonal origin, we are presented here with a manipulable system with which to study the origins of tumour cell heterogeneity. However, only when the subpopulations are cloned, can the production of a heterogeneous tumour by a particular subpopulation be irrefutably stated to be of clonal origin. It is then that the putative factors controlling the origins of heterogeneity *in vivo* be investigated. Similar treatment to that performed on the original primary carcinoma, which yielded the parent cell line and subpopulations, would need to be exercised on the tumours formed by the cloned subpopulation. This would involve establishing a new parental cell line, isolating subpopulations if they exist and characterising them in the same way as the HCA-7 subpopulations. It would be of great interest to note if phenotypic and genotypic differences once again presented themselves. Heterogeneity of tumours *in vivo* has been observed after the implantation of a cloned murine lymphoma cell line, where it was suggested that the host stroma may allow or contribute to the generation of tumour cell diversity (Chow and Greenberg, 1980). Indeed damage to host stroma, by radiation, has been shown to lead to the competitive exclusion of the minority subpopulation of artificially heterogeneous colorectal tumours suggesting an involvement of host tissues in the maintenance of heterogeneity (Leith *et al.*, 1988 ; Leith *et al.*, 1989). However, in this study there seemed to be no correlation between the amount of stroma present in the tumours of the HCA-7 cell lines and the degree of heterogeneity within the tumours.

Regarding the histological features of the xenografts of the HCA-7 cell lines studied here, it was also attempted to correlate the histology of the xenografts to their tumourigenicity and the in vitro characteristics of the cell lines. Once again little correlation was observed between these parameters. However of interest was col 3 which demonstrated a 100% tumour yield and formed tumours in 49 days. These tumours were found to be the most consistently aggressive and undifferentiated of all the tumours produced by the subpopulations. In vitro, col 3 demonstrated the least reactivity to the 1A3, 4D4 and 5D5 antibodies and the penultimately lowest reactivity to the CR3/43 antibody. In contrast col 3 was found to have the greatest expression of CEA. This finding is in agreement with Brattain et al. (1981) and Drewinko et al. (1984) where undifferentiated in vitro characteristics correlated with an increased CEA secretion however in these cases the cell lines were non-tumourigenic. It is also of interest that col 1 but cell which are the most tumourigenic express the least CEA and is the fastest growing in vitro. Brattain et al. (1984) also found that low doubling times and low CEA secretion in vitro positively correlate with increased tumourigenicity. However, from this study, only isolated in vitro characteristics of individual subpopulations can be correlated to their tumourigenic capacities and xenograft histology. Growth in soft agarose appears to be the only partially reliable indicator of tumourigenic capacity. Further investigation must be performed if a predictor of a tumour subpopulation's malignant capability is to be unearthed.

Subpopulation Interactions.

Having established a series of subpopulations from a single colorectal carcinoma cell line and found them phenotypically diverse, it remained to be determined how one subpopulation would behave when introduced to another. That different subpopulations exist within the same neoplasm is a well recognised phenomenon. However, it has been clearly demonstrated, both in this study and by other groups (Calabresi et al., 1979 ; Dexter et al., 1981 ; Brattain et al., 1981), that subpopulations derived from a parent cell line do demonstrate different growth rates in culture. We must then ask the question as to what enables a tumour to remain heterogeneous if its constituent cell types grow at different rates and/or are responsive to different growth controls? Surely simple logic would dictate that the faster growing subpopulation would outgrow its contemporaries. This is obviously not the case in a primary tumour. We must then look to the mechanisms which enable a tumour to maintain its heterogeneous nature.

It is possible that the processes which generate tumour cell diversity in the first instant do not cease when a certain level of heterogeneity is reached but continue throughout the life of the tumour. Such mechanisms would include an increased genetic instability, somatic cell hybridisation, differentiation of tumour cells and involvement of the host environment, all of which are not necessarily mutually exclusive options. However the possibility of tumour cell interaction is a theory receiving increased credulity and attention (Heppner, 1982 ; Miller, 1983 ; Poste et al., 1984). That tumour subpopulations may exist in such close proximity to one another yet exert no influence on the behavior and survival of other groups is almost an inconceivable thought. Therefore the mechanisms by which subpopulations interact and what effect these interactions incur remain to be elucidated.

Interactions may exert changes in the phenotypic characteristics of a cell subpopulation which enable it to survive where otherwise it would have become extinct in that particular neoplasm. Using subpopulations isolated

from established tumour cell lines, cellular interactions have been postulated to be the cause of changes in immunogenicity (Nowotny and Grohsman, 1973; Miller and Heppner, 1980), clonal instability (Poste et al., 1981), metastatic ability (Miller, 1983), sensitivity to drugs (Miller et al., 1981), in vitro growth (Heppner et al., 1980 ; Isacke and Deller, 1983 ; Miller et al., 1984) and in vivo growth (Miller et al., 1980 ; Leith et al., 1987).

The subpopulations isolated from the HCA-7 cell line provide a unique system with which to study the possibility and diversity of interactions occurring between cell types, as the extensive range of phenotypic variants available is unprecedented. As the putative effects of clonal interactions are so numerous, as are the possible combinations of subpopulations, it was chosen to initially investigate the effect of interactions on the growth of the subpopulations. To this end, two subpopulations were grown individually and in an equal mixture to ascertain whether inhibition or acceleration of growth of one subpopulation was induced by the other.

The growth of the co-culture was predicted by taking the mean of the DNA values of the two subpopulations at each time point and constructing a growth curve from these values. This method of predicting growth was not ideal however it provided an effective method of screening the subpopulations for possible interactions.

The growth of the majority of combinations of subpopulations examined did not deviate to any great extent from the predicted path of growth. Although, in these cases there was no alteration in the growth of the cells, this does not mean to say that interactions were not taking place which may have altered other phenotypic characteristics of the subpopulations. However the co-culture of HCA-7 cells and col 3 cells showed an increase in growth rate above that predicted from their individual growth rates. As col 3 were the faster growing cells, it was assumed that they were interacting with the HCA-7 cells in a way which increased the growth rate of the parent line cells. To confirm this theory it would have been ideal to have a marker with 100% or

0% expression by the cells in order to determine which cell type was affected by the interaction. Unfortunately no such marker was available.

It then remained to elucidate how these cells interacted to alter the combined growth rate. It is possible that the interaction was contact-mediated as the subpopulations mingled with each other in the co-culture. Contact-mediated interactions have been demonstrated in a metastatic mouse mammary tumour cell line (Miller *et al.*, 1983) which may affect growth and drug sensitivity within a tumour. Lowenstein (1980) has discussed the possibility that cell-to-cell channels are routes for the passage of molecules necessary for growth control in normal cells. This theory may be applied to explain how malignant cells communicate with each other to maintain tumour heterogeneity.

Alternatively, it could be that the col 3 cells release a soluble mediator which exerts an effect on the HCA-7 cells. To test these theories, each cell type was 'fed' with medium which had been in contact with one or the other cell line, so that the only communication between the cell lines was via the conditioned media. This, however, was not an ideal method for testing for the presence of soluble factors, as the conditioned media had to be first filtered to remove whole cells and cellular debris. Although filters were primed by passing through foetal calf serum in order to occupy any possible binding sites present, it is feasible that any soluble factor within the medium could be lost on the filter. In addition, if such a soluble factor exists it may only be transiently active so that activity would be lost in the time taken to prepare the conditioned media. Another problem was that of loss of nutrients in the conditioned media. As the medium had been in the presence of cells for 24 hours prior to the application to the test cells, it is conceivable that a considerable amount of essential nutrients could be lost. In an attempt to combat this, conditioned media was mixed with fresh media before filtering and applying to the test cells. However a deficiency in nutrients would still remain in the conditioned media compared to that in fresh media.

Even with these problems in tow, significant differences were observed in the amount of DNA present at the end of the time course in wells of both HCA-7 cells and col 3 cells fed with conditioned media compared to those when fed with fresh media. The amounts of DNA present in wells of HCA-7 cells were less when fed with media conditioned by either cell type compared to fresh media. However, more DNA was present in wells fed with col 3 conditioned media than those with HCA-7 conditioned media. When these media were placed on col 3 cells an increase in DNA was observed in the presence of HCA-7 conditioned media and a decrease with col 3 conditioned media. It would therefore appear that inhibition of the growth of HCA-7 cells occurs in the presence of conditioned media though the effect is less so when using col 3 conditioned media. However the growth of col 3 cells is increased by HCA-7 conditioned media suggesting that a factor is released by HCA-7 cells which is beneficial to col 3 cells but detrimental to its own growth. These results may suggest that, in contrast to the proposed theory that col 3 cells interact with HCA-7 cells to increase the growth of the parent line, it is in fact the HCA-7 cells which exert an effect which further increases the growth of col 3 cells. In order to obtain a clearer picture of these events the utilisation of transwells came into force whereby cells could be grown contemporaneously in the same well without contact of the two cell types. Transwells have previously been used in permeability studies, as these polycarbonate membranes allow nutrients and other factors to reach the basolateral surface of the cells in the transwell inserts (Hidalgo *et al.*, 1989).

In this study the results from the transwells confirmed the results obtained from the conditioned media experiments very accurately. The only discrepancy which occurred was when col 3 cells were grown in the presence of HCA-7 cells whereby a lower amount of DNA was present compared to the same cells grown in the absence of any other cells. However there was still a greater amount of DNA when in the presence of HCA-7 cells compared to its own cell type.

Further experimentation is now needed to determine what factor(s) is released by the HCA-7 cells which increase the growth of col 3 cells, and by what mechanism(s) it exerts its effect. Also further examination of the remaining subpopulations is needed to determine whether or not any one of these releases such a factor as to produce similar effects. It must be remembered at this stage that the HCA-7 parent line is a mixture of many different cell types. It remains to be demonstrated whether the cells which interact to produce such an effect have been isolated from the parent line. It may be the case that it is one particular subpopulation of the HCA-7 cells which releases this soluble factor which was not tested or indeed has not been isolated in this present study. It could also be that contact with other cell types is needed for a particular subpopulation to release growth enhancing or inhibiting factors which exert their effects on other subpopulations. It is however blatantly obvious that the mechanisms involved in growth control within a heterogeneous tumour are complex and probably multitudinous.

Colorectal Carcinoma Cell Heterogeneity.

That cellular variants occur both between individual colorectal tumours and within them is now a well known and accepted fact. This cellular diversity is one possible reason for the lack of success in the treatment of the disease. It is with this thought in mind that several groups have directed their research towards unravelling the mysteries of what mechanisms are involved in the generation and maintenance of heterogeneity.

The vast majority of these investigations have involved the study of cell lines which have been derived from primary colorectal carcinomas. The use of such in vitro systems is obviously not ideal due to both the introduction of factors and conditions not present in the in vivo situation and also the absence of the influence of host factors. Setting aside these disadvantages of the in vitro system, we are left with an extremely manipulable model of colorectal tumours which allows close examination of the characteristics of individual cells and their behaviour. How well these in vitro features extrapolate back to the behaviour of cells in human tumours is uncertain. However, in vitro systems are increasingly being used to investigate the effects of cytotoxic treatments. The colorectal carcinoma-derived cell line HCT-8 has been used to assess the antagonism which occurs between 5-fluorouracil and mitomycin C in the clinic which has lead to further clinical trials testing a different regimen of administration (Russello et al., 1989). Similar work on the HT-29 cell line has investigated the efficacy of 5-fluorouracil and N-methylformamide in combination therapy (Zupi et al., 1988).

In order to bridge the gap from the in vitro to the human situation, the use of immunodeficient animal hosts has been invaluable. In this situation the colorectal carcinoma cell lines are in an environment more closely related to that from which they came, allowing more insight into the complexity of their natures. Using such a model Leith et al. (1988) demonstrated that the composition of artificially heterogeneous tumours, produced by the inoculation of two subpopulations of a colorectal carcinoma cell line, is altered after

treatment with mitomycin C. The contribution of in vitro and consequently in vivo systems in predicting the effect of clinical therapies of primary colorectal carcinomas is quite apparent.

Not only have cell lines been utilised in the investigations of colorectal carcinoma biology and treatment of the disease, they have also provided a model system with which to study the mechanisms controlling normal cell differentiation in the colon as the majority of cell lines retain some of the properties exhibited by the normal colorectal epithelium. Therefore, although not ideal, in vitro cell lines derived from colorectal carcinomas have provided us with models to investigate both normal and neoplastic cell behaviour.

Many colorectal carcinoma cell lines have now been documented. However, precious few of these exhibit the heterogeneity characteristic of many primary colorectal carcinomas, limiting the bounds to which our knowledge of heterogeneity can reach. Until now the number of subpopulations isolated from a single primary colorectal neoplasm or cell line has only reached two (Dexter et al., 1981 ; Brattain et al., 1983) or three (Brattain et al., 1981. Using flow cytometry, Hiddemann et al. (1986) studied the heterogeneity in DNA stemline number of 88 human colorectal carcinomas. Their results indicated that approximately 30 - 40% of all carcinomas contained two or more stem lines. Therefore the need for a more extensive range of subpopulations is quite apparent.

This study has presented such a model system. From one parent cell line, HCA-7, derived from a single human primary colonic adenocarcinoma, eleven subpopulations have now been isolated, nine of which have been extensively characterised. Not only have these subpopulations been shown to differ in their in vitro morphology, on which characteristic they were isolated, but also their modal chromosome numbers, in vitro growth characteristics, antigen expression, transport properties and in vivo behaviour as xenografts in nude mice.

That the HCA-7 cell line is heterogeneous at early passage, which is later lost, suggests that the parent cell line represents a co-culture of the cell types present in the original tumour which itself was morphologically heterogeneous. This also suggests that the subpopulations, which were isolated from early passage HCA-7 cells, are also representative of the cell types present in the original tumour and not a product of adaptation to in vitro conditions. It is possible that the original tumour, from which the cell line was derived, was itself of polyclonal origin, lending a simple explanation to the results presented here in. However, if monoclonal origin is assumed, we are then presented with the resulting cell types of the generation of cellular diversity.

With regard to the generation of heterogeneity, the HCA-7 cell line and subpopulations have provided indications of the mechanisms involved. The simple fact that these cell lines have different modal chromosome numbers supports the theory of increased genetic instability. The cells of each subpopulation also displayed different degrees of differentiation, suggesting that factors akin to those controlling normal cell differentiation may be involved in the generation of diversity.

Possibly more important is the potential of some of these subpopulations to produce a morphological heterogeneity in the resulting tumours when xenografted into nude mice. Interpreting this observation, it is possible, if a little presumptuous, to suggest that the HCA-7 subpopulations represent a potentially invaluable model to study the origins of tumour cell heterogeneity. It is obvious, however that more work is needed to develop such a model system from these subpopulations.

The HCA-7 cell line and subpopulations have also provided evidence that non-contact mediated interactions occur between cell lines which affect their growth. Such interactions may play an important role in the maintenance of heterogeneity once it has been established. The different antigenic properties of the subpopulations may indicate different immunogenicities of the cells,

also providing an insight into the mechanisms by which a tumour retains its heterogeneous character.

It is noteworthy, that in all the characterisations performed on the parent line and subpopulations, no one feature is expressed equally by all the cells of each subpopulation. Regarding the treatment of colorectal neoplasms with respect to heterogeneity, would not a better approach to be to investigate the possibility of a common feature of the subpopulations in order to direct therapy at such a target. The search for a characteristic expressed by all the subpopulations of a tumour may be futile if no such peculiarity exists and indeed results obtained from the HCA-7 model suggest that the extensive diversity present within tumours predict that such a search would be long and arduous, if success is to be obtained. Another approach to therapy receiving increasing recognition is the induction of differentiation of carcinoma cells (Pierce and Speers, 1988 ; Otaka et al., 1989). Again the HCA-7 cell line and subpopulations provide a range of cell types at different states of differentiation where such approaches could be tested.

The potential of the model of colorectal carcinoma cell heterogeneity developed in this study is enormous. It has been possible so far to extensively characterise some of the different cell types present within a single neoplasm. It has also been established that non contact-mediated interactions can occur between individual subpopulations that alter their growth rates. What is clear is that this system has contributed much to the understanding of the extent of tumour cell diversity and may in the future aid in the explanation of the mechanisms involved in the generation and maintenance of heterogeneity within colorectal neoplasms.

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Copied from :- The Journal of Pathology, February 1989, Volume 157, Number 2, Page 170A

Heterogeneity of a human colonic adenocarcinoma cell line.

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Colorectal carcinoma is one of the most common cancers in the U.K. and like many other carcinomas can display cellular heterogeneity. The HCA-7 cell line established from a primary human colonic adenocarcinoma retains this characteristic heterogeneity and provides a model system with which to study this phenomenon. The aim of this work was to isolate some of the subpopulations observed in the HCA-7 cell line and investigate their biological characteristics. Nine subpopulations designated col 6, col 11, col 17, col 23, col 24, col 26, col 27, col 29 and col 30, were established from the HCA-7 cell line. This was achieved by sparsely seeding HCA-7 cells and selecting colonies of differing morphologies which were individually grown as cell lines and have each maintained their characteristic morphology *in vitro* for at least 15 passages. The number of subpopulations isolated from the HCA-7 cell line now totals 11 including the previously established col 1 and col 3 lines. *In vitro* growth rates and saturation densities of the subpopulations differed from the parent line. The majority of the subpopulations had a faster growth rate than the parent cell line but col 30 grew slower than the HCA-7 cells. Xenografts of the subpopulations were established in athymic nude mice (NIMR). These tumours were removed and processed for light and electron microscopy. A wide variation in tumourigenicity was demonstrated, the time taken for tumours to form ranged from between 2 weeks for col 1 cells and 27 weeks for col 6 cells. Marked differences were observed in the structural organisations of the xenograft tumours produced. Some xenografts contained many gland-like structures whilst others were poorly differentiated. Therefore, in addition to their differences in morphology, the nine new subpopulations have been shown to differ both in their *in vitro* growth characteristics and behavior as xenografts in nude mice. Further characterisation of these cell subpopulations and study of interactions between them will increase our understanding of the generation and maintenance of heterogeneity within carcinomas.