THE UNIVERSITY *of* EDINBURGH

Title	In vitro augmentation of carcinoembryonic antigen expression in colorectal cancer cells
Author	Collie M. H. S.
Qualification	MD
Year	1999

Thesis scanned from best copy available: may contain faint or blurred text, and/or cropped or missing pages.

Digitisation notes:

- Page number 38 skipped in original.

Scanned as part of the PhD Thesis Digitisation project
http://libraryblogs.is.ed.ac.uk/phddigitisation

IN VITRO AUGMENTATION OF CARCINOEMBRYONIC ANTIGEN EXPRESSION IN COLORECTAL CANCER CELLS

M H S COLLIE

ROYAL FREE HOSPITAL

POND STREET

LONDON

Presented for: DOCTOR of MEDICINE

UNIVERSITY of EDINBURGH, 1998

DECLARATION OF ORIGINALITY

I hereby declare that the work presented in this thesis is original, and was undertaken by MHS Collie under the supervision of Professor MC Winslet.

Signed MHS Collie MBChB FRCS

MD THESIS ABSTRACT

Introduction: Carcinoembryonic antigen (CEA) is an oncofoetal antigen heterogeneously expressed by colorectal cancer cells, which may provide a useful target for antibody guided tumour localisation and therapy. The efficacy of tumour localisation would be improved by increased, more homogeneous CEA expression. Colorectal cancer cell lines are known to show augmentation of CEA expression on exposure to various chemical agents or to changing physicochemical environments.

Aim: The aim of this work was to find chemical or environmental factors which successfully induced increased CEA expression in 3 different colorectal cell lines, Lovo, Ht29 and Colo, which are respectively high, low and non-expressors of CEA. Studies of differentiation were also undertaken, by observing concomitant changes in cell proliferation and morphology.

Methods: The three cell lines were cultured in medium containing differentiating agents (Butyric acid, gamma-Interferon and 5-Azacytidine and Theophylline), or a range of commonly used cytotoxic drugs at a set dose, or in conditions of altered environmental factors (pH, temperature, oxygen supply, radiation, and serum content of medium). According to the observed effects of these factors used singly, combinations of chemical and physicochemical factors were also tested for additive or synergistic reactions.

The growth, differentiation characteristics and CEA expression parameters were measured by a combination of Electron Microscopy, Immunocytochemistry and Fluorescein Activated Cell Sorting. Membrane CEA expression and total CEA content were compared. In addition, the degree of release of CEA by the growing cells into their medium was measured by Radioimmunoassay.

A limited investigation of the genetic events which accompanied observed differentiation and CEA expression was conducted by immunostaining for oncogene products known to be associated with proliferation, apoptosis or tumour progression, e.g.,p53, Bcl-2, K-ras, and c-myc.

Results: The differentiating agents were found to induce different effects in the three cell lines, with only the Ht29 cells showing increased CEA levels with each agent. It was noted that CEA expression was seen to rise in the Colo cells with two agents, using the more sensitive FACS analysis (Wilcoxon Rank Sum test).

5-Fluorouracil, hypoxia, serum-depletion and radiation induced increased CEA in some cells and not in others; Chloroquine, Methotrexate and Taxol were universally successful CEAinducers. Of the combinations, Butyric Acid plus Interferon was particularly effective, causing potentiated augmentation of CEA in Lovo and Ht29 cells.

Increases in CEA were frequently accompanied by significant growth inhibition (T-test, Correlation co-efficients –0.4, -0.54 & -0.57 for Lovo, Ht29 and Colo respectively); but were not associated with significant increases in CEA release into the supernatant, as measured by Radio-immunoassay.

Electron Microscopy studies showed mixed responses to the various agents, with signs both of cell damage and of improved differentiation.

No striking changes in oncogene expression by Ht29 cells exposed to successful CEA-inducers were observed; very slight changes in Bcl-2 or c-myc with 3 of the agents were noted.

Conclusions:

Apparent non-CEA expressing cells may be induced to express CEA using various agents.

Different colorectal cell lines respond to chemical and environmental changes to different extents, via different mechanisms.

Potentially useful CEA-inducing agents include Butyric Acid, Interferon, Theophylline, 5-Azacytidine, Chloroquine, 5-Fluorouracil, Methotrexate and Taxol.

The combination of Butyric Acid and Interferon is particularly powerful in CEA augmentation.

Increases in CEA expression are often accompanied by decreases in cell proliferation, but not by increases in CEA release.

Augmentation of CEA expression may be accompanied by morphological features of cell damage or of improved differentiation.

CHAPTER ONE: GENERAL INTRODUCTION

COLORECTAL CARCINOMA

The impact of colorectal carcinoma can scarcely be underestimated: 20 000 people die from it every year in the UK alone (*260,124*). The average age of the patients is 60 years and the incidence increases with age. Men and women are equally susceptible. The geographical distribution of the disease makes it predominantly a disease of the Western World. There is a high incidence in the UK, Western Europe and North America, a low incidence in Asia and South America and only rare occurrences in Africa and Japan. It has been found that migrants acquire the respective risks of the areas to which they move. It has also been found however, that some genetic populations have very different risk levels than others in the same environment, e.g. Maori's and nonMaori's in New Zealand (*5,78,133*).

ONCOGENESIS OF COLORECTAL CARCINOMA

The Neoplastic Process

Neoplasia as defined by Willis (*272)* is a combination of disturbances in cell proliferation, differentiation and the relationship between a cell and its surroundings. The mechanisms by which abnormalities in these three areas can arise, instigatiing the growth of a tumour, are multiple and

include inherited gene mutations, random mutations, and viral or physico-chemical induced oncogenesis.

The genetic model of Colorectal Carcinoma proposed by Fearon and Vogelstein (*91*) provides a clearly understandable theory of carcinogenesis as it occurs in the colon and rectum. In order to construct the full model, the following concepts need to be accepted:

1. There is a progression from adenoma to carcinoma with most if not all carcinomas starting as adenomas.

This concept was first explored in detail in 1975 by Muto et al (*201*). He studied 2231 benign polyps and 275 malignant carcinoma specimens and the associated clinical histories, and noticed that:

a. There was a development from mild to severe epithelial atypia, with an increased incidence of malignancy in specimens showing severe epithelial atypia.

b. There was an increasingly frequent incidence of benign tumour around the spreading edge of invasive tumour.

c. Patients with the autosomal dominant condition Familial Adenomatous Polyposis (FAP) showed a progression from having small benign polyps to invasive carcinomas over a period of 12 years on average. (The genetics of FAP will be more fully discussed later in this introduction).

d. Performing a total colectomy, or removing rectal polyps by fulguration prevented the development of rectal carcinoma.

e. The risk of having colorectal carcinoma varied directly with the number of polyps present.

He concluded that although not all polyps become invasive carcinomas, all carcinomas originate from polyps. This conclusion was ratified by several authors including Kelvin et al (*149*), who quantified the risks of invasion in adenomatous polyps as:

> 10% if diameter > 2cm

5 - 10 % if diameter 1 - 2 cm

Negligible if diameter < 5 - 6 mm

It should be noted that the risks of invasion in villous polyps were greater than in tubular polyps. Pertinent to the science underlying these figures may be the findings of Vogelstein et al in 1988 (*262*), that the number of ras oncogene mutations increases in proportion to the diameter of adenomas; and those of Fearon et al in 1987 (*93*) that monoclonal patterns of chromosome inactivation may be identified in both adenomas and carcinomas. This leads onto the second concept:

2. Colorectal Carcinoma arises due to the mutational activation of Oncogenes plus the mutational inactivation of Tumour Suppressor Genes.

Oncogenes and Tumour Suppressor Genes

It is now recognised that a single malfunctioning gene may upset the systems of cell cycle control, cell growth and differentiation *(145,263*): aberrant regulatory genes may initiate and maintain cell transformation (oncogenes, first described in 1910 by Peyton Rous (*140*) or may fail to suppress the transformation of the cell (the normal function of tumour supppressor genes, first described in 1971 by Knudson (*155*). This balance between positive and negative genetic stimuli was first forecast by Boveri in 1914 (*45*).

Those cellular genes with the potential to transform the cell are known as proto-oncogenes, and the aberrant genes they become on activation as oncogenes (*37*). They can be subdivided into nuclear or cytoplasmic according to their mechanism of action, and may be activated by viral, chemical or physical agents. Additionally, it is possible to have a hereditory predisposition for activation of oncogenes (*140*).

Viral Activation: (I) **DNA**, eg papilloma viruses. These encode a protein which binds to a growth regulatory peptide, disabling it.

(ii) **RNA**. These may transduce cellular proto-oncogenes, cause insertional mutagenesis or transactivation of a cellular gene.

(iii) **Retro** viruses, eg HTLV, HIV. These may initiate an autocrine stimulatory system.

Chemical/ Physical Activation: Various chemical agents and other factors such as UV and X-Ray radiation may damage cellular DNA, leading to mutated geno- and pheno- types. Alternatively, gene amplification may occur, with intra- or extra-cellular influences inducing overexpression of an encoded oncogene product.

Hereditory predispositions: these include abnormalities of chromosome number or form.

One of the important oncogenes in colorectal carcinoma is ras. Mutated ras oncogenes were reported to be capable of transforming primary cultured cells by Bos et al in 1987 (*42*). Hybridisation work performed by the same team found ras mutations in 11/27 colorectal tumours studied. These findings were repeated by Vogelstein et al in 1988 (*262)*, who found that single base pair ras mutations could cause cell transformation, and were seen in 40-50% of colorectal tumours.

Tumour Suppressor Genes:

The evidence for the existence of tumour suppressor genes was gathered from the study of cell hybrids and of familial tumours:

Hybrids of tumorigenic and non-tumorigenic cells did not necessarily result in immortality in vitro or tumour formation on transfection, suggesting an internal tumour regulation system, dominant over genes inducing malignant transformation.

Familial tumours including Familial Adenomatous Polyposis, Retinoblastoma and Neurofibroma 1 were found to have in common a mutation on Chromosome 17. Loss of heterozygosity on 17p was found to commonly occur in Breast, Lung and Colon carcinoma, suggesting the loss of a cell regulatory gene important to suppress tumours in various types of cell (*189*).

Tumour suppressor genes should induce 3 responses: G1/S phase arrest, end-stage post-mitotic differentiation and apoptosis (*140*), by activation of transcription or by blocking the interaction of DNA polymerases with the rest of the DNA replication complex. Loss or inactivation of tumour suppressor genes prevents the cell from following one of these pathways when appropriate.

In the colorectum, the most crucial tumour suppressor gene found to date is p53, which was first identified by Baker et al in 1989 (*15,16*). She described a common allelic loss on 17p in > 70% of colorectal carcinomas, with a very different p53 protein associated with these tumours than that found in normal cells. The conclusion was that a mutant, ineffective form of p53 was present in the tumours, with resulting non-suppression of transformed cells.

Another important regulatory gene in the colorectum is DCC ("Deleted in Colorectal Carcinoma") which was cloned by Fearon et al in 1990 (*92*), is found on chromosome 18q and normally encodes a cell adhesion molecule (CAM). This cell surface CAM regulates cell behaviour through interactions with surrounding cells; deletions on 18q therefore remove this control on cell behaviour, leading to the possibility of invasion. A high proportion (73%) of colorectal carcinomas has been found to have allelic deletions in chromosome 18 (*262*).

A further significant site of allelic deletions in colorectal cancer is on Chromosome 5, which is particularly prevalent in Familial Adenomatous Polyposis (*262*). Bodmer et al in 1987 used DNA probes to identify the specific gene associated with FAP, on chromosome 5q21-22 (*40*). Some 20% of colorectal cancers, without FAP, also show allelic deletions on chromosome 5.

3. Four - five genetic changes are required to form a tumour, occurring in any order.

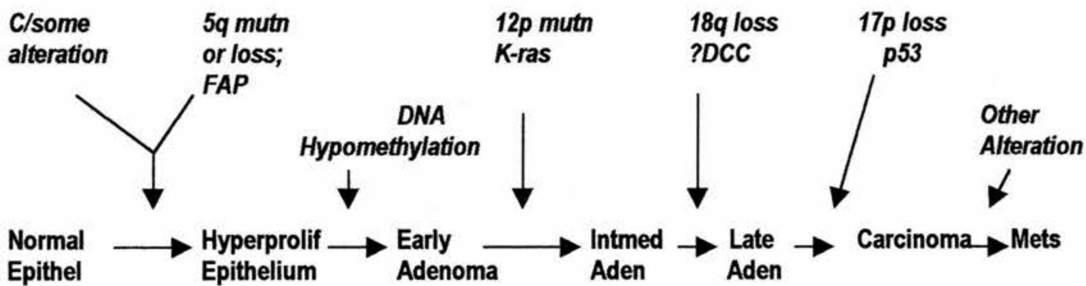
Carcinogenesis in general has been found to be a multistep process (*17*). In the colon and rectum, over 90% of carcinomas have previously been shown to have ≥ 2 genetic alterations (*262*). The form

of these genetic alterations may be at the nucleotide or chromosomal level, termed microsatellite and chromosomal instability respectively (*169*). The two types of genetic instability may both be present in one tumour.

Studies of genetic mutations by Sasaki et al in 1989 found evidence of loss of heterozygosity on 15 different chromosomes in patients with FAP, and on 8 different chromosomes in patients with non-Polyposis associated Colorectal Carcinoma (*234*). It has already been noted above that the activation or amplification of oncogenes such as ras may be necessary for the development of full-blown carcinoma in Polyposis patients.

An interesting study by Delattre et al in 1989 found that distal colorectal carcinomas showed a far greater preponderance of deletions or mitotic abnormalities, (e.g. hyperdiploidy), on chromosomes 5, 17 and /or 18, than was evident in proximal colorectal carcinomas, although ras mutations occurred consistently throughout the colon and rectum (*70*).

In summary, the constructed model is as follows:



EPIDEMIOLOGY of COLORECTAL CANCER

There is much debate over the epidemiological factors which may or may not alter the risk of developing colorectal carcinoma. Bowel flora variations in particular have been studied: high risk populations have a greater proportion of Bacteroides to aerobic bacteria, but the increased degradation of bile salts this causes has not been shown to be carcinogenetic per se (*5,201,138*).

Several conditions are known to confer a higher risk of colorectal cancer, including Familial Adenomatous Polyposis, multiple Adenomas, Gardner's Syndrome and Ulcerative Colitis (*149*). In FAP, the risk of developing cancer borders on 100%. Adenomas in patients who do not have FAP have a variable risk level for invasion (*149,201*). The risk varies most significantly with the size of the adenoma, but also with histological type - villous adenomas, which are the least common, carry a higher risk of invasion than tubular adenomas. Gardner's Syndrome is characterised by the presence of polyps of multiclonal origin (*144*).

The risk of developing adenocarcinoma of the colorectum in patients with Ulcerative Colitis increases with the time-span of the disease, becoming greater than 12% at 20 years, on average.

Other less common predisposing factors in the development of colorectal carcinoma include radiation and ureterosigmoidostomy.

CLINICAL PICTURE:

Macroscopically, colorectal cancer may take the form of a protuberant mass, an ulcer or a solid penetrating tumour. The typical presentation varies with the position of the cancer: right-sided tumours tend to present as a mass, or with anaemia, left-sided tumours with obstruction and rectal tumours with fresh bleeding or tenesmus (*78*).

Microscopically, by far the most common type of cancer is the adenocarcinoma, which is the histological tumour type studied throughout this work.

SPREAD:

As with most tumours, colorectal cancer may spread in several ways: directly, via the blood stream, portal or systemic, via the lymphatic system or across the peritoneal cavity. Often there is an element of all of these at presentation. The degree of spread through the bowel wall and into the lymphatic system correlates with the prognosis, as first observed by Dukes (*85*). More precise recognition of small hepatic and extrahepatic metastases has led to an improvement in the accuracy of this staging system (*268)*.

TREATMENT:

Surgery is the best treatment option for this disease. The first rectal excision for carcinoma was performed by Von Volkermann in 1878. Unfortunately, in recent years there has been no change in the overall survival for patients with colorectal cancer, being only 50% at 5 years (*200*). This is partly because of the relatively small proportion of tumours picked up when still locally confined and thus "curatively" excised - 30% of patients present initially with advanced metastatic disease (*244*). An additional problem is that in the USA, one third of patients who have a so-called curative resection suffer a recurrence of the disease (*10*). Non-surgical treatment, i.e. Radiotherapy and Chemotherapy can slow the progression of the disease, but has not yet been shown to improve overall survival.

A different approach to treatment may be called for, in order to specifically target the tumour cells - thus pursuing the predictions for cancer treatment in this century made by Ehrlich, with his "Magic Bullet Theory" (*67*). In order to consider cancer treatment at a cellular level, an indepth study of individual neoplastic cells is required:

Normal cell development involves differentiation from stem cells to mature cells. In neoplasia, differentiation is suppressed genetically, so that the cells are "frozen" at an immature stage. This may cause the expression of an altered phenotype. Differentiated cells express and secrete cytostructural proteins. Neoplastic cells express particular cell-surface proteins or antigens. Some of these antigens are promoters of adherence, which may be a necessary property of invading cells (*188*). Carcino-embryonic Antigen is an example of a cell-surface antigen which is commonly expressed by moderate to well-differentiated colorectal carcinoma cells and has a very important role to play in cancer detection and therapy.

CARCINO-EMBRYONIC ANTIGEN

Carcino-embryonic Antigen, (CEA), was first described by Gold and Freedman in 1965 (*117*). Using the Ouchtertony technique of double diffusion, they demonstrated a high degree of antigen cross-reactivity between normal rabbit sera with pooled human sera and rabbits immunised with human tumour extract. They repeated the technique to look for the antigen in 322 different tissues - normal, fetal and malignant(*116*). The antigen was positively identified in fetal tissues and cancers originally derived from the areas of the gastrointestinal tract which are of stomatodeal origin. They therefore coined the name Carcino-embryonic Antigen. Since that time, CEA has been extensively studied and has also been identified in non-endodermally derived cancers and normal tissues, including lung and breast and in non-cancerous tissues from the gastrointestinal tract (*270,197,193*). Serial grafting of tumour cells in animal hosts has proved that the CEA expressed in these tumour cells arises from the tumour cells, and not the hosts (*123*).

Improved methods of detection of CEA have been developed (*104,279*) and include:

Farr or ammonium sulphate technique

Z-gel technique

Radioimmunoassay - specific, non-specific or cross-reactive

CEA can be purified for close study by a combination of perchloric acid/ lithium diiodosalicylate extraction, column or ion exchange chromatography and electrophoresis (*104*).

Structure:

Purified CEA has been subjected to a battery of investigations including acid hydrolysis, enzymatic hydrolysis, periodic oxidation and neuraminadase oxidation (*104*). These methods have allowed a detailed knowledge of the structure of CEA to be built up. CEA is a complex glycoprotein of 180

000d molecular weight (*256*). It displays heterogeneity in terms of its protein:carbohydrate ratio, and the concentrations of amino acid residues, sugar residues and sialic acid (*17*). Colonic carcinoma CEA varies from other tumour CEA in these areas.

In general, the carbohydrate portion forms 50 - 65% of the total molecule. This further divides into:
N-acetylglucosamine (v40%)

Fucose, mannose, galactose, sialic acid (variable amounts)

N-acetylgalactosamine (minimal amount) (*104,23,17*)

These sugars are structured into linear or branched oligosaccharide chains of variable length. The polypeptide part forms 35 -50% of the molecule. The amino acid residues are in chains with 7 extracellular domains, each having a sequence similar to immunoglobulin and intercellular adhesion molecule domains. The sidechains of the amino acids have terminal COH and OH groups (*23,112*).

Colonic carcinoma CEA has a carbohydrate fraction of 49.5 - 63.7% and a fixed amino acid fraction. Compared to gastric carcinoma CEA, there is only one fifth as much sialic acid and a higher concentration of N-acetylglucosamine (*17*).

The carbohydrate and polypeptide parts of CEA are linked by peptide bonds or O-glycosidic linkages. In addition, there are intra-oligosaccharide chain disulfide bonds (*23,277,112*).

Location:

CEA is found free in serum, bile and intestinal contents and attached to various cells (*256,139,175*). Its exact cellular position when it is expressed, has been determined by a number of methods: Ultraviolet light studies of fluorescein-conjugated rabbit anti-CEA serum demonstrated that CEA localised to the plasma membrane (*114*); studies with ferritin conjugated goat anti-CEA antiserum confirmed the localisation of CEA to the plasma membrane and further specified its location to be on the glycocalyx coating the cell surface membrane (*182,113*).

There is a heterogeneity of CEA expression in seemingly identical cells. This has been illustrated by studies of HT29 colorectal carcinoma cells where homogenous anti-CEA antibody penetration was shown by freeze-drying, fixation and autoradiography, and yet the binding of these antibodies to the cell-bound CEA was very heterogenous *(55)*. Several foci of genes are thought to control the degree of expression of CEA, with variation at transcriptional, post-transcriptional and DNA methylation stages *(44)*. The variation produced in the phenotype is mainly in the membrane-bound CEA fraction, rather than in intracellular CEA *(239)*. An important discovery in studying tumour cell CEA expression, was that expression varies with tumour cell differentiation: there is a higher degree of CEA expression in well-differentiated cancer cells compared to poorly differentiated cells *(41,72,125,41)*. The relation between CEA expression and differentiation is complex.

Circulating CEA was first demonstrated in 1969 by DMP Thomson et al *(253)*, using a radioimmuno-assay technique. Normal levels of serum CEA in humans are usually taken to be 0 - 2.5ng/ml, and do not vary with sex, race, age or diurnal rhythm *(276,110)*. Levels are raised in many disease states - both benign and malignant. As a general rule, a level of circulating CEA of 5.0ng/ml has been taken to mark a cut-off point between benign and malignant disease, but this is not without exceptions.

<u>Disease States with Raised CEA:</u>

<u>Benign</u>: Inflammatory Bowel Disease (92% patients with extensive Ulcerative Colitis) *(256,106,192,276)*

Gastritis and Peptic ulcer disease *(256)*

Pancreatitis, especially alcoholic *(256,173,276)*

Alcoholic liver disease and other liver diseases *(256,276)*

Renal disease and Uraemia *(197)*

Benign Prostatic Hypertrophy *(256)*

Bronchitis and Emphysema (*255*)

Benign Colorectal Polyps (5 - 10% only) (*256,276*)

Malignant: Carcinoma of the Gastrointestinal tract, Lung, Breast, Pancreas, Prostate, Thyroid and female genitourinary tract (*256,174,197,173,71,276*).

In addition, serum CEA levels have been noted to be raised in cigarette smokers, without necessarily having any of the pathological conditions listed above (*256*).

The raised levels of serum CEA in various liver diseases is thought to be due in some part to a slowing of the metabolism of CEA, which is generally accepted to occur via the liver, with a normal half-life of 1 - 8 days (*256,175*).

Antibodies to CEA have also been detected in the circulation - in approximately 70% of patients with gastrointestinal cancer. Large volume tumours have been reported to have lower levels of circulating anti-CEA antibodies, which may be due to saturation by the CEA released by the tumour tissue, but other studies have reported no difference in circulating CEA antibodies with differing tumour size (*125,274*).

Function of CEA

CEA is thought to have a role to play in intercellular recognition and adhesion. Cell lines studied for fluorescein labelled CEA were found to display properties of aggregation in direct relation to their degree of CEA expression, and to be prevented from aggregating by anti-CEA antibodies (*34*). CEA is also thought to be important in the intercellular organisation of architecture (*34*), particularly in malignant cells. Overproduction of CEA has been shown to be associated with disruption of the normal lateral adhesion within cell layers and with disordered cell architecture (*240*).

Clinical Relevance of CEA:

CEA is a tumour marker. It has an important role to play in cancer detection and treatment as such, although it can not be described as an ideal tumour marker *(24)*. Features of the ideal tumour marker include:

1. The tumour marker is produced or expressed by the diseased cells only, and is not found in health or in benign diseases.
2. It is readily detectable in body fluids.
3. It is present at an early stage in the disease process.
4. It is present frequently enough for efficient screening pick-up of disease.
5. Levels of the tumour marker vary with the amount of tumour bulk
6. Levels of marker alter according to disease response to therapy.

Serum CEA levels have been extensively studied pre- and post- tumour excision, for long term follow-up periods. It has been shown that the pre-operative serum CEA level varies proportionately with tumour spread, and inversely with differentiation and 5 year survival *(275,264,175)*. Throughout therapy, whether it be surgical, by radiotherapy or chemotherapy, CEA levels have been found to mirror almost all disease regressions and most disease progressions *(137,264,248)*. The pattern of CEA levels post-operatively has been found to be particularly informative: stable, elevated CEA levels are highly predictive of incomplete excision, recurrent or metastatic disease; a transient rise is not indicative of residual disease; and a fall to normal levels is strongly predictive of complete excision of disease *(246,182,141,249)*. CEA levels have been found to be more accurate in diagnosing recurrent disease even than CT scanning *(27)*. The National Health Symposium in 1981 declared that serial plasma CEA assays correlated to the stage of colorectal cancer, and were useful in monitoring therapy *(119)*.

With long term follow-up, it has been found that an increase in CEA levels tends to precede clinical evidence of recurrent disease by an average of 9 months. Rising CEA levels have been used as an indication for second look laparotomy in several clinical trials. The results of the recent MRC study are currently awaited.

CLINICAL APPLICATIONS OF CEA - RAID AND RIGS

RADIOIMMUNODETECTION (RAID)

The expression of CEA on tumour cells can be used in the clinical context. Tagging the CEA antigen with labelled antibody leads to the possible identification of tumour deposits which may not be picked up by conventional investigations - X-ray, Ultrasound and Computed Tomography, and therefore to the possibility of cell-specific therapy.

Historically, antibodies were first described as proteins in 1934 (*188*). Ground breaking work with antibody-antigen localisation was performed by Pressman et al. in 1947, who succeeded in preparing anti-tumour sera where Hericourt and Richet had failed in 1895 (*219*). They demonstrated that innoculating rabbits with rat kidney extract and then injecting radiolabelled serum from these rabbits back into rats caused localisation of radioactivity in the rat kidneys. Similar work with mouse Wagner osteosarcoma extract was performed by the same team in 1953. The localisation of labelled antibodies raised against the mouse tumours (in rabbits) to the tumour, which did not happen with non-tumour rabbit anti-mouse antibodies led to the suspicion that tumours bear specific antigens (*68,218,202*).

In 1975, Kohler et al further developed the technique of producing appropriate antibodies from live animal sources to cell culture lines - thus improving the specificity of the purified antibodies to the tumour antigens (*218,158*).

Radiolabelling of antibodies was initially performed using ^{131} Iodine. Subsequently, ^{125} Iodine and ^{111} Indium have replaced ^{131} Iodine. ^{125} Iodine has been found to give a better tumour:tissue ratio than ^{131} Iodine, and ^{111} Indium has been found to be more efficacious in identification of hepatic metastases (*207,26,77*).

The technique of immunodiagnostic scanning was first developed in 1967, when ^{131} Iodine labelled anti-fibrin antibody was injected and external scans used to successfully locate 75% of a mixture of tumours (*210,245*).

Goldenberg et al in 1978 and 1980 first successfully demonstrated radioimmunodetection of colorectal cancer in two series of 18 and 37 patients. The patients were given ^{131} Iodine labelled goat anti-CEA antibody, then scanned by external gamma scintillation photometry. Background radiation was accounted for by digital subtraction. The results of the scans were then compared to the results of conventional investigations and the operative findings. Radioimmunodetection was found to have a sensitivity of 85% and a specificity of 98% in these series (*120,122*). A larger series of 170 patients confirmed these findings, with 93% of tumours successfully located. Tumours which were negative for CEA expression were not identified by RAID, but serum negativity for CEA did not affect tumour localisation (*121*).

The favourable results of Goldenberg were not initially matched by other groups (*180,183*). The main problem was that background radiation was too high and false positives resulted from the excretion of radiolabelled antibody in the reticuloendothelial system. Digital subtraction produced improved scans, but toxicity problems were centred around the high uptake of radioactivity in the reticulendothelial system.

Fragmented antibodies were developed in order to combat the toxicity problems. Removing the Fc portion of the antibody enzymatically to leave F(ab')2 or Fab' fragments meant that there was less non-specific binding of the antibody to Fc receptors and the lower weight of the fragments lead to more rapid uptake by tumours, more rapid excretion by the kidney and thus shorter half-life in the body.

Mach et al in 1981 successfully performed radiolocalisation of tumour using radiolabelled F(ab') fragments (*180*). Subsequent xenograft and clinical trials confirmed the specific localisation of tumours by whole and fragmented antibodies using autoradiography, and demonstrated that there is

improved localisation, decreased toxicity and overall improved efficacy with fragments (*51,52,147,163,166*).

Comparisons between $F(ab')_2$ and Fab' fragments did not reveal significant differences in toxicity or accuracy (*118*). Fab' is cheaper and more readily available, however, and further studies have revealed differences in pharmacokinetics: Fab' distributes more quickly into a larger distribution volume, has a higher interstitium:plasma ratio and is cleared many times faster than whole antibody, with $F(ab')_2$ fragments lying somewhere between the two (*63*).

Recent work has utilised ^{76}Bromine in place of ^{125}Iodine, with good results on PET scans (*177*). Another new antibody imaging agent being researched is "CEA-Scan", which is an anti-CEA Fab fragment labelled with technetium- 99m-pertechnate. External scintigrams taken using this agent were found to give improved images of the pelvis and the extra-hepatic abdomen (*229*). Another approach in immune-guided tumour detection has been to use fluoresceinated monoclonal antibodies and laser immunophotoscanning. A preliminary study with primary colon carcinomas in 6 patients showed successful tumour localisation in each patient with this method (*100*), demonstrating the feasibility of immunophotodiagnosis for tumour detection clinically.

The uptake of radiolabelled whole or fragmented anti-CEA antibody by tumour cells has consistently been found to be heterogenous (*99,2*). Factors affecting uptake were vascular permeability, vascular volume and CEA accessibility. The size of tumours was also found to correlate with fragment uptake (*52,59*), which may be due to these factors and to the rising interstitial pressure in larger tumours, which leads to a pressure gradient from the centre of the tumour outwards, and compromises the vasculature. Altering tumour blood flow with Tumour Necrosis Factor alpha was not found to significantly alter radiolabelled antibody localisation (*213*), although the recent development of a bispecific antibody for CEA and TNFα may improve the localisation and immuno-absorption of TNFα, overcoming some of the problems with its short half-life and toxicity (*228*). Uptake of radiolabelled antibody was also shown to be improved by the administration of mannitol 15 minutes pre-antibody injection in an animal model (*2*).

Studies of human adenocarcinoma spheroids in culture with varying concentrations of ^{131}I anti-CEA antibody revealed a concave curve of increasing radiation with increasing concentration of ^{131}Iodine. A resistant population of cells was thought to account for this - possibly the innermost, potentially hypoxic cells (*164*).

In comparison with conventional investigative measures, radioimmunolocalisation performs well, if the tumours express the appropriate antigens. This may be more of a problem in poorly differentiated tumours, but RAID has been found to pick up poorly diffentiated tumours masquerading as fibrosis on Computed Tomography (*269*). Importantly, comparisons with CT scanning found that positive retroperitoneal lymph nodes, less than 1cm in diameter, which were too small to be picked up by CT, were identified using [^{131}I] monoclonal antibody F(ab' $)_2$ imaging (*63,241*).The pharmacokinetics, dosimetry and accuracy of RAID has been shown to be altered in patients with liver metastases or raised liver enzymes, presumably due to altered hepatic clearance of injected antibody (*32*).

RADIOIMMUNOGUIDED SURGERY (RIGS)

The development of small, hand held gamma detector probes has also improved the efficacy of radioimmunodetection of tumours, and has allowed the introduction of this technique into the operating theatre (3,*76,190*). The probe has a role to play pre-, peri- and post-operatively: Radio-immunoguided surgery means that a presumed "curative" resection of disease as estimated by the human eye or hand may be extended if necessary if small, clinically occult lesions are picked up with the probe. One series found that use of the probe altered the planned surgery in 8/31 patients and another, in 3/16 patients (*207,242*). A trial by Arnold et al found that extra information, especially in staging, was provided by the probe in 29/36 patients (*7*). A combination of pre-operative gamma scan and intra-operative probe scanning has been found to be more accurate than ultrasound, CT and clinical, including operative, findings (*160)*.

Post operative follow-up patients who are noted to have a sudden rise in serum CEA may be suitable for second-look surgery, in which scenario, particularly when there is an absence of clinical symptoms or signs, RIGS is helpful in identifying site of recurrence.

The relation between the expression of CEA by tumours, the uptake of radiolabelled antibody and seropositivity for CEA has been studied. One trial showed no relation between the amount of antibody uptake and the serum CEA (*47*), but a multicentre trial found that 80.3% of CEA positive patients compared to 63% of CEA negative patients had lesions identified by immunodetection (*241*).

THERAPY

Drug or radiation therapy can theoretically be specifically directed to cancer cells by attaching them to appropriate antibodies.

In mouse models bearing human colon carcinoma tumours, ^{90} Yttrium labelled anti-CEA antibodies improved survival and increased cell kill (*146, 54*). ^{90}Yttrium is a trace element which is a beta emitter, and the tumour dose estimated to be delivered in these trials was 34 Gray. A clinical trial where patients with B cell lymphoma were given ^{131} I labelled antibodies against B cells, showed a favourable response in 16/19 patients.

The development of bispecific antibodies has allowed improved efficiency of therapy delivery. The antibodies bind to CEA and also to trace elements, eg Bismuth 211 (*185*), to cytotoxic drugs, eg Melphalan, Etoposide and Methotrexate (*235,214,254*) or to immunologically active chemicals. Animal trials have been promising (*29*); one clinical trial showed subjective improvements only (*53*). In vitro trials of pre-immunising cells with biotinylated anti-CEA monoclonal antibody prior to administration of cytotoxic bound anti-CEA Mab have dramatically reduced the required dose of cytotoxic drugs; animal and clinical trials have yet to be performed, but there is a potential for improving the efficacy of antibody targetted cytotoxic drug delivery (*203*).

Conjugating antibodies to non-competing antigens which induce antibody dependant cell-mediated cytoxicity has been investigated: a clinical trial of 189 patients showed 30% improved survival and 27% fewer recurrances (*19,226,98*). Delayed type hypersensitivity reactions have also been induced using recombinant bacillovirus human CEA (*33*).

A monoclonal antibody with dual specificity for CEA and anionic boron cluster compounds has also been developed. This antibody allows the selective accretion of a high concentration of Boron-10 in tumours for Boron neutron capture therapy (*221*).

Improving the uptake of anti-tumour antibodies is crucial to developing cell-specific therapy, and a first step in acheiving this may be to increase the degree of expression of tumour specific antigens, using differentiating agents.

DIFFERENTIATION

The goal of Differentiation Therapy has been succinctly defined by Ferrari et al (*96*) as: "To restore the physiological balance between growth and differentiation signals, so that tumour cell lose their self-renewal capacity and progress to terminal differentiation or death by apoptosis". Agents which induce differentiation, whether they be natural, synthetic or recombinant compounds, act on the signalling pathways which control the cell's commitment to cell proliferation or terminal differentiation, (mutually exclusive events (*209*)). Work by Leo Sachs on myeloid leukaemia cells showed that it is possible to suppress malignancy using differentiating proteins, without necessarily having to reverse genetic mutations (*231*). Using differentiation therapy as an alternative to conventional cytotoxic strategies is not likely to be a viable option, since the response rate of tumour cells will not be 100%, and remission would not therefore be achievable (*28*). The use of differentiation therapy in combination with other treatment regimes such as tumour targetting and cytotoxic therapy, however is a viable option.

DIFFERENTIATING AGENTS

Several workers have evaluated exposing various tumour cells to various of these differentiating agents, (including polar solvents, Hexamthylene Bisacetamide (HMBA), Butyric acid, Retinoic acid, Interferon and Transforming growth factor), to cytotoxic agents and to changing environmental stresses. This work was initiated in 1971, when mouse erythroleukaemic (Friend) cells exposed to Dimethyl sulfoxide (DMSO) were noted to undergo erythroid differentiation (*103*). The agents which have subsequently been shown to induce MELC differentiation are as follows (*187, 96*):/

POLAR COMPOUNDS:	DMSO, HMBA
FATTY ACIDS:	Butyric Acid
DNA INTERCALORS:	Actinomycin
MODIFIED BASES:	Azacytidine
PHOSPHODIESTERASE INHIBITORS:	Methylisoxanthine
ION-FLUX AGENTS:	Ouabain
ANTIPROLIFERATIVE AGENTS	Interferon, Cytotoxic Drugs
PHYSICAL AGENTS:	Ultraviolet & X-ray radiation

The differentiation in the MELC cells was measured by terminal cell division; the presence of heme-synthesising enzymes and membrane-associated erythroid specific enzymes; increasing α and β - globin mRNA; altered proportions of Hb^{maj} and Hb^{min}; and decreased expression of p53. The translocation of the cells into nude mice caused death unless there was evidence of differentiation in the cells by this criteria.

The National Cancer Institute now recommends a disease-orientated human tumour cell line panel for testing of multiple potential differentiating agents, which may induce a variety of responses in the different target cell types, and may interact with one another in multiple patterns (*101*).

Polar Solvents

Since 1971, the effects of **Dimethyl Sulfoxide** on leukaemia cells and of DMSO and other polar solvents on colorectal cancer cells have been further explored. Friend cells were induced to express an erythrocyte membrane antigen by exposure to DMSO in 1973 (*75*). The Human Acute Myeloid Leukaemia cell line HL60 grown with DMSO in 1978 was found to show increased functional activity (phagocytosis activity was elevated from 10 to 90%); with changes in morphology to terminally differentiated cells, and growth inhibition at concentrations of DMSO of >1.5% (*61*). HL60 cells

grown in the presence of DMSO have also been shown to undergo genetic changes, showing decreased expression of MPO and c-myc oncogenes, which are associated with differentiation and proliferation respectively (*4*).

Human colon cancer cells were first grown in the presence of N,N dimethyl formamide (DMF) by Dexter et al in 1978 (*75*). Cell lines DLD-1 and HCT-15 were used. DMF was found to inhibit clonagenicity, induce changed morphology and increase the membrane CEA expression, as measured by fluorescent antibody assay. In addition, xenograft inoculation studies were performed and cells pretreated with DMF found to show much less tumorogenicity than nontreated cells. Subsequent studies agree with the growth inhibitory properties of these polar solvents, but variable effects on CEA expression have been observed (*102*).

The human colorectal cancer cell line HRT-18 has also been grown in the presence of DMSO, (*243,258*). In both of those studies, DMSO was found to inhibit growth but also to result in decreased CEA expression. Similar effects on cell growth were seen on exposing cell lines SW480 and SW620 to DMSO.

<u>Hexamethylene Bisacetamide</u>

Friend cells were first tested with Hexamethylene Bisacetamide, by Reubens et al in 1976 (*225*), and were found to undergo erythroid differentiation. Further experiments in 1977 studied the effect of Hexamethylene Bisacetamide on the human malignant mesenchymal cell line CBT-glioblastoma. The cells were seen to have increased procollagen synthesis, and an increased ratio of Type I : Type II procollagen. On xenograft implantation, different types of tumour altogether were produced - fibrosarcomata as opposed to glioblastomata.

Butyric Acid

Butyrate and its analogues have been found to be potent inducers of differentiation in both neoplastic and non-neoplastic cells (*205*). Butyrate is a naturally occuring metabolite, produced in the colon from bacterial fermentation. It is found in fruit and vegetables, and particularly in milk fat.

Butyric acid was found to induce similar changes to DMSO in Friend cells by Leder et al in 1975 (*168*). In addition, Butyric acid has been found to induce growth inhibition and morphological changes in HeLa cells, neuroblastoma and Chinese hamster ovary cells. Another hamster tumour line, the aggressive fibroblast BP6T line was found to show decreased clonagenicity, proliferation and intracellular fibrinolysis, inhibited anchorage independent colony formation and a changed, more uniform morphology and cell orientation on exposure to Butyric acid (167).

Human colorectal cancer cell line HRT-18 grown with Sodium butyrate has shown decreased growth and increased CEA expression in repeated studies (*243,258*). The cell lines SW480 and SW620 also show decreased growth on Sodium butyrate exposure. A comparison of normal colonic epithelial cells and LIM 1215 colon cancer cells found that butyrate inhibited growth and induced increased CEA expression in the neoplastic cells only (*211*).

DNA and RNA probes have been used to investigate the mechanism of CEA induction by Sodium butyrate (*255,234*) and increased transcription and post-transcription rates have been found.

Retinoic Acid

Retinoic acid derivatives were first used in cancer therapy in the treatment of Basal Cell Carcinoma in the early 1960s (*48*). Since then, they have been used to treat Acute Myeloid Leukaemia, T-cell

Lymphoma and bronchial tumours. The action of Vitamin A as a growth inhibitor, with concomitant effects on epithelial differentiation has been well documented, but is not fully understood (*176,1*).

HRT-18 treated with retinoic acid has been shown to result in reduced growth and variable suppression of CEA expression (*243,258*).

Interferon

Interferon was first noted to have anti-neoplastic effects in 1970, when it was shown to inhibit the multiplication of mouse neoplastic cells (8). Human colorectal carcinoma cell lines have since been repeatedly shown to have dose-dependent growth inhibition and increased CEA expression on exposure to Interferon (*8,128,273*). Comparisons of the various different interferons have identified gamma-Interferon as the more potent growth inhibitor (*159*), and it has been confirmed as modulating CEA mRNA levels in colorectal cancer cells (*194*). A comparison of 7 different human colorectal carcinoma cell lines grown in the presence of gamma-Interferon found growth was inhibited in all 7 lines, suggesting a common receptor for Interferon. Interestingly, one of the cell lines which was previously thought to be a non-expressor of CEA was induced to express CEA by exposure to gamma-Interferon.

Studies of polarised (suramin-treated) HT29.D4 human colorectal cancer cells demonstrated that the administration of gamma-Interferon to the basolateral rather than the apical compartment resulted in increased CEA expression, suggesting that the Interferon receptors lie in the basolateral membrane (*14*).

A clinical trial of 6 colorectal cancer patients given alpha-Interferon preoperatively compared the pretreatment biopsy tissue to the treated, resected specimens. CEA expression was found to be increased in the patients who had some degree of CEA expression pre-treatment (*186*).

Interferon has also been found to increase the expression of other membrane antigens by various tumours: human lymphoblastoid cells and fibroblasts express increase HLA-A3, B2microglobulin and HLA-B5 (*94,22*); melanoma cells express increased melanoma-associated antigen, B2m and HLA-A,B,C and DR (*108*); and breast cancer cell line MCF7 expresses increased cell surface antigen and HLA (*127*).

Xenografts of human breast tumour treated with gamma-Interferon for 4 days showed increased radiolocalisation on administration of radiolabelled anti-HLA-DR antibody (*232*).

Growth Factors

The significance of growth factors in the neoplastic process has not been fully elucidated. Studies of biopsied gastric malignancies show evidence of increased growth factor production compared to normal tissue (*35*). However, Transforming Growth Factor (TGF) beta has been found to inhibit lymphoid, myeloid and epithelial cell growth (*201*). In MOSER colorectal cancer cell lines, it has been found to cause increased CEA production, and increased expression of acidic and basic epithelial keratins - a sign of differentiation (*57*). TGF-beta is thought to target B23 protein, the synthesis of which signifies a commitment to mitogenesis.

Studies of Insulin-like Growth Factor (IGF) effects on HT29.D4 cells by Remacle-Bonnet in 1992 concluded that the IGF-I receptor on these cells is an important control of differentiation (*225*).

Miscellaneous Differentiating Agents

Vaso-active Inhibitory Peptide and cyclic AMP have been shown to cause longer doubling times of colorectal cancer cells in vitro and increased release of CEA into the cell supernatant (*142*).

Xenograft studies with cAMP treated colorectal cancer cells demonstrated increased CEA expression and increased localisation of radiolabelled anti-CEA antibodies (*131*).

CYTOTOXIC DRUGS & RADIATION

Human HL-60 leukaemia cells were exposed to a range of cytotoxic drugs by Collins et al in 1980 (*60*), including Actinomycin-D, Adriamycin, Daunomycin, Cytosine arabinoside, Vincristine and Hydroxyurea. All the agents had a growth inhibitory effect but only actinomycin-D slightly induced cell differentiation. In another study, AML cell lines grown in the presence of Actinomycin, Methotrexate, Cytosine, Vincristine and Daunomycin showed characteristics of improved differentiation including lysosome secretion, enzyme activity, bacterial killing and increased adherance (*157*).

Isolated studies exposing colorectal cell lines to 5-Fluorouracil, Doxorubicin and Docetaxol have shown respectively increases in CEA expression, reduced proliferation rates or both (*220,89,263*). Methotrexate has also been observed to induce significant differentiation in human colon cancer cells (*170*).

Combining actinomycin-D or Cyclohexamide with gamma-Interferon has not been shown to increase the augmented CEA expression seen with gamma-Interferon alone in studies of HT29 colorectal carcinoma cells (*254*).

5-Fluorouracil (5FU) given in combination with gamma-Interferon in vitro has been shown consistently to cause synergistic growth inhibition, but studies disagree on whether the effects on CEA expression are synergistic or independant of each other (*179,69*). One study on mice transfected with human colon cancer cells confirmed that the combination of 5-Fluorouracil and Interferon produced a better tumouricidal effect than the agents given alone (*198*).

Radiotherapy

Radiotherapy alone has not been found to induce significant differentiation of HL-60 leukaemia cells (*60*); however, studies of gastric cancer MKN45 cell lines did find that radiotherapy induced dose-related increased CEA expression (*135*). A study of the effects of pre-irradiation on radiopharmacological localisation in human colon tumour xenografts using mono- and bi-specific antibodies found significant increases in localisation of both antibodies after the radiation (*129*).

PHYSICO-CHEMICAL ENVIRONMENT

Temperature

Wong et al heated colorectal cancer cell LS174T cells in 1989. They found significant cell death occurred at temperatures over 43°C and a 40 - 50 % increase in CEA expression after one hour of heating to 42'C (*271*). Xenografts bearing T380 colorectal cancer tumours were heated to 41.5°C for 45 minutes and a significant increase in radiolabelled antibody localisation was noted by Gridley et al in 1991 (*130*).

Combining heat treatment (40°C for 30 minutes) with retinoic acid on gastric carcinoma MKN-45 cells produced more growth inhibition and CEA expression than with retinoic acid alone (*156*).

Early clinical trials of heat treatment for various cancers involved a high mortality and morbidity, with the most responsive tumour type being melanoma (*56*). Heat treatment for colorectal cancer has not been further clinically developed due to the adverse side effects and poor response.

Starvation

Porro et al in 1986 grew several colorectal cancer lines including HT29 in serum free medium and found growth inhibition and increased CEA production compared with growing in whole medium (*216*). Substituting glucose with galactose had similar growth inhibitory and CEA augmentation effects on HT29.D4 cells studied by Fantini et al in 1989 (*90*).

COMBINATIONS OF DIFFERENTIATING AGENTS

The interactions between differentiation agents may be additive, synergistic or antagonistic. If the agents act on the cell through the same pathway, the results should be additive. Synergistic and antagonistic interactions are more complex, with various converging and diverging mechanisms (*101*).

The use of combinations of differentiating agents and cytotoxic drugs has been advocated to increase the number of cells committed to programmed cell death. Work done on mouse erythroleukaemia cells (MEL) using Trans-retinoic acid and Cytosine Arabinoside showed enhanced differentiation, suggesting that the cytotoxic agent may generate a "differentiation- responsive state" (96).

COLORECTAL CANCER CELLS IN VITRO

The first human colorectal cancer cells to be cultured in vitro were **Ht29** cells, in 1964, by Fogh et al (*97*). They were developed from a primary adenocarcinoma, removed from a 44 year old caucasian woman. Ht29 cells were described as showing an epithelial-like morphology, and were observed to have abnormalities of chromosome number and structure. Specifically, Ht29 cells are aneuploid with variable losses and gains of different chromosomes, e.g. 15% show a loss of chromosome 7, 34% a loss of chromosome 15 and 12% a gain of chromosome 15 *(169*). They are known to express mutant p53. Work written up by Zweibaum et al in 1991 (*280*) provided more information about the characteristics of Ht29 cells: they were described as being poorly differentiated, with an absence of tight junctions, domes, apical brush borders, mucus secretion and CEA expression. It was particularly noted that the differentiation of the Ht29 cells could however be modulated, for example by growing in glucose deprived medium or in the presence of Sodium Butyrate and that the cells could successfully be transfected into nude mice.

Lovo cells were first reported to be established in culture by Drewinko et al, after four years of working with them. The cells were originally taken from a metastatic nodule of Colorectal adenocarcinoma (*83*). They were found to have an aneuploid karyotype (modal number 49), but with variation in chromosomal content between cells in one clone (as also seen in Ht29 cells). They showed chromosomal and microsatellite instability. Wild-type p53 expression was noted, precluding mutated p53 as the cause for the genetic instability. Morphologically, they were seen to be cuboidal, columnar or signet ring cells, which formed acinar structures. They characteristically had desmosomes and terminal bars at the cell-cell junctions, and microvilli and a glycohalyx at the free border. Internally, there were many microfibril bundles and ribosomes, but few lysosomes and no Golgi apparatus. They had a large nucleus with a single nucleolus. It is relevant that CEA was observed to be produced by the cells, a property which was retained in transfected cells (*81*).

Colo (Colo 320) cells are quite different to Ht29 and Lovo cells. They were established in 1979 by Quinn et al (*223*), having been grown from a 55 year old woman's undifferentiated sigmoid

carcinoma. Their modal chromosome number was 51, with multiple copies of chromosome 20 commonly appearing in metaphase, and chromosome 5 frequently deleted. Morphologically they were spheroid cells, with much convoluted Golgi, many ribosomes and polysomes, large lipid vacuoles and rarer clear or dense vacuoles. They did not have microvilli, and desmosomes were only occasionally seen. Repeated radioimmunassay for CEA was negative, although the cells did produce small amounts of PTH, ACTH, Serotonin, Adrenalin and Noradrenalin. These properties make them akin to the Amine Precursor Uptake and Decarboxylator (APUD) family, and a good model for the more unusual types of Colorectal Cancer that exist.

The three cell lines Colo, Ht29 and Lovo represent a range of CEA secreting properties and differentiation characteristics, and will be studied throughout this work.

AIMS OF THIS WORK

The primary aim of this work is to find a chemical or environmental factor or combination of factors which can successfully induce increased CEA expression in 3 different colorectal cell lines – Lovo, Ht29 and Colo. Additive or synergistic augmenting effect by combinations of CEA-augmenting agents will be sought.

The differentiating agents included in the study are Butyric Acid, gamma-Interferon and 5-Azacytidine and Theophylline. The cell culture environment will be changed by altering pH, oxygen supply and serum content of medium, and by exposing the cells to radiation. A range of commonly used cytotoxic drugs at one fixed dose will also be studied. According to the observed effects of these factors used singly, combinations of successful chemical and physicochemical factors will be tested.

CEA expression will be measured using Immunocytochemistry and Fluorescein Activated Cell Sorting. Membrane CEA expression and total cell CEA content, reflecting cytosolic CEA, will be compared.

Additional information on cell growth and differentiation characteristics will be gathered, by cell counting and Electron Microscopy.

The amount of CEA released by the growing cells into their medium will be measured by Radioimmunoassay of culture medium. Changes in CEA expression will be compared to changes detected in CEA release.

In order to investigate genetically determined changes in the cell cycle which accompany perceived changes in CEA expression and differentiation, a limited study of the changes in oncogene expression in CEA-augmented will be conducted. Treated cells will be immunostained for oncogene products known to be associated with apoptosis or tumour progression, e.g. p53, Bcl-2, c-myc, and K-ras.

CHAPTER TWO: METHODOLOGY

CELL CULTURE

Bringing On

The three cell lines, Lovo, Ht29 and Colo-320 were obtained courtesy of the European Collection of animal Cell Cultures (ECACC). They were stored in Nunc Cryotubes (GIBCO) in liquid Nitrogen at −160°C, preserved in a 10% solution of Dimethyl Sulfoxide (SIGMA), at a concentration of $5x10^6$ cells per millilitre.

When required, the Cryotubes were removed from the Nitrogen store and were rapidly defrosted in a water bath at 37°C. The cell solutions were transferred by sterile pipetting under an incubator hood into sterile universal containers, and diluted by the addition of 15 mls of Whole Medium, i.e. 500ml Dulbecco's Modified Eagle's Medium (NBL), 50mls Fetal Bovine Serum (GIBCO), 5 mls L-Glutamine 200mM (SIGMA)and 5 mls Penicillin 10 000U & 10 mg Streptomycin per ml in 0.9% Sodium Chloride Solution (SIGMA).

This solution was then centrifuged at 90rpm for 7 minutes, producing a cell pellet. The supernatant fluid was discarded, the pellet flicked and resuspended in more Whole Medium, to a concentration of 10^6cells per ml. One ml of this suspension plus another 4mls of Whole Medium was pipetted into sterile $25cm^2$ vented tissue culture flasks (MARATHON), and placed in a humidified incubator set at 37°C, and 5% CO_2 . The incubator was cleaned with Hycolin 2% Phenolic disinfectant (DEPUY HEALTHCARE), an anti-fungal antiseptic, before and after each use and also exposed to Ultraviolet light. Initially in the course of the work, the cells occasionally became infected with fungi, and were

discarded, along with the Whole Medium currently in use. No mycoplasm infections were isolated at any point.

According to the factor under investigation, the Whole Medium was altered by the addition of a differentiating agent or cytotoxic drug at a specified concentration. The environmental factors were investigated by altering the pH of the medium, adding Chloroquine, exposing the flasks to x-radiation or growing the flasks in a sealed module (see Chapter 4 for specific methodology).

Feeding

The cells in the culture flasks were fed with more Whole Medium every 2 - 3 days. Initially, feeding was required every three days only, but as the cells approached confluence (reached on average at 5 days), they needed more frequent feeding. All discarding of the old medium and pipetting of fresh whole medium into the flasks was performed under sterile conditions in the incubator hood.

Harvesting

Under sterile conditions in the incubator hood, waste medium was discarded from the culture flasks and 5mls sterile Phosphate Buffered Saline (PBS - SIGMA) pipetted into each flask to wash the cells. The PBS was then removed and 2mls Trypsin-EDTA (SIGMA), was added to each flask, to unstick the adherant monolayer. The flasks were then incubated at 37°C for 7 - 10 minutes (Lovo cells requiring the longer incubation in Trypsin), agitated and the cells seen to have been lifted from the flask base. Further Whole Medium was added (10mls per flask) and a 1ml sample of this solution was counted using the Hemocytometer (see below), with the remaining solution being centrifuged at 90rpm for 7 minutes. The supernatant was then discarded, the pellet flicked and resuspended in fresh Whole Medium at the desired concentration, according to the cell count.

Counting

Trypsinised cells in whole medium were counted pre-Centrifuging using a Neubauer Counting Chamber. 800µl of the cell solution was removed, added to 100µl of Trypan Blue (SIGMA) and well mixed. A drop of the resulting solution was placed in the counting chamber and the cells present in a marked $1mm^2$ area counted under light microscopy. The number of cells counted was then extrapolated to the total number of cells by multiplication, according to the volume of cell solution. The most efficacious mix of cell solution and Trypan Blue was determined using a Serial Dilution experiment, described in Appendix 1.

Freezing Down

Harvested, counted cells in pellet form were resuspended at $5x10^6$ cells/ml in Whole Medium/ 10% Dimethyl Sulfoxide. The supensions in cryotubes were gradually lowered (at a rate of 2 centimetres per 15 minutes) into liquid Nitrogen in a freezing jar, before being transferred to stocks in the Nitrogen banks.

Cytospins

Twin-frosted glass microscope slides (WESTERN LAB SERVICES) were pretreated with Poly-L-Lysine (SIGMA). The slides were lined up with filter paper (SHANDON) and placed in a Shandon Cytospin. Aliquots of 100µl of cell solution, after the cells had been trypsinised, counted, centrifuged and resuspended in whole medium at a concentration of 10^6cells/ml, were placed in each cytospin well, then spun at 800rpm for 5 minutes. The slides were dried at room temperature overnight. They were then stored, uncovered, at –20°C.

IMMUNOCYTOCHEMISTRY

The presence of CEA may be assumed by indirect two stage immunocytochemistry, first described by Mason et al in 1969 (*194*). This involves staining CEA-expressing cells by tagging CEA with anti-CEA antibody and further tagging that compound with a second peroxidase-labelled antibody. The second antibody reacts with the immunostain, colouring the cells. The primary antibody used throughout these experiment was A5B7, a CEA-specific mouse Immunoglobulin, first characterised by Harwood et al in 1986 (*136*) and further validated as being suitable for in vitro and in vivo use by Boxer et al (*47*). It was supplied by the Department of Clinical Oncology, Royal Free Hospital, Pond Street, London.

Materials and Methods:

The flasks of growing colorectal cancer cells were harvested by washing, trypsinising and centrifuging the cell suspension to produce pellets of cells. The cells were counted and re-suspended in Whole Medium to a dilution of 10^6 cells per ml. Cytospins of each cell sample were made, as described above, producing a circle of cells adherent to the glass slide. The cell circle was encircled with a Paraffin pen (DAKO), and the slides left to dry overnight.

The slides were next fixed by immersion in Acetone (MERCK) at 4°C for 10 minutes. The acetone was washed off with PBS x 3.

A5B7 anti-CEA antibody in 0.1% Bovine Serum Albumin (BSA - SIGMA), at a 1:2000 dilution was laid onto the slides, with 2 negative controls treated with PBS only. A further negative isotype-specific control using mouse IgG_1 immunoglobulin at 10μl/ml was prepared. (The BSA was required to override background protein staining in the cells under investigation.) The slides were incubated in a humidified chamber at room temperature for 45 minutes, before unbound antibody was washed off with PBS x 3.

Peroxidase labelled Rabbit Anti-mouse peroxidase-conjugated secondary antibody (RAMP - DAKO), at a concentration of 13μg/ml in 10% normal human serum (courtesy of self), was then applied to every slide except one of the negative controls per set (which was treated with PBS to prevent drying out) , and the slides again incubated in a humidified chamber at room temperature for 45 minutes, before being washed in PBS x 3.

The slides were then immersed in 100ml TRIS/HCL buffer (SIGMA) containing 60mg 3,3'-Diaminobenzidine Tetrahydrochloride (BDH), 1ml of 0.1M Imidazole (BDH) and 40μl 30% Hydrogen Peroxide (SIGMA) for 5 minutes, to develop the immunostain. The stained slides were washed in distilled water x 3, and counterstained with Mayer's Haemalum (HD SUPPLIES) for 5 minutes.

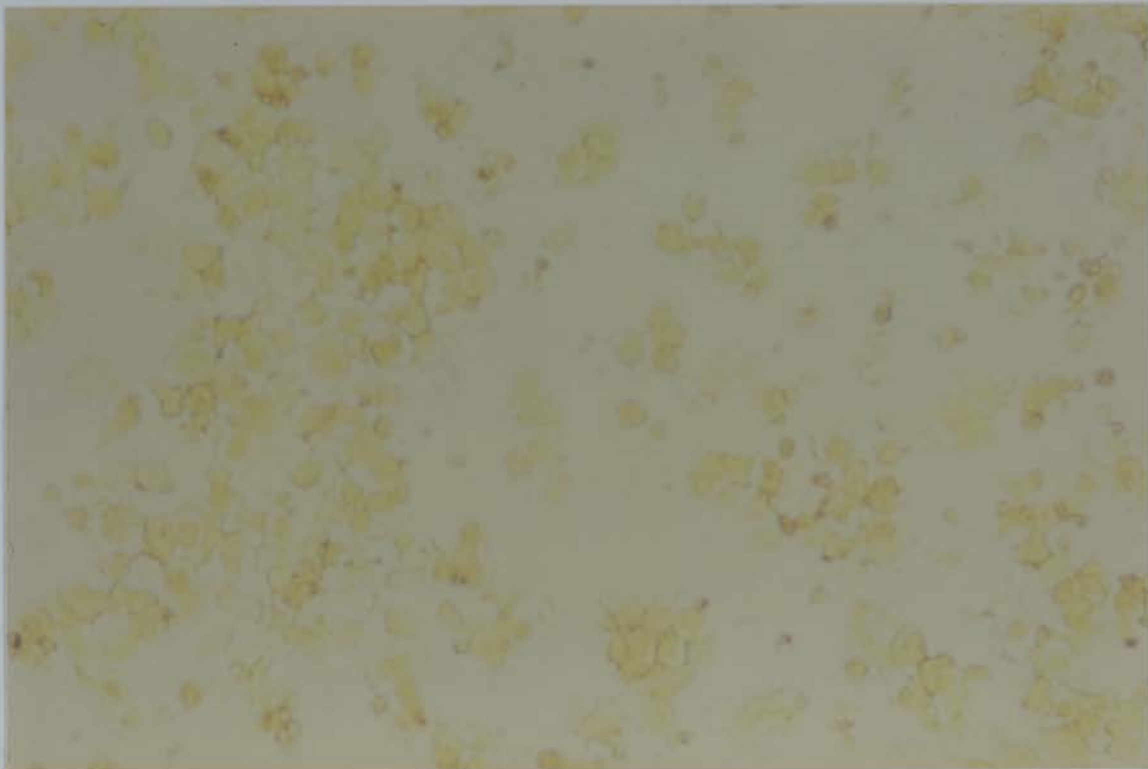
The above recipe was found to be the most efficacious, after trying variations of antibody concentrations, antibody-exposure times, DAB-exposure times and deleting the Acetone fixation step. The titration experiments are described in Appendix 1.

The slides were again washed in distilled water x 3, dried in Analar Methylated Spirits (BDH) x 3, followed by Analar Xylene (BDH) x 3 and finally mounted with coverslips (MARATHON) using DePeX fixative (GURR).

The slides were finally examined under light microscopy and graded according to the degree of brown CEA-staining of the cells. An observer-based system of grades was used, with a range of arbitrary units awarded from 0 for no staining to 10 for maximal 100% staining. This non-computerised system was previously compared to a computer-analysed system, and a cell-bound ELISA, developed in this department *(13)*, and found to be as accurate and as reproducible. The observer-based grading system was used in this work, after comparative validation studies grading the slides produced in this work. Photographic examples of the grades awarded are shown on the next page.

Grade 1 (Ht29 Control cells)

Grade 9 (Lovo control cells)



FLUORESCEIN ACTIVATED CELL SORTING

Introduction

Fluorescein Activated Cell Sorting (FACS) involves directing a laser beam of light through a suspension of cells. The pattern of scattering of the light beam allows the number, size and fluorescence of the cells to be accurately calculated and allows a sub-population within a mixed population to be separated and characterised *(237)*. Human colorectal cancer cells have a residual low fluorescence. It is possible to highlight surface and cytoplasmic cell associated antigens, such as CEA, by binding them to fluorescein-conjugated antibodies. The degree of expression of these cell associated antigens can then be measured according to the degree of fluorescence seen with FACS.

The highlighting of the cell associated antigens is achieved by two stage immunostaining. A primary anti-CEA antibody is first applied to the cells, followed by a fluorescein-conjugated secondary antibody, which binds to the primary antibody.

Method

Three human colorectal cell lines, (HT29, Colo and Lovo) were grown in standard tissue culture for 5 days. The cells were then harvested by trypsinising, counted and re-suspended in whole medium at a concentration of 3x 10^6 cells per ml. Aliquots of 100μl of each cell suspension were pipetted into separate FACS pyrex tubes (FALCON), one aliquot from each cell suspension for surface antigen measurement and one for cytoplasm antigen measurement. A negative control tube for each cell line was also prepared. When there were insufficient cells to make up aliquots of 3x 10^5cells each, the cell suspensions were made up to 200μl, and fewer cells were read through the FACS Scanner (a minimum of 1000 rather than 3000).

The samples for cytoplasmic plus membrane antigen measurement were then permeabolised: 2 mls of Ortho Permeafix (ORTHO DIAGNOSTIC SYSTEMS) at 0.5 concentration with distilled water was added to the tubes which were incubated for 30 minutes at room temperature. The samples were then centrifuged at 2000rpm for 5 minutes, the supernatants discarded and the pellets flicked. The cells were then washed twice in PBS.A (PBS, Albumin 0.2%, Azide 0.2% - SIGMA) and re-spun at 2000rpm for 5 minutes after each wash. The albumin was used to prevent background cell protein staining, and the azide as an anti-bacterial agent.

All the samples were then immunostained - the permeabolised and the non-permeabolised cells. The primary mouse anti-CEA antibody, A5B7, at 10μl per ml in PBS.A was applied in 100μl aliquots. The negative control - mouse IgG_1 immunoglobulin at 10μl/ml was applied to negative control tubes. The concentration of primary anti-CEA antibody was chosen after a titration experiment described in Appendix 1. No positive controls were successfully identified, but high staining Lovo control cells were included in every staining experiment as an internal positive control.

The samples were incubated for 30 minutes at room temperature, then washed twice in PBS.A, being spun at 2000rpm for 5 minutes in between washes. The second, fluorescein conjugated antibody, Goat anti-mouse Fluorescein Isothionate (FITC- SOUTHERN BIOTECHNOLOGY ASSOCIATES) was then applied in 100μl aliquots at a concentration of 1:100 in PBS.A.

The samples were again incubated at room temperature for 30 minutes, then washed twice with PBS.A and spun at 2000rpm for 5 minutes. They were then fixed in Paraformaldehyde (1%), and stored at 4°C prior to being put through the FACS scanner. One to three thousand cells per sample were exposed to the laser within the FACS scanner. Graphs showing the distribution of fluorescence of the samples were obtained and analysed. The average value of the one to three thousand cells in each sample was noted.

ELECTRON MICROSCOPY

Specimen Processing Method

Principle

Cells in tissue culture have to be converted initially into cell suspensions, and eventually into a cell pellet, which may be sectioned and then studied under the electron microscope.

Method

The adherent cells were separated from the culture flask using a cell scraper, and Gluteraldehyde/Paraformaldehyde fixative was added to them. The fixed cell suspensions were transferred to centrifuge tubes and centrifuged at 10g for 5 minutes. The cells were then washed three times in PBS for 10 minutes, centrifuging in between each wash. After this, the cells were fixed in Osmium Tetroxide (transferring solutions in fume cupboard) for one hour and 30 minutes. They were then washed in water three times for 5 minutes each time.

A process of dehydration was then undertaken, through graded alcohols 30%, 50% for 5 minutes; 70% and 90% twice each for 5 minutes. The cells were then exposed to absolute alcohol three times, for 10 minutes each time.

LEMIX resin was removed from the freezer, allowed to reach room temperature, and made up to a 50:50 mixture of resin/100% alcohol, which was infiltrated into the cells overnight.

A second batch of 100% resin (at room temperature) was then allowed to infiltrate the cells for 5 - 6 hours.

The samples were then centrifuged in fresh 100% resin and polymerized at 70°C overnight to form a cell pellet, from which sections could be cut.

This processing and sectioning work was performed by the Electron Microscopy laboratory technicians. The final stage of studying the sections under the Electron Microscope and taking photographs of relevant cells was undertaken by myself.

RADIOIMMUNOASSAY FOR CEA

An acceptably accurate method for measuring the amount of free CEA in a solution (such as plasma in the clinical environment) is that of Radioimmunoassay (*192*). In this work, radioimmunoassay was used to measure the amount of CEA being released into the supernatant medium by the cultured colorectal cancer cells under investigation.

Procedure Principle

A solid phase two-site immunoradiometric assay kit called ELSA2-CEA was used (CIS BIO-INTERNATIONAL). Monoclonal antibodies were prepared against sterically remote antigenic sites on the CEA molecule: the first one is coated on the ELSA solid phase, the second one, radiolabelled with Iodine 125, is used as a tracer.

The CEA molecules attached themselves to the solid ELSA-bound antibodies and in turn the radiolabelled tracer antibody was bound to this compound, to form an antibody-antigen-antibody sandwich. The unbound tracer was removed and the residual radioactivity measured taken as a measure of the amount of CEA present.

Method

All reagents were raised to room temperature, and a water bath was heated to 45°C.

300µl of ^{125}I labelled anti-CEA monoclonal antibody was dispensed into all groups of ELSA-tubes.

100µl of the standards, control serum or the experiment samples was added to appropriately labelled ELSA-tubes.

Each ELSA-tube was gently mixed.

The tubes were incubated for 3 hours at 45°C.

The tubes were then washed; the contents were aspirated, 3ml of Tween 20 washing solution added and left for 2 minutes and then re-aspirated. This procedure was repeated twice.

The radioactivity bound to the ELSA was then measured with a gamma scintillation counter.

Results Analysis

A curve was drawn using the measured data from the standard concentration solutions supplied (ng/ml CEA v cpm x 10^3). The concentrations of CEA in the experiment tubes were read from this curve, according to their cpm values.

CHAPTER THREE: RESULTS - DIFFERENTIATING AGENTS

Introduction

Colorectal cancer cells have been studied for signs of differentiation on exposure to various of the differentiating agents (*244,258,152,142,,75*). The expression of CEA by human colorectal cancer cells has been observed as being augmented by certain of the differentiating agents, particularly Butyric Acid, (*244,258*) and γ- Interferon,(*8,128,272,*).

Preliminary studies were performed in the University Department of Surgery at the Royal Free Hospital, comparing the effects of Butyric Acid, gamma-Interferon, Theophylline, 5-Azacytidine, Retinoic Acid and phorbol esters on the colorectal cancer cell lines Lovo and HT29. Growth rate and CEA expression as measured by Immunocytochemistry was measured over a 7 day time scale. It was found that only Butyric Acid and gamma-Interferon caused significant augmentation of CEA expression Lovo and HT29 cells, with the maximum effects occurring by day 5 (*12,13*). The most efficacious concentrations of each agent, from a serial dilution experiment (*13*), were: Butyric Acid 1mM; Theophylline 10mM; 5-Azacytidine 1μM; Interferon 10ng/ml. Colo cells were not studied, and FACS analysis was not undertaken.

Aim

The aim of this experiment was to further explore the CEA augmentation effects previously seen with Butyric Acid, Interferon, Theophylline and 5-Azacytidine using the more sensitive Fluorescein Activated Cell Sorting analysis in addition to immunocytochemistry. Lovo, H29 and also Colo cells were studied to give a fuller range of CEA-expressing properties. Additional information of intracellular effects of the differentiating agents was sought with Electron Microscopy and Radioimmunoassay of supernatant CEA (see chapters 7 & 8).

Methods

For each cell line, two control 25cm^2 tissue culture flasks containing 10^6 cells were grown continually in standard Whole Medium at pH 7.4 in an incubator at 37°C for 5 days. Five days of growing was used in each experiment, as confluence was reached in the control samples at that point. Four identical experiment flasks containing 10^6 cells were also grown in the incubator at 37°C, but in Experimental medium containing the differentiating agent. The concentrations of differentiating agents were identical to those found to be most successful in previous published work from the department (*13*), that is: Butyric Acid 1mM, Interferon 10ng/ml, Theophylline 10mM, 5-Azacytidine 1μM.

On day 5, one of the experiment flasks was removed for Electron Microscopy. The remaining experiment flasks and the control flasks were each harvested and counted.

The cells were then analysed by immunostaining with peroxidase or fluorescein-conjugated anti-CEA antibody for Two-stage indirect Immunocytochemistry or Fluorescein Activated Cell Sorting.

The density of staining for CEA in the experimental samples was compared to the pooled control samples.

Analysis of the immunocytochemistry and FACS results was undertaken using parametric and non-parametric tests respectively and taking account of dissimilar variances between the control and experiment samples.

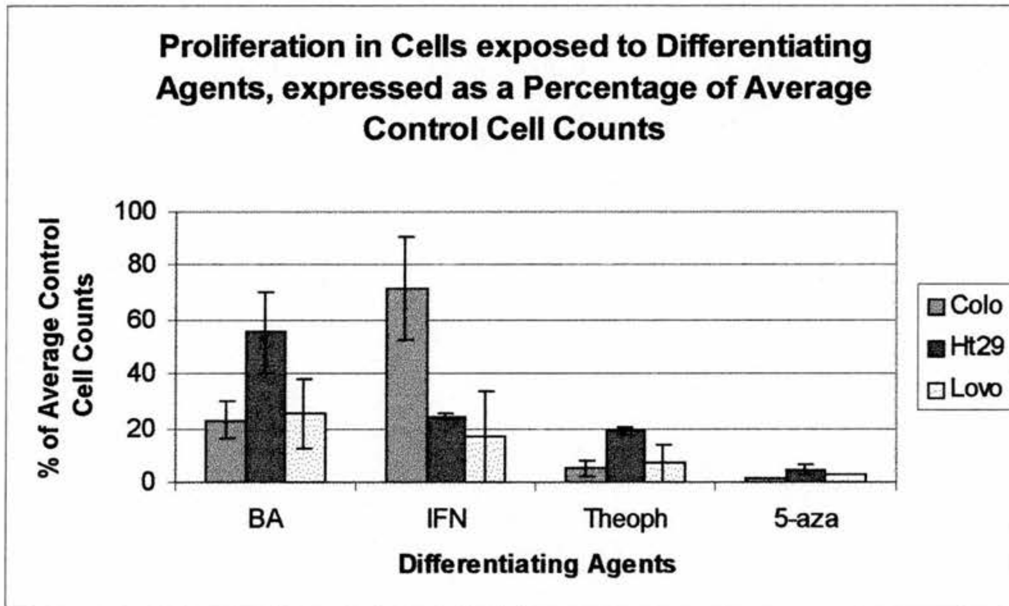
RESULTS

The numerical values for the results illustrated in the graphs below may be found in Appendix 2.

The 4 differentiating agents, Butyric Acid, Interferon, Theophylline and 5-Azacytidine affected the 3 cell lines in differing ways as follows:

GROWTH

The changes in cell proliferation were measured by calculating the percentage of each experimental cell count to the averaged control cell count, as illustrated below.

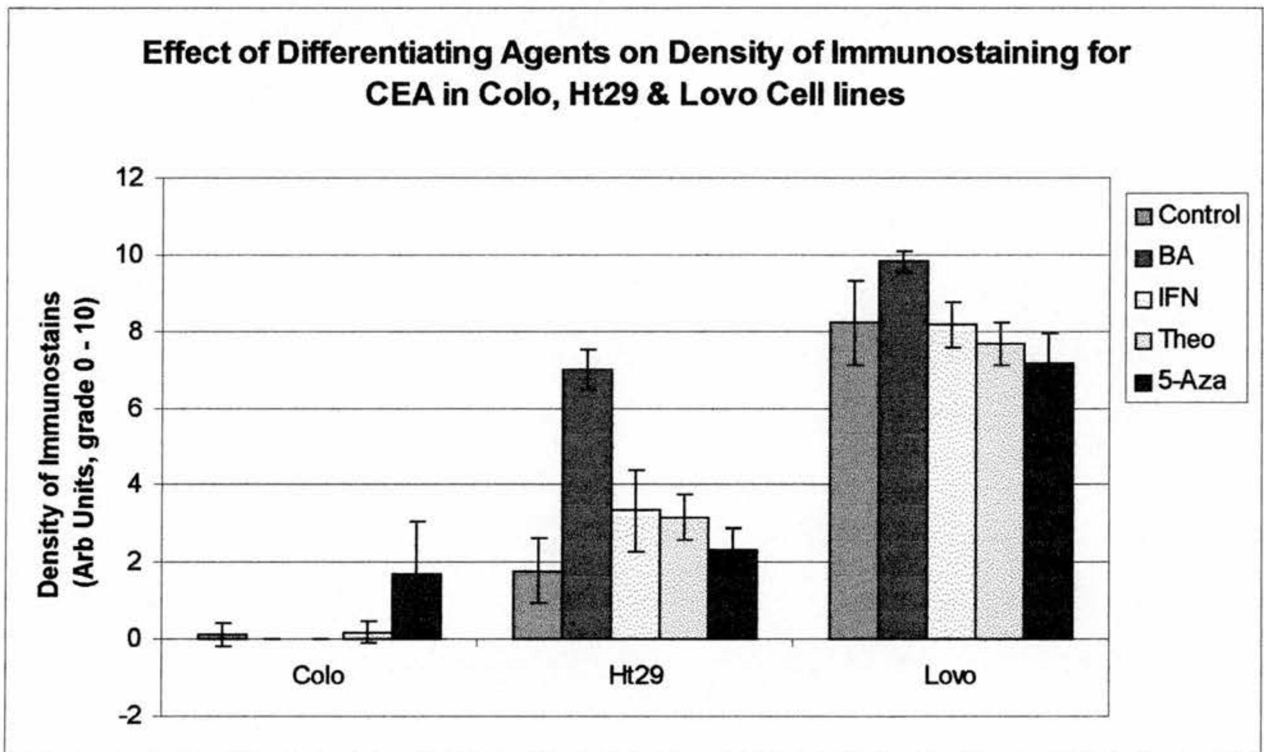


Analysis of the results using the t-test was performed. Significant growth inhibition (at $p \leq 0.05$) was seen in the Colo cells exposed to Butyric Acid, Theophylline and 5-Azacytidine; in the Ht29 cells exposed to Interferon, Theophylline and 5-Azacytidine; and in the Lovo cells with all 4 agents.

CEA EXPRESSION:

IMMUNOCYTOCHEMISTRY

The density of staining for CEA was measured in triplicate samples of cells exposed to each differentiating agent, and compared to the average density of control samples from all of the immunocytochemistry runs from all of the experiments.

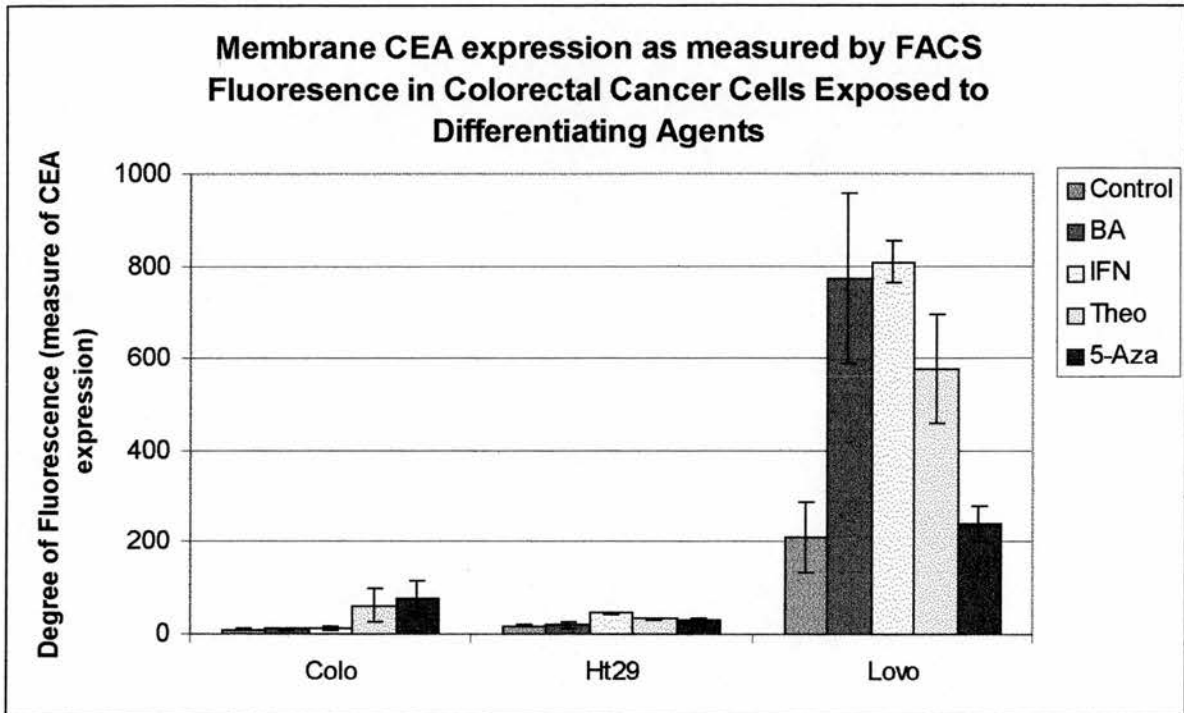


Analysis using the t-test showed significant augmentation in the Colo cells with 5-Azacytidine, the Ht29 cells with Butyric Acid, Interferon and Theophylline, and the Lovo cells with Butyric Acid only.

FLUORESCEIN ACTIVATED CELL SORTING

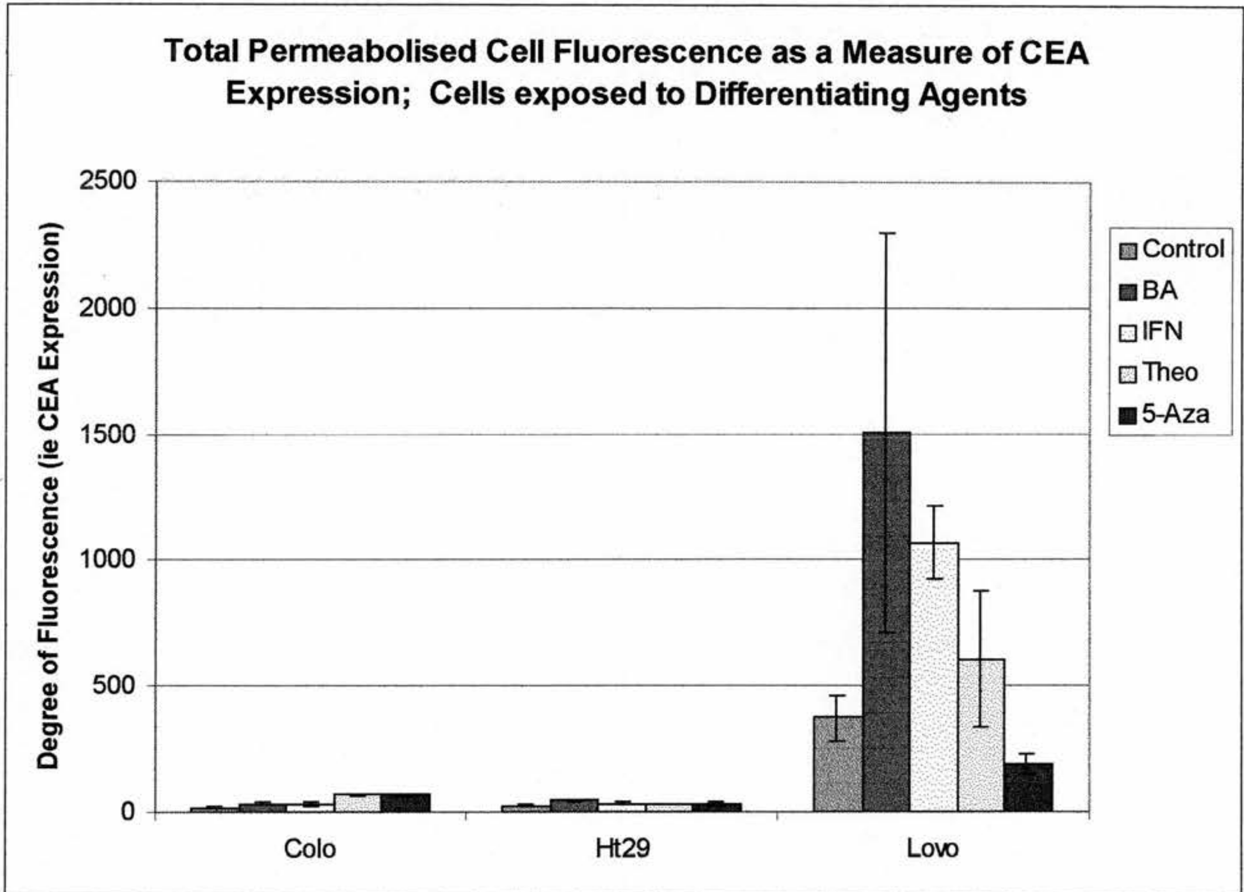
Both membrane-bound and total cell CEA were measured and compared in triplicate in the experiment samples. Changes in CEA expression were identified by comparing to a bank of all control cell FACS results, from all of the experiments.

FACS OF MEMBRANE-BOUND CEA:

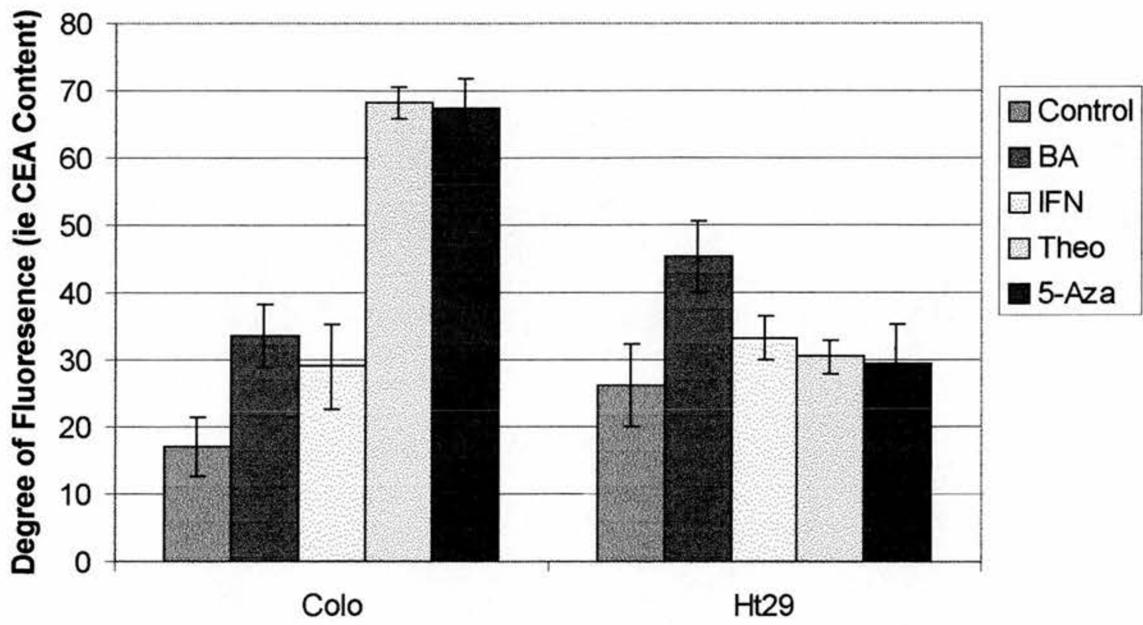


Analysis using the Mann Whitney-U test (at $p \leq 0.05$) showed significant increases in membrane CEA in Colo cells exposed to Theophylline and 5-Azacytidine, and in Ht29 and Lovo cells exposed to Butyric Acid, Interferon and Theophylline.

TOTAL CELL CEA CONTENT (Permeabolised Cells)



It is obvious from the above graph that the changes in the Lovo cells far outstrip those seen in the Ht29 and Colo cells. Statistical analysis with the Mann Whitney-U test at $p \leq 0.05$ confirmed significant increases in fluorescence in Lovo cells exposed to Butyric Acid and to Interferon. Significant increases, although of a lower magnitude, were also seen in Colo cells with Theophylline and 5-Azacytidine, and in Ht29 cells with Butyric Acid, Interferon and Theophylline. These are more clearly demonstrated in the following graph, which omits the Lovo cell results:


Total Permeabolised Cell Fluorescence as a Measure of CEA Content; Cells exposed to Differentiating Agents
Degree of Fluoresence (ie CEA Content)
80
70
60
50
40
30
20
10
0
Colo
Ht29
Control
BA
IFN
Theo
5-Aza

SUMMARY OF RESULTS

Growth was significantly inhibited in the Lovo cells by all four differentiating agents; in Ht29 cells by Theophylline, 5-Azacytidine and Interferon; and in Colo cells by Butyric Acid, Theophylline and 5-Azacytidine (t-test; $p \leq 0.05$).

Immunocytochemistry showed positive CEA augmentation (t-test; $p \leq 0.05$) in Lovo cells exposed to Butyric Acid only; and in Ht29 cells with Butyric Acid and Interferon. No change in CEA staining was observed in the Colo cells with any of the 4 agents.

FACS measurement of membrane CEA staining showed positive CEA (Mann Whitney-U test; $p \leq 0.05$) in Lovo and Ht29 cells with Butyric Acid, Interferon and Theophylline; and in Colo cells with Theophylline and 5-Azacytidine.

FACS testing for total cell CEA content showed significant CEA increases in Lovo cells with Butyric Acid and Interferon; in Ht29 cells with Butyric Acid, Theophylline and Interferon; and in Colo cells with Theophylline and 5-Azacytidine.

DISCUSSION

All four differentiating agents successfully augmented CEA expression in one or more of the cell lines. It is noted that the cells responded in different ways to the various agents. It is interesting that the agents which were less strongly inductive of CEA in Lovo and Ht29 cells, i.e. 5-Azacytidine and Theophylline, were particularly successful with the Colo cells. These results concur with the preliminary studies undertaken in this department (12,13), and with other sporadic studies (244,258,272). Previous work on these differentiating agents in colorectal cancer cells has been limited to single cell lines exposed to Interferon or Butyric acid; cell lines with variable natural CEA expression have not been simultaneously exposed to the four differentiating agents tested in this work for comparison of effects.

Augmentation in Colo cells has not been previously documented at all with the differentiating agents studied in this work. This may be due to increased sensitivity of FACS analysis, compared to immunocytochemistry.

The cell growth-inhibitory effects of the agents did not exactly mirror their CEA-augmentation properties, but positive CEA augmentation was associated with growth inhibition in all cases except Ht29 cells in Butyric Acid.

The varied patterns of response to the differentiating agents may be indicative of different pathways to CEA expression within the colorectal cancer cells. Using a combination of these agents is a potentially efficacious way of inducing increased CEA expression in a typical heterogeneous colorectal cancer, different mechanisms of augmentation being one of the requirements for synergistic reaction between agents. It is also possible that a combination of agents could induce additive or synergistic augmentation within one type of cell, if CEA is being induced via different pathways. Because each of the differentiating agents studied had positive effects on one or more of the colorectal cancer cells, it was decided that dual combinations of each of them should be tested on the cells (see Chapter5).

CONCLUSIONS

1. Colorectal cancer cells may be induced to express CEA by differentiating agents, even if they are do not express CEA in their natural state.

2. The differentiating agents Butyric Acid, Theophylline, 5-Azacytidine and Interferon have variable effects on different colorectal cancer cell lines, suggesting more than one mechanism for CEA induction.

3. CEA augmentation in colorectal cancer cells by differentiating agents is usually, but not invariably, accompanied by inhibition of cell replication.

CHAPTER FOUR

RESULTS (ii): EFFECTS OF ALTERED ENVIRONMENT

Introduction

Sporadic experiments on isolated human colorectal cancer cell lines involving alterations in physical environment have been performed over the past decade: Wong et al heated colorectal cancer cell LS174T cells in 1989 and found significant cell death occurred at temperatures over 43°C with a 40 - 50% increase in CEA expression after one hour of heating to 42°C (*269*), although poor results from subsequent clinical trials have resulted in a loss of interest in heat in colorectal cancer (*56*).
Porro et al in 1986 grew several colorectal cancer lines including HT29 in serum free medium and found growth inhibition and increased CEA production compared with growing in whole medium (*216*). Substituting glucose with galactose had similar growth inhibitory and CEA augmentation effects on HT29.D4 cells studied by Fantini et al in 1989 (*90*).

Preliminary studies in this department on the effects of starvation, hypoxia and altered pH on growth and CEA expression of HT 29 human colorectal cancer cell lines suggested that changes in nutrition and pH may cause decreased cell growth and increased CEA expression (*12*).

Aim

To further explore the effects of altering environment on cell growth and CEA expression on the three colorectal cancer cell lines, Lovo, Ht29 and Colo, known to be high, low and non expressors of CEA respectively, in their natural state. The alterations to cell environment studied were malnutrition, pH changes, hypoxia and radiation.

Methods

1. Altered Nutrition:

Serum depletion effectively means markedly reducing the protein content of the culture medium, which would be expected to affect the growth patterns of the cells. It should be emphasised that serum depletion is a form of malnutrition, not complete starvation.

Four 10^6 cell samples of each cell line were grown in monolayer cell culture in protein-depleted medium (Dulbecco's Modified Eagle's Medium with **0.5%** Fetal Calf Serum, L-Glutamine, penicillin and Streptomycin), alongside two samples of each cell line grown in whole medium (Dulbecco's Modified Eagle's Medium, **10%** Fetal Calf Serum, L-Glutamine, Penicillin and Streptomycin). After 5 days, one flask per cell line was removed for Electron Microscopy and the remaining cells were harvested. counted and stained for immunohistochemistry and FACS scanning.

2. Altered pH

The culture medium used for all the cell culture work had a pH of 7.4. Initial studies on pH were performed, altering the medium pH to 7.2 or to 7.6, by the addition of concentrated Hydrochloric acid and Sodium Hydroxide respectively.

A further pH study was undertaken, using **Chloroquine**. Chloroquine is known to cross biological membranes and to enter lysosomes. Once inside the lysosome it becomes protonated, is then no longer free to traverse the membranes and so accumulates in the lysosome, mopping up Hydrogen ions (*74*). The cytoplasmic pH being robbed of its hydrogen ions would thereby be expected to rise.

The three human colorectal cancer cell lines Lovo, HT29 and Colo were grown in standard monolayer cell culture as described in Appendix 1, with triplicate samples of each grown in physiological, acidic

medium, alkaline medium or medium containing 10^{-5} M Chloroquine. Simultaneously, two control flasks were grown in unaltered whole medium. After 5 days, one of each of the experimental flasks per cell line was removed for Electron Microscopy and the other experimental flasks and the control flasks processed as described in Chapter 2: the cells were trypsinised, counted and stained for Immunocytochemistry and FACS measurement of fluorescence.

3. Hypoxia

The initial study of the responses of colorectal cancer cells to hypoxic conditions involved almost total oxygen deprivation. The positive results achieved in terms of CEA expression led on to two further experiments, with conditions of 5% and 12% Oxygen. These percentages would be more realistic reflections of the situation in vivo and of the potential results of using capillary blocking microspheres in vivo to cause hypoxia. It is not possible in vivo to achieve prolonged hypoxic conditions at oxygen tensions of less than 5%; nevertheless, it is of interest to study the evolving pattern of changes in cell behaviour caused by progressive decreases in oxygen tension.

For each of the three oxygen concentrations tested, four experimental flasks, fitted with air filters, per cell line were grown in standard monolayer cell culture, but in a sealed module which was flushed with one of the three gas mixtures (pure 100% Nitrogen gas, or a mixture of 5% Oxygen and 95% Nitrogen, or a mixture of 12% Oxygen, 83% Nitrogen and 5% Carbon Dioxide), over 10 minutes to expel residual oxygen from the module and from the flasks. After 5 days, one flask was removed for Electron Microscopy and the remaining cells were harvested, counted and stained for Immunocytochemistry and FACS scanning, along with the cells from duplicate control flasks for each cell line.

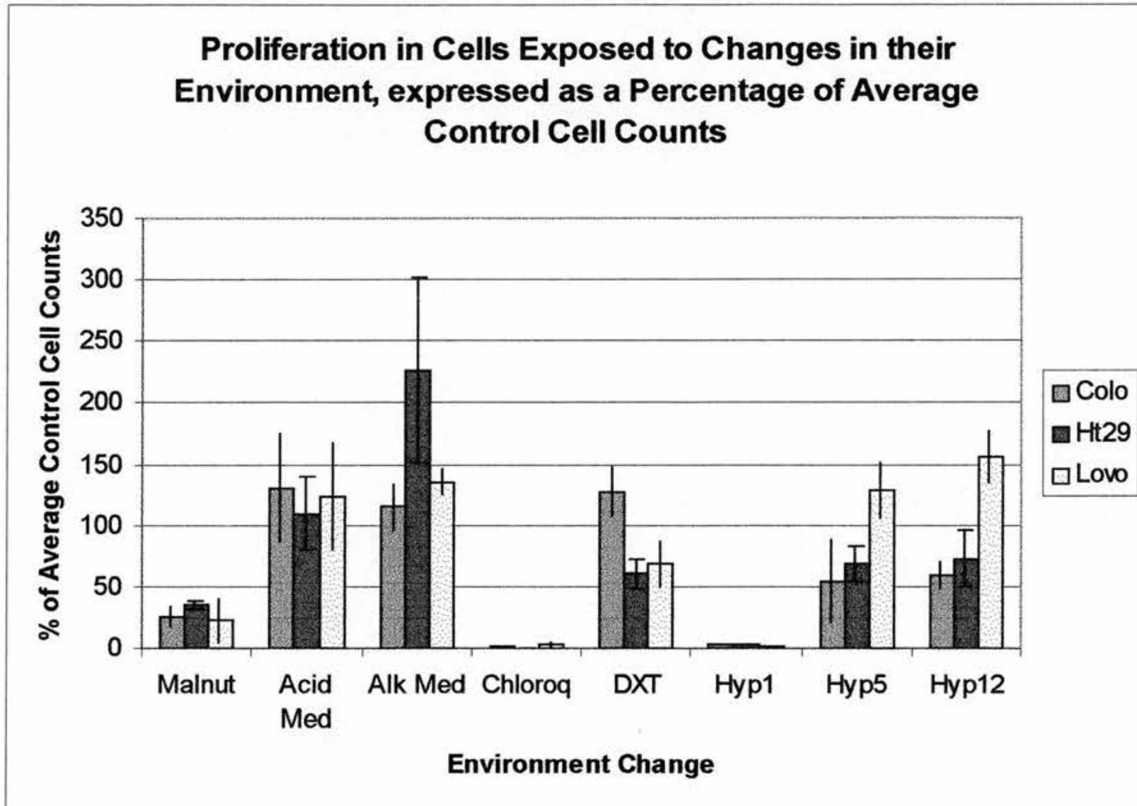
4. Radiation

The effects of X-radiation on cancer cells in terms of growth retardation are well established. The effects of X-radiation on tumour associated antigen expression are less well investigated. This study aimed to firstly find a dose of radiation which was inhibitory to the growth of human colorectal cancer cells in vitro over a period of 5 days, and secondly to look for any associated change in CEA expression at that dose. A preliminary titration experiment with Ht29 cells only was undertaken, and identified 5Grays of radiation as an effective dose, with the maximum change in the cells observed 72 hours after irradiation (see Appendix 1).

Four samples each of the Lovo, HT29 and Colo cells were exposed to 5 Grays of X-radiation, on Day 2 of a 5-day standard monolayer culture. Along with duplicate control flasks per cell line, the cells were then harvested, counted and stained for immunocytochemistry and FACS scanning assay of CEA expression. (see Appendix 1). One flask per cell line was processed for Electron Microscopy.

Results: Growth

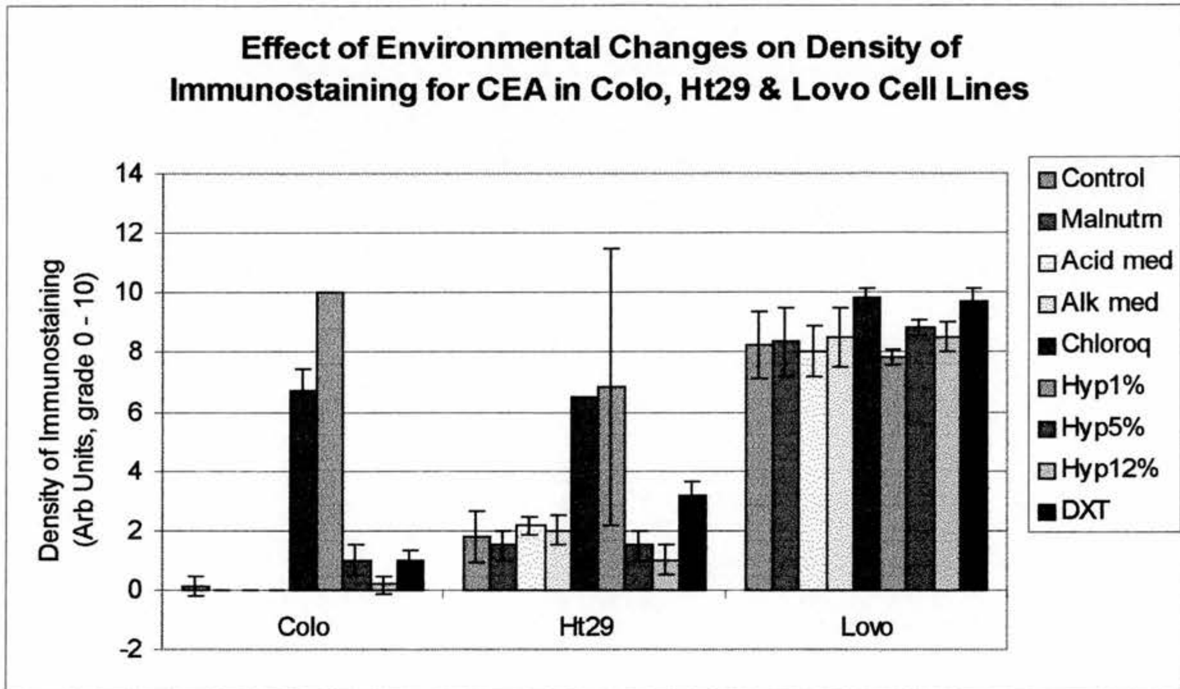
The changes in cell numbers are depicted below, shown as percentages of the average numbers of control cells.



As predicted, cell growth was inhibited by serum depletion. Altering the pH of the cell culture medium had no appreciable effect on growth in any of the three cell lines, but altering the pH of the internal cell environment by treatment with Chloroquine did inhibit cell growth, in all cells. Hypoxic conditions of 1% Oxygen were very effective at decreasing cell growth, but there was no significant effect at higher concentrations of Oxygen. 5Grays of X-radiation inhibited cell growth in the Ht29 cells only. (All results were analysed using the t-test, at $p \leq 0.05$)

CEA Expression: Immunocytochemistry

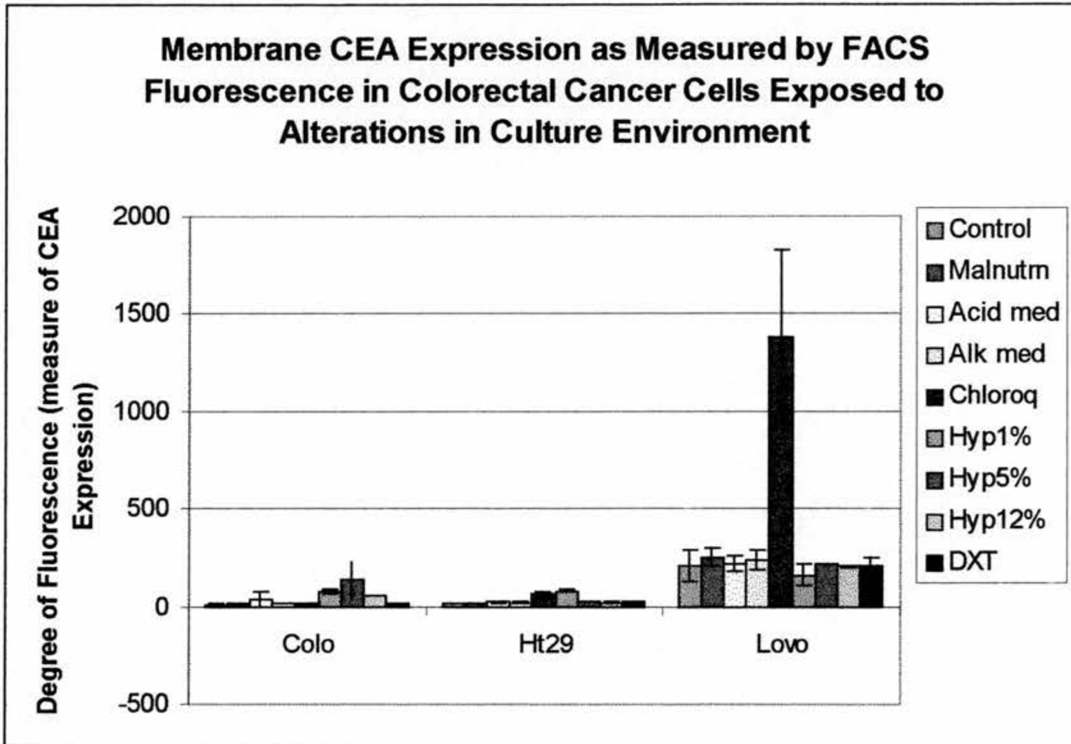
Changes in the density of immunostaining for CEA are shown below:



Augmentation of CEA expression was seen in Ht29 and Colo cells with Chloroquine, in Colo cells with <1% Oxygen only. Malnutrition, alterations in medium pH, X-radiation and Hypoxia at concentration of Oxygen of >1% had no recordable effect on density of staining for CEA using immunocytochemistry. All results were analysed using the t-test.

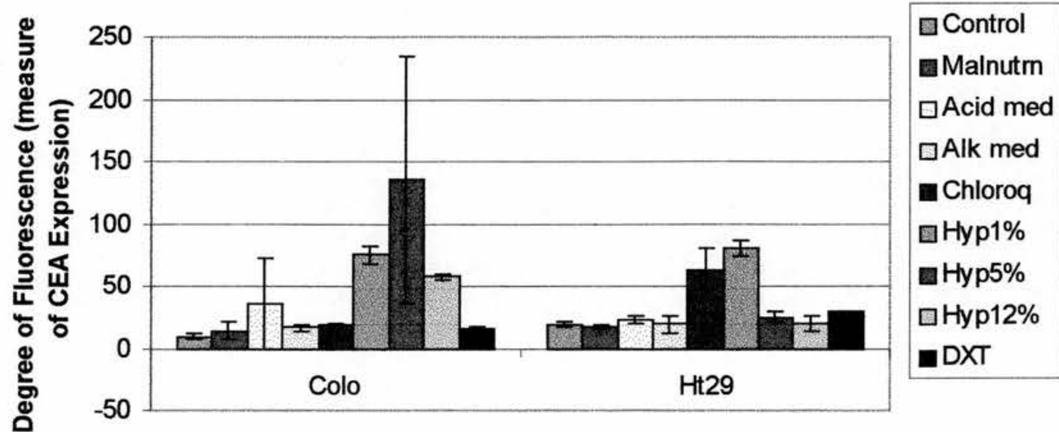
FLUORESCEIN ACTIVATED CELL SORTING

The effects of changes in cell culture environment on FACS fluorescence, staining for **membrane** CEA were as follows:



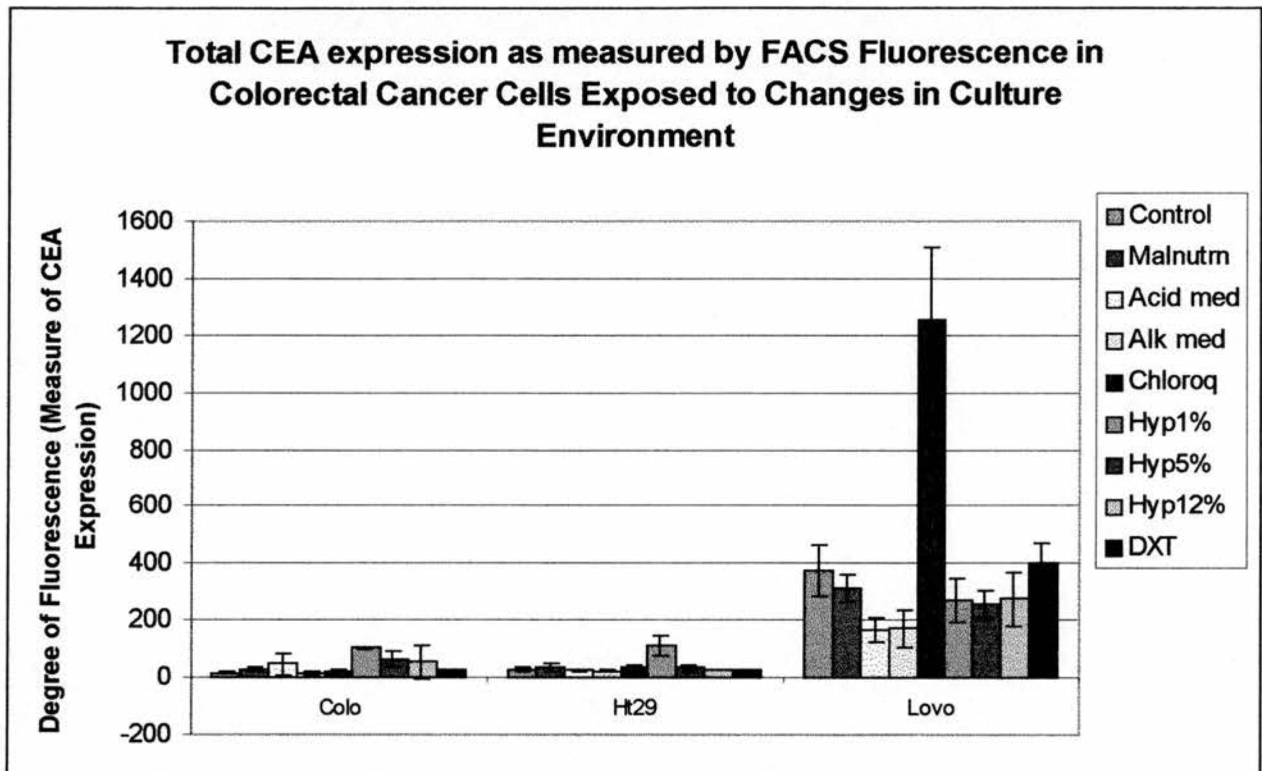
Using the Mann Whitney-U test, ($p \leq 0.05$), significant increases were observed in Colo cells exposed to <1% or 5% Oxygen, to Ht29 cells exposed to <1% Oxygen, Chloroquine or 5Grays of X-radiation, and to Lovo cells only on exposure to Chloroquine.

Changes in the Ht29 and Colo cells are more clearly shown in the following graph:

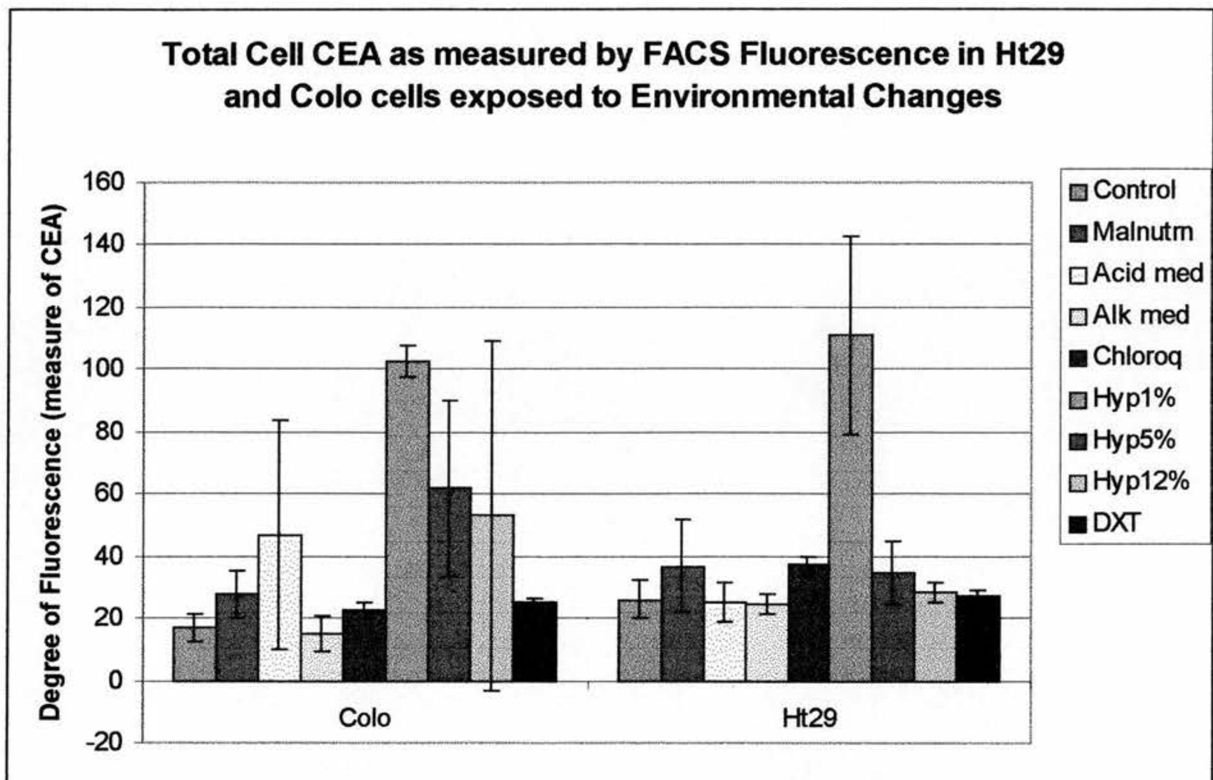

Membrane CEA Expression as Measured by FACS Fluorescence in Ht29 and Colo Cells on Expsosure to Alterations in Culture Environment
Degree of Fluorescence (measure of CEA Expression)
250
200
150
100
50
0
-50
Colo
Ht29
Control
Malnutrn
Acid med
Alk med
Chloroq
Hyp1%
Hyp5%
Hyp12%
DXT

FACS OF PERMEABOLISED CELL CEA

After permeabolising, FACS fluoresence values as a measure of total cell CEA showed significant increases in Colo cells with <1% and 5% Oxygen, in Ht29 cells with Chloroquine and with <1% Oxygen, and in Lovo cells with Chloroquine only (Mann Whitney-U test; $p \leq 0.05$):



Changes in the Ht29 and Colo cells are more clearly seen in the following graph:



Discussion

1 Starvation:

Starvation caused inhibition of cell growth and proliferation, but had no effect on tumour associated antigen expression. It is important that CEA augmentation was not found to be an inevitable consequence of the cell growth inhibition and increased cell death seen in this experiment, and conversely, that the increases in CEA expression seen in other experiments would appear not to be due simply to a process of cell death.

2. Altered pH

The results show that Chloroquine has effects on a range of colorectal cancer cell lines, causing growth inhibition and CEA augmentation, irrespective of the natural CEA-expressing status of the cells. Chloroquine is known to have a major effect on the pH of the internal cellular environment (*74*), and this change may be related to the change in cell behaviour in terms of CEA expression. It

is possible, of course, that Chloroquine's actions on the cells are unrelated to its pH-changing properties. An in-depth study of the intracellular action of Chloroquine which produced the recorded changes in CEA is beyond the range of this work. Altering the external environment of the cells in terms of pH had no such effect on CEA expression. Further information on the response of colorectal cancer cells to Chloroquine may be gained by exposing the cells to combinations of Chloroquine and differentiating or cytotoxic agents (Chapter 6).

3 Hypoxia

The results indicate that conditions of hypoxia, with less than 1% Oxygen are effective inhibitors of cell growth and inducers of CEA expression in colorectal cancer cell lines which do not normally express a high degree of tumour associated antigen. The increased CEA is not simply a by-product of cell death, as the Trypan blue cell check/count established the presence of live cells. It was noted in the previous experiment involving malnourishment of cells, that increased cell death was not automatically accompanied by increased CEA expression. Lovo cells, which have a high background level of CEA expression, were not induced to increase that level. The reason for the particular response of Colo cells to hypoxia is not known; no direct comparisons of the responses of Colo and Lovo cells to hypoxic environmental stresses or changes have been documented.

The relatively poor results with Oxygen concentrations of 5 and 12% does not bode well for trials of hypoxia in vivo for CEA augmentation, as achieving levels of Oxygen below 5% would pose great practical difficulties. Transient levels of Hypoxia of less than 5% may be achievable using techniques of embolization with starch microspheres. The duration of hypoxia required to produce significant CEA expression augmentation has not been investigated here.

4. X-Radiation

Irradiating the three colorectal cancer cell lines Lovo, Ht29 and Colo cells produced only slight growth inhibition of the Ht29 cells, and a slight increase in CEA expression, again only in Ht29 cells. It is possible that a higher dose of radiation might augment CEA, but the 5 Gray dose given here

which was sufficient to cause significant growth inhibition in the Ht29 cells produced only slight, although significant, increases in the membrane CEA expression in these cells.

Conclusions

1. Malnutrition is effective in curbing cell growth, but has no significant effect on CEA expression in colorectal cancer cells in vitro.
2. pH changes per se produce little effect on CEA expression in colorectal cancer cells in vitro.
3. Chloroquine is an effective CEA-inducing agent in this in vitro study, which may be related to its intracellular pH-changing properties; more in vitro studies and further work leading towards in vivo applications are warranted.
4. Hypoxic conditions may inhibit colorectal cancer cell growth and increase CEA expression, but only at extremes of hypoxia (<1%), and only in selected colorectal cancer cell lines.
5. Radiation does not appear to be a practical method of augmenting CEA expression in colorectal cancer cells.

CHAPTER FIVE

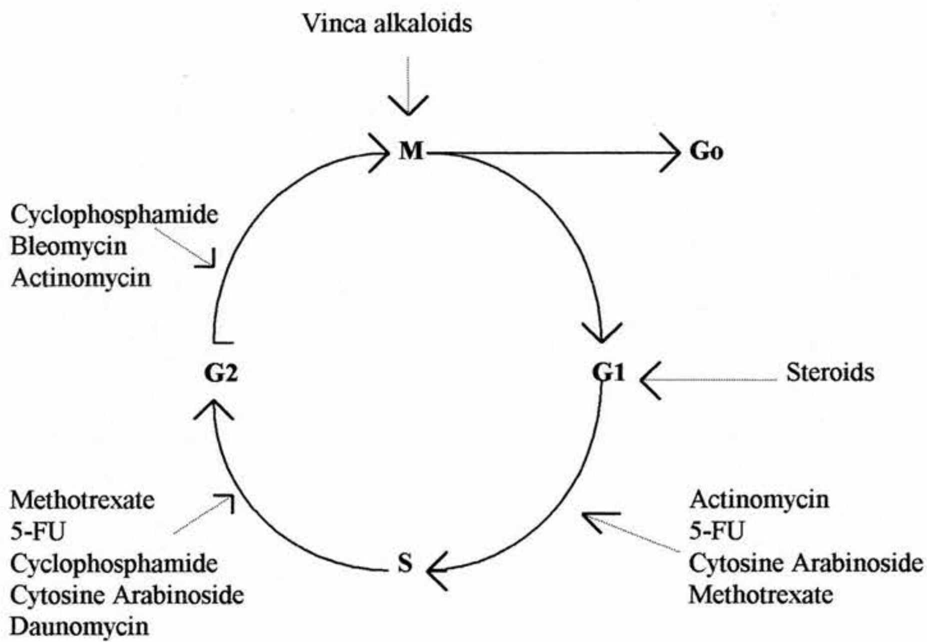
RESULTS: CYTOTOXIC DRUGS

Introduction

Cytotoxic (literally - cell-killing) drugs may be classified in terms of their mechanism of action on the growing cells within the cell cycle, and in terms of their chemical derivation. Some agents act on a specific phase of the cell cycle only; others are non-cycle dependent. The cell-killing abilities of each agent vary, and the proportion of normal cell deaths to neoplastic cell deaths also changes for each agent. A further source of variation is present in tumour type - the rate of growth of tumours alters their sensitivity to cell cycle interruptions by cytotoxic drugs. This means that seemingly more aggressive, faster growing tumours may be more effectively treated by phase-specific agents. There are five main groups of agents:

1. Antimetabolites
2. Plant alkaloids
3. Alkylating agents
4. Antibiotics
5. Random synthetic enzymes and hormones

Their actions on the cell cycle may be summarised in the following diagram:



The agents used in this series of experiments are classified as follows:

1. Antimetabolites: Methotrexate, 5-Fluorouracil, Taxol, Azathioprine

These compounds may be incorporated into new nuclear material or may combine irreversibly with cellular enzymes, thus preventing normal cell division from occurring. Methotrexate prevents DNA synthesis by inhibiting the enzyme dihydrofolate reductase; 5-FU blocks thymidylate synthetase. These actions are cell-cycle dependent, occurring only during the G1/S or S phases.

2. Vinca Alkaloids: Vincristine, Etoposide, Carboplatin

These plant alkaloids are derived from the periwinkle and May apple plants respectively. Vincristine acts by interfering with microtubule assembly which prevents mitosis, causing arrest of the cell cycle at the metaphase stage. Etoposide also blocks mitosis, but via a mechanism unconnected to the microtubule disruption.

3. Alkylating Agents: Cyclophosphamide

The alkyl groups attached to these agents react with nucleic acids, disabling them and thus damaging the cell DNA. In addition, the agents induce bond formation between DNA and RNA strands, which impedes DNA replication and RNA translation.

4. Antibiotics: Doxorubicin, Mitomycin, Bleomycin

These antibiotics act by preventing DNA and RNA synthesis, by binding to DNA, uncoiling the DNA helix, or fragmenting the strands of DNA. Their actions on RNA are indirect, mediated by the disabling of DNA.

Relatively little work has been done on the effects of cytotoxic agents on CEA expression by colorectal cancer cells in vitro. Studies of the effect of Fluorouracil on CEA expression have produced conflicting results (182,183). Studies of other cytotoxic agents (Cyclohexamide, Actinomycin-D) have demonstrated inhibition of growth of Ht29 colorectal cancer cells, but no differentiating effects (181). The antimetabolites are the drugs used in current clinical practice; 5-Fluorouracil has been shown to reduce recurrence of colorectal cancers, and post-operative intra-hepatic regimes have shown a survival advantage of 5% at 5 years. Methotrexate is licensed as a second line drug, and Taxol is awaiting approval, again as second line therapy.

Aim

To study the effects of the examples of commonly used cytotoxic drugs in each of the categories outlined above, on colorectal cancer cells in vitro, particularly searching for changes in cell growth, differentiation and CEA expression.

Methods

In this study, the above-listed cytotoxic agents from each class of agent were separately added to culture medium. Samples of 10^6 cells of each of the three colorectal cancer cell lines Lovo, Ht29 and Colo, were grown in standard culture conditions in the altered culture medium, alongside duplicate control samples for each cell line and each cytotoxic agent. Changes in cell growth, differentiating features, CEA expression and CEA production analysed by Electron Microscopy, Immunocytochemistry, FACS scanning and Radioimmunoassay, as outlined in Chapter 2. The results for the Radioimmunoassay and Electron Microscopy studies may be found in Chapters 7 and 8 respectively.

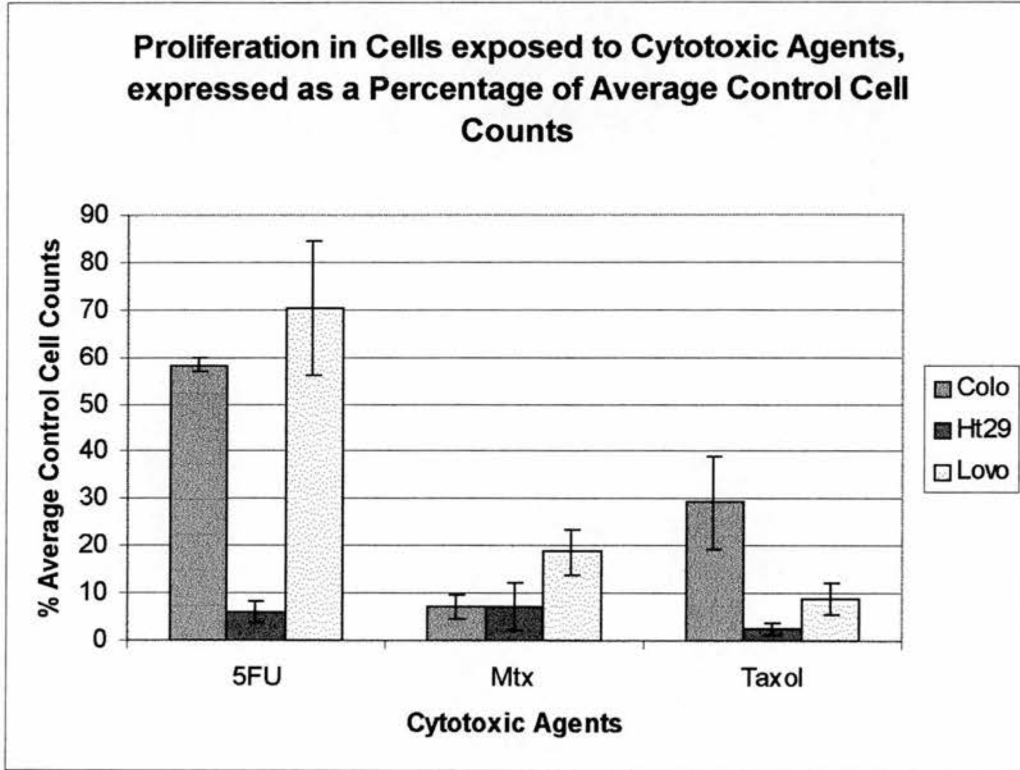
The concentrations of cytotoxic agents were selected according to limited data available on Vincristine, Etoposide and 5-Fluorouracil, advocating doses in the range of 1 - 20ng/ml (*264,265*). It is difficult to extrapolate from an in vitro concentration to a realistic approximation of a clinical dose range; the limited data available on Vincristine, Etoposide and 5-Fluorouracil suggested that these concentrations were relevant to the clinical scenario, but it is recognised that other agents have different cell-killing concentration properties, and undergo different metabolism clinically. Full titrations of doses for each cytotoxic drug were felt to be beyond the range of this initial study; therefore a fixed dose of 10ng/ml of each agent was used.

Results

Of all the agents listed above, only 5-Fluorouracil, Methotrexate and Taxol showed appreciable growth inhibitory and CEA augmentation effects. There were also minor increases in CEA in the Lovo cells only, with Doxorubicin, Vincristine and Etoposide. The main positive results are printed below.

Growth

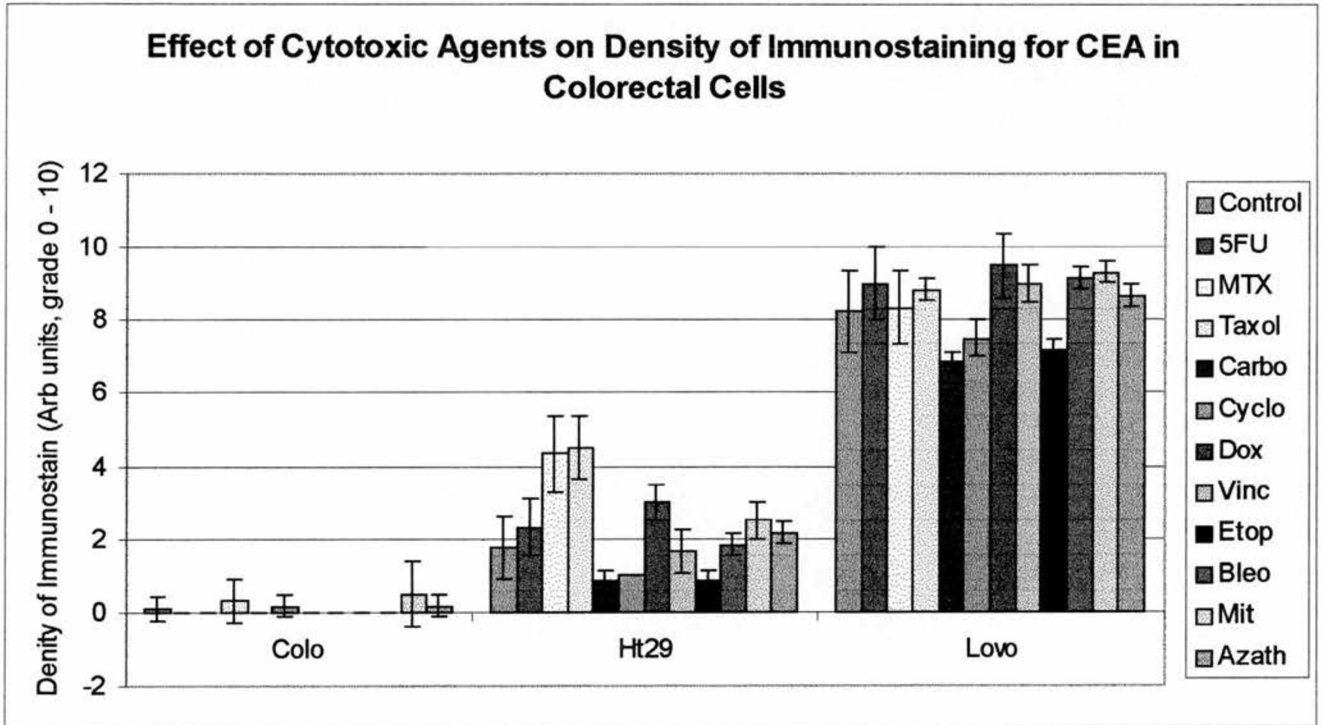
The effects on average cell counts in the cells grown in 5-Fluorouracil, Methotrexate and Taxol compared to control cells are shown below.



CEA EXPRESSION:

IMMUNOCYTOCHEMISTRY:

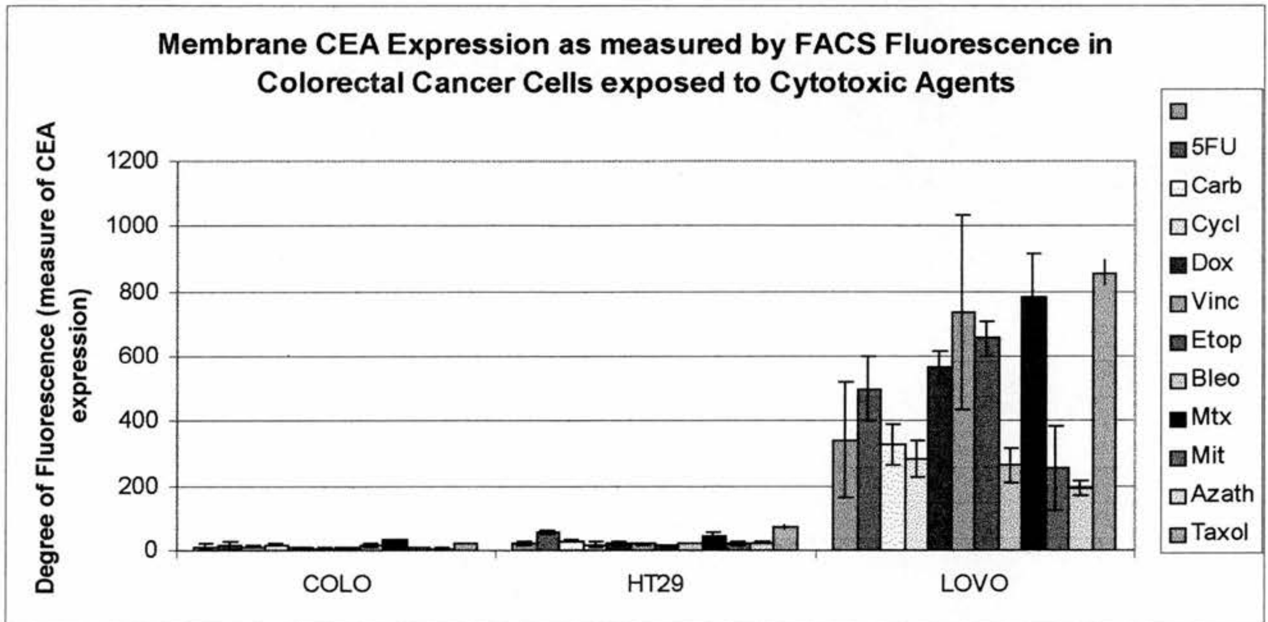
The effects of the various cytotoxic agents on the density of immunostaining for CEA are shown below.



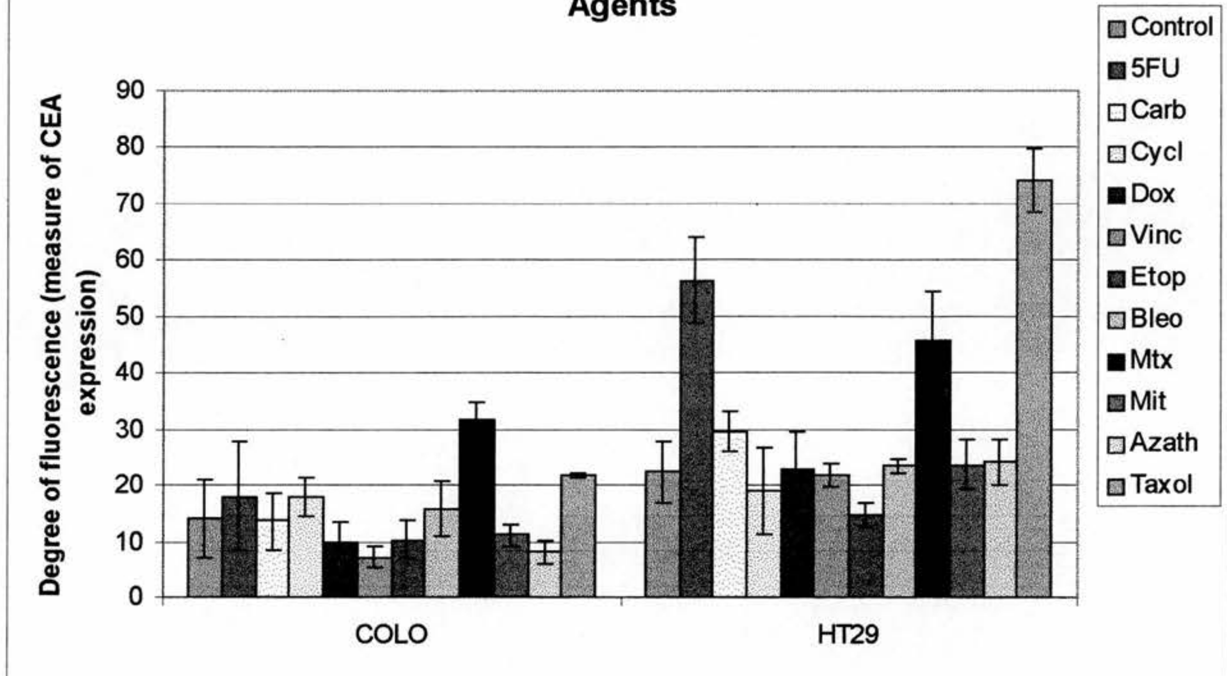
Statistical analysis using the t-test, and comparing against all of the control samples revealed significant increases in immunostaining for CEA in Ht29 cells exposed to Methotrexate, Taxol and Doxorubicin. None of the agents had any noticeable effect on staining for CEA in the Colo or Lovo cells.

FACS FLUORESCENCE: MEMBRANE CEA

The changes in membrane CEA expression on exposure to the various cytotoxic agents are chown below:



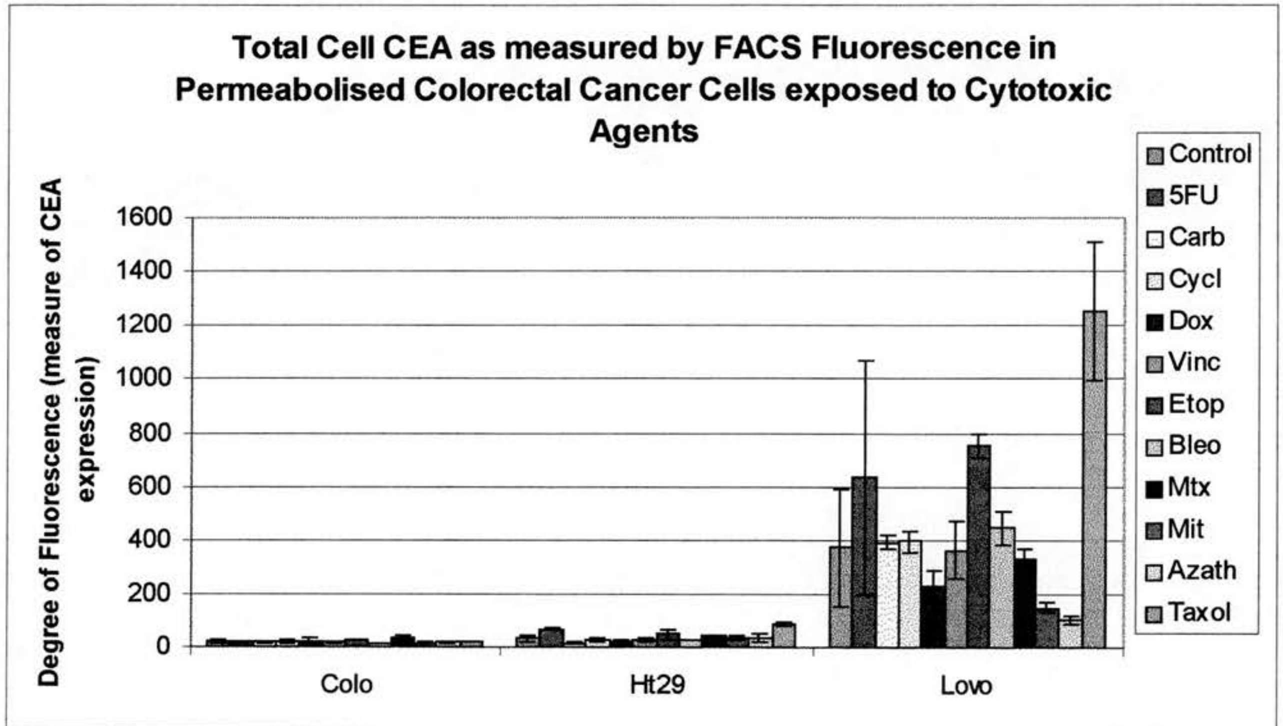
The results in the Colo and Ht29 cells are more clearly shown in the following graph, without the Lovo cells:



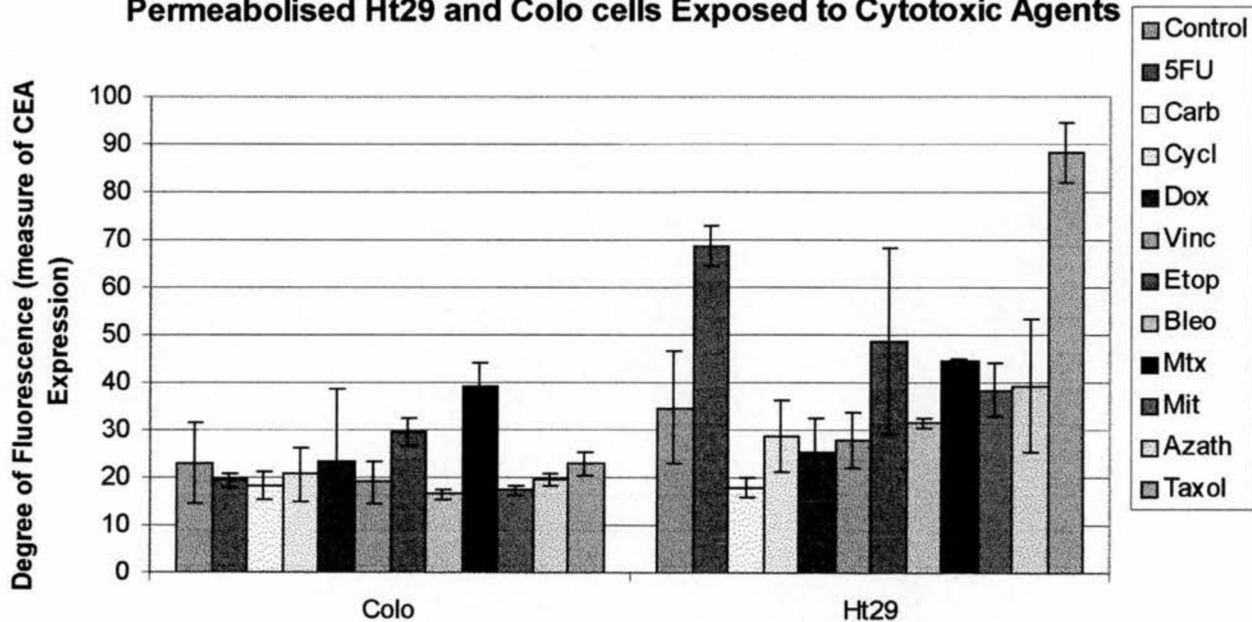
Analysis using the Mann-Whitney-U test revealed significant CEA augmentation in the Colo cells on exposure to Methotrexate and Taxol; in the Ht29 cells with 5-Fluorouracil, Methotrexate and Taxol; and in the Lovo cells with Methotrexate, Taxol, Doxorubicin, Vincristine and Etoposide.

FACS FLUORESCENCE: TOTAL CEA CONTENT

The changes in total cell CEA content on exposure to the cytotoxic agents are shown below:



The results in the Colo and Ht29 cells are more clearly shown in the following graph:/



The Mann-Whitney-U test showed significant increase in total cell CEA content in the Colo cells with Methotrexate only, in the Ht29 cells with 5-Fluorouracil, Methotrexateand Taxol; and the Lovo cells with Taxol and Etoposide.

Discussion

Of all the categories of cytotoxic agent studied, the anti-metabolites have consistently shown the greatest effects with 5-FU, Methotrexate and Taxol all inhibiting growth and inducing CEA expression in one or more of the colorectal cancer cell lines at the single tested dose of 10ng/ml. These three drugs are commonly used in the treatment of colorectal cancer in vivo, having tumouricidal or at least tumouristatic effects. They are known to interfere with protein synthesis at a nuclear level, so it may appear surprising that the colorectal cancer cells respond to them with increased CEA glycoprotein synthesis. If there is an improvement in the differentiation of the cells, however, selective inhibition and stimulation of various biochemical processes intracellularly would be expected.

5-Fluorouracil

5FU had effects on each of the cell lines - it depressed growth in the Ht29 and Colo cells, increased CEA staining according to immunocytochemistry in the Lovo cells, and increased FACS fluorescence for CEA in the Ht29 cells.

There was an increase in CEA staining by immunocytochemistry in the Ht29 cells, but the effects were not statistically significant. However, the effects on CEA in Ht29 cells according to FACS were consistent with both parametric and non-parametric statistical analysis, and do point to a CEA-augmentation effect of 5-FU in these cells. The total cell CEA was increased by 5-FU, but did not significantly outstrip the increased level of membrane CEA. It is not expected that 5-FU should induce increased intracellular synthesis of CEA, since its known mechanism of action is to prevent DNA synthesis and thus prevent intracellular protein synthesis. The mechanism by which 5-FU induces increased CEA expression is not clear.

The apparent increase in CEA staining in Lovo cells seen on immunocytochemistry was not confirmed with the more accurate FACS analysis, and is therefore not taken as a sign of a definite CEA increase.

It is noted that growth inhibition in the Colo cells was not accompanied by increased CEA, one marker of differentiation. Growth inhibiting effects are not necessarily expected to be associated with improved differentiation.

Methotrexate

Methotrexate showed inhibitory effects on cell growth in each cell line, and this was accompanied by an apparent increase in membrane CEA expression, according to FACS analysis. The lack of corresponding significant increases seen on immunocytochemistry may reflect the reduced sensitivity of this method compared to FACS. Increases in total cell content of CEA were seen in Ht29 and Colo cells, but not in Lovo cells, which may imply an increased movement of intracellular CEA to membrane sites, rather than an increase in CEA production by the cells; indeed, the action of Methotrexate in blocking DNA synthesis, makes increased production of CEA most unlikely. How Methotrexate does affect CEA expression is not clear; it may be an indirect effect of improved differentiation induced by the agent; or may reflect a change in the population of cells, the more quickly reproducing, poorly differentiated cells being wiped out by the Methotrexate to leave well differentiated, CEA expressing cells. The latter possibility does not account for the changes in the Colo cells, which have changed character from non-CEA expressing to CEA-expressing.

Taxol

Taxol was effective as an inhibitor of cell proliferation in all the cell lines studied, and induced impressive augmentation of tumour-associated antigen expression in the Lovo and Ht29 cells. It was also effective to a lesser extent in the Colo cells, which are naturally non-expressors of CEA, showing some significant membrane CEA augmentation. Being a relatively new drug, Taxol has not featured in the studies of CEA augmentation by cytotoxic agents reported to date. The above results can therefore not be compared with historical data, but it is certainly exciting to find that Taxol has

definite effects on colorectal cancer cell differentiation in vitro; it should be included in future in vitro and in vivo studies.

Taxol is from the family of drugs which includes 5-Fluorouracil and Methotrexate. It might be presumed that all three compounds have a similar mechanism of action; however, it is noted that they show heterogeneity in their effects on the three different cell lines.

The only other drugs to have any effect on the colorectal cancer cells at this dose, were Etoposide, Vincristine and also Doxorubicin, which induced a minor degree of CEA augmentation in Lovo cells. Considering the degree of heterogeneity present even within one colorectal tumour in vivo, the narrow range of activity of these three agents is likely to preclude them from attaining a useful role in tumour-associated antigen targetting. Previous studies of Vincristine particularly have shown differentiating actions on human AML cells (*157*). Further studies of the Vinca alkaloids at a range of concentrations are needed before a role for them in CEA augmentation may be outlined.

Conclusion

1. 5-FU is effective as a growth inhibitor in Ht29 cells and Colo cells, and as an inducer of CEA expression in Ht29 cells. It does not appear to be a broad range CEA-inducer in different colorectal cancer cell lines.

2. Methotrexate is effective in producing growth retardation and increased membrane CEA expression in a range of colorectal cancer cells in vitro, whether they naturally express high levels, low levels or no CEA.

3. Taxol shows both growth inhibitory and significant CEA-augmentation effects in colorectal cancer cell lines, particularly those which naturally express CEA to a variable degree.

4. Etoposide, Vincristine and Doxorubicin have limited activity in CEA augmentation in colorectal cancer cell lines.

CHAPTER SIX

Results (iv): Combination Experiments

INTRODUCTION

The effects of the variations in environment, exposure to differentiating agents and to cytotoxic drugs on the three colorectal cancer cell lines may be loosely summarised as follows:

Increased CEA (membrane) with:

LOVO: Butyric Acid, Interferon, Chloroquine, Methotrexate, Taxol, and 5-Fluorouracil and, (to a lesser extent), Doxorubicin, Etoposide and Vincristine .

HT29: Butyric Acid, Theophylline, Interferon, Chloroquine, Hypoxia, Irradiation, 5-Fluorouracil, Methotrexate, and Taxol.

COLO: Theophylline, 5-Azacytidine, Hypoxia, Chloroquine, Methotrexate and Taxol,

Decreased growth with:

LOVO: Starvation, Chloroquine, Hypoxia, Butyric Acid, Theophylline, 5-Azacytidine, Interferon, 5-Fluorouracil, Methotrexate and Taxol.

HT29 : Theophylline, 5-Azacytidine, Interferon, 5-Fluorouracil, Methotrexate, Taxol, Chloroquine, Hypoxia and Irradiation.

COLO: Butyric Acid, Theophylline, 5-Azacytidine, 5- Fluorouracil, Methotrexate, Mitomycin, Taxol, Chloroquine, Starvation and Hypoxia

The discovery of augmented CEA expression in Colo cells is particularly interesting since they were previously thought to be non-expressors of CEA - by immunocytochemistry analysis. The immuncytochemistry results were generally found to follow the FACS results, but to be much less sensitive (See Chapter 6).

As a result of these preliminary findings, further experiments were set up to look for possible synergistic effects between the various factors which had positive results on CEA expression in the preceding experiments. As mentioned in Chapter one, combinations of differentiating or cytotoxic agents may have additive, inhibitive or synergistic effects on tumour-associated antigen expression and differentiation (*101*). Additive effects imply independent actions of two different agents, synergistic and inhibitive effects, positive or negative interaction between agents.

Limited studies on combining differentiating and cytotoxic agents have been performed; mouse erythroleukaemia cells (MEL) exposed to Trans-retinoic acid and Cytosine Arabinoside showed enhanced differentiation (96). Little work on the effects of such combinations on colorectal cancer cells has been published, and no trials of combining environmental changes with chemical differentiating or cytotoxic agents have been reported.

AIM

To look for additive or synergistic differentiating effects on growth inhibition and augmented CEA expression in the three colorectal cancer cell lines Lovo, Ht29 and Colo, on exposure to combinations of the chemical agents and environmental changes found to successfully induce differentiation when used singly. The range of combinations of effective agents was chosen based on successful agents, and potential practical application, and was tested on all three cell lines.

The combinations studied were:

1. DIFFERENTIATING AGENT + DIFFERENTIATING AGENT

a. Butyric Acid + Theophylline

b. Butyric Acid + 5-Azacytidine

c. Butyric Acid + Interferon

d. Interferon + Theophylline

e. Interferon + 5-Azacytidine

2. DIFFERENTIATING AGENT + CYTOTOXIC DRUG

a. Butyric Acid + 5Fluorouracil

b. Theophylline + 5Fluorouracil

c. 5-Azacytidine + 5Fluorouracil

d. Interferon + 5Fluorouracil

e. Interferon + Methotrexate

f. Butyric Acid + Methotrexate

g. Theophylline + Methotrexate

h. 5-Azacytidine + Methotrexate

3. DIFFERENTIATING AGENT + ENVIRONMENT CHANGE

a. Butyric Acid + Hypoxia

b. Butyric Acid + Chloroquine

c. Butyric Acid + Alkaline Medium

4. ENVIRONMENT CHANGE + CYTOTOXIC DRUG

a. Chloroquine + 5-Fluorouracil

b. Chloroquine + Methotrexate

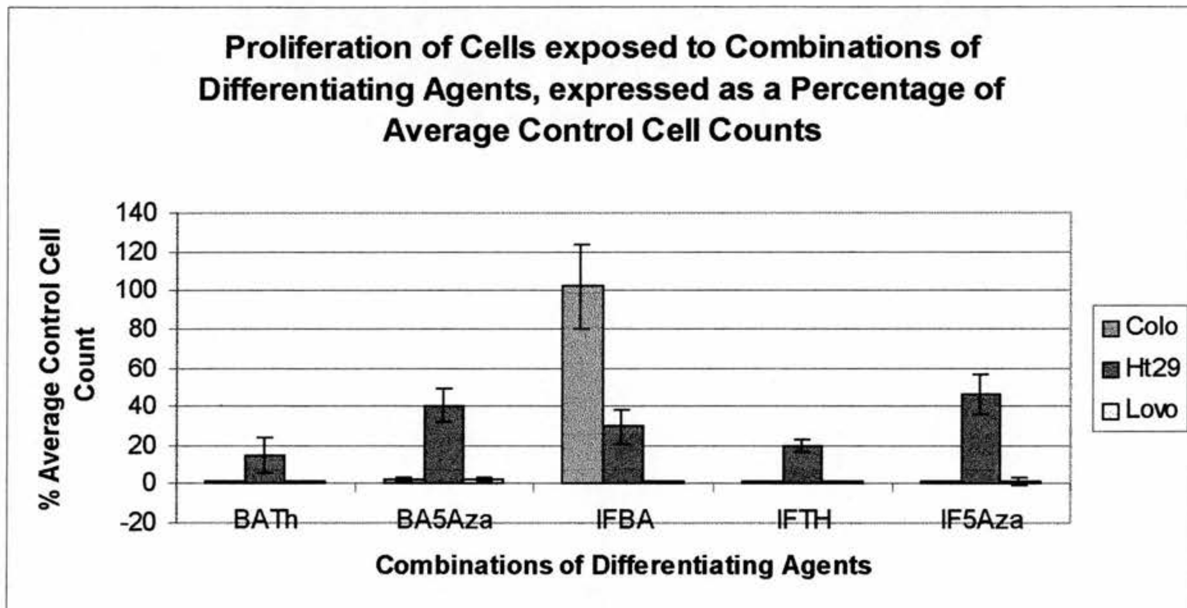
METHODS

As in the previous chapters, and as described in Chapter 1, triplicate samples of each cell line were grown for 5 days in medium altered by the addition of the 2 agents under investigation, in the same concentrations as used in the single agent experiments. The cells were then counted, studied under electron microscopy and stained for CEA measurement by immunocytochemistry and FACS.

RESULTS

Growth

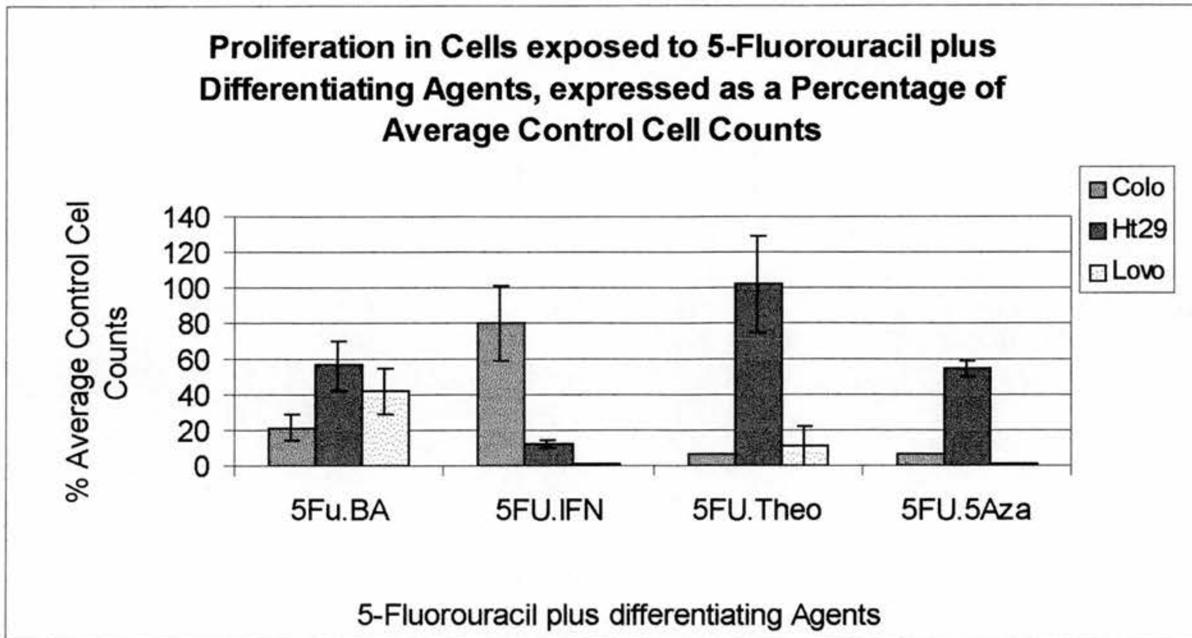
1. Differentiating Agent + Differentiating Agent



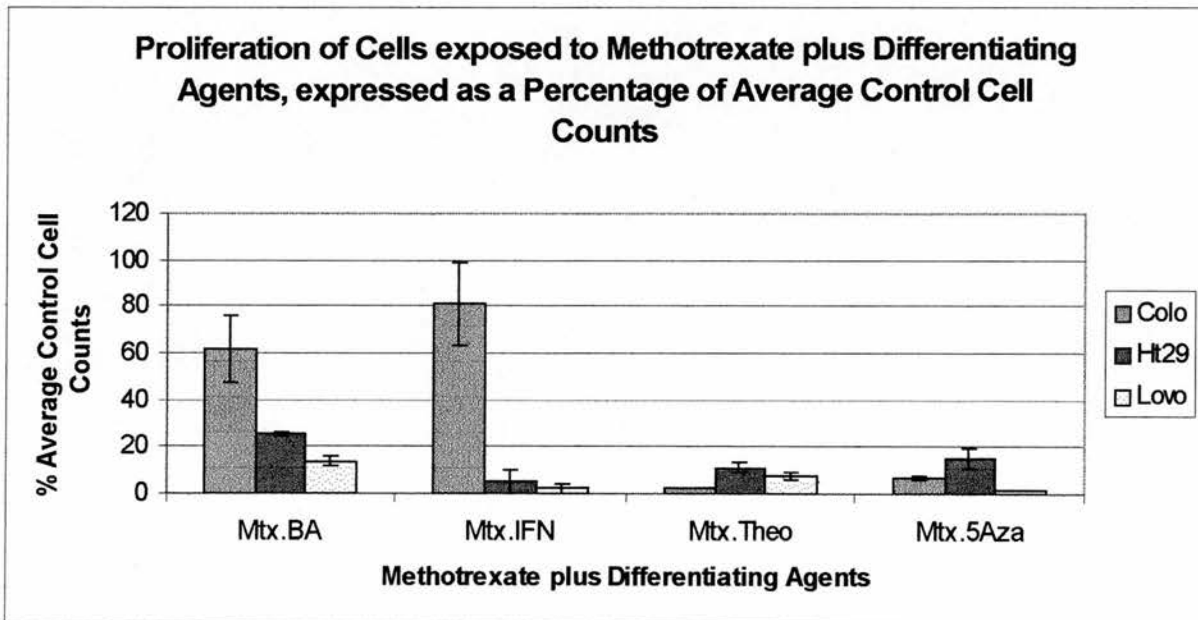
Significant growth inhibition (t-test; $p \leq 0.05$) was seen in all cell lines with each combination of 2 differentiating agents, except the Colo cells with Interferon plus Butyric acid.

2. Differentiating Agent + Cytotoxic Agent

The effects of combining 5-Fluorouracil or Methotrexate with each of the differentiating agents are shown in separate graphs below:



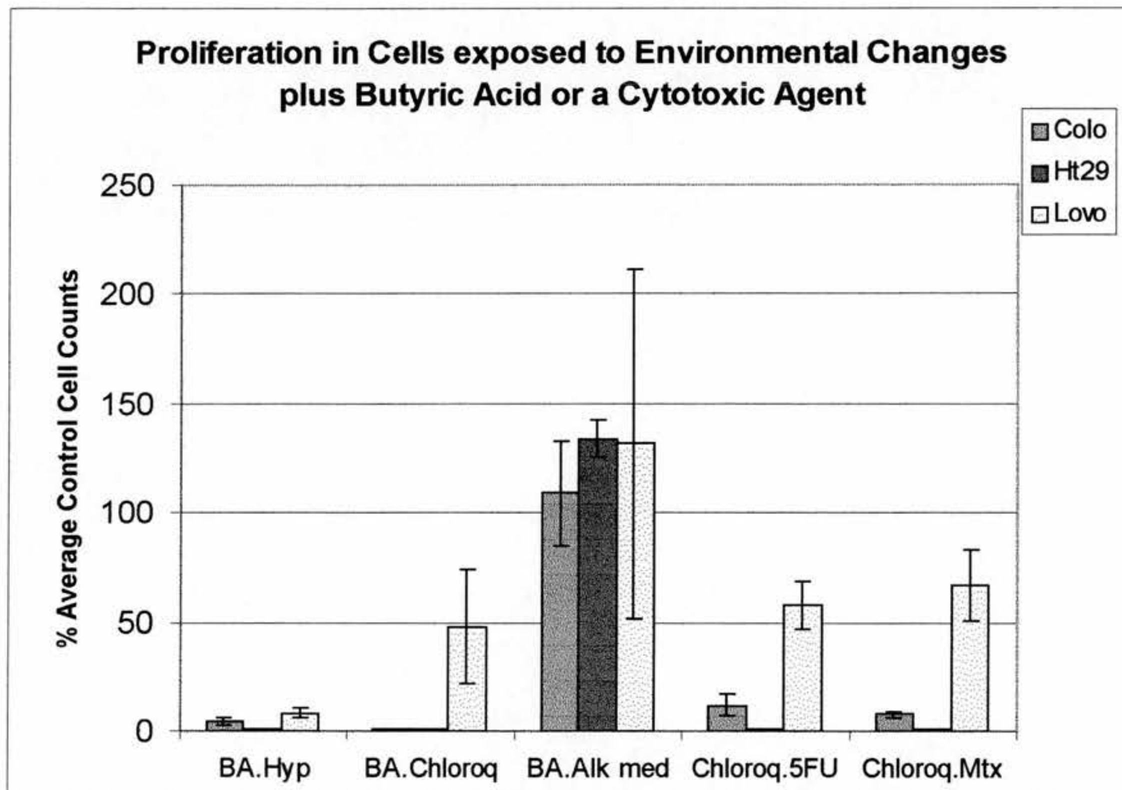
Significant growth reduction (t-test; p≤0.05) was seen in the Lovo cells with all combinations, in the Colo cells with all combinations except 5-Fluorouracil plus Interferon; and in the Ht29 cells with 5-Fluorouracil plus Interferon only.



Methotrexate plus any of the differentiating agents induced significant growth inhibition in all of the cell lines, except in Colo cells when combined with Interferon (t-test, p≤0.05).

3. Environmental Factor+ Differentiating or Cytotoxic Agent

Combinations of altered oxygenation, medium pH and intracellular pH (Chloroquine) were tested with Butyric Acid. Chloroquine was also tested with 5-Fluorouracil and Methotrexate.



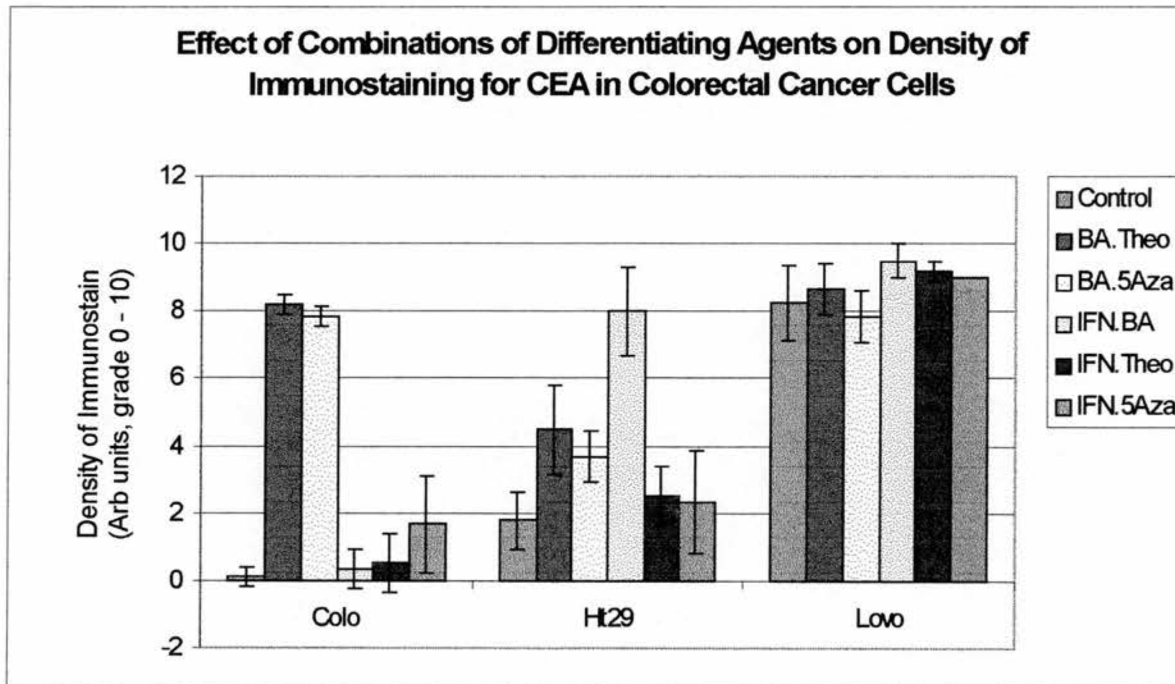
Butyric Acid plus hypoxia induced growth inhibition in all the cells. With the exception of Chloroquine plus Methotrexate in Lovo cells, all of the combinations involving Chloroquine caused growth inhibition (t-test, $p \leq 0.05$). Butyric Acid with alkaline medium was ineffective in all cell lines.

CEA EXPRESSION

IMMUNOCYTOCHEMISTRY

Statistical analysis was performed using the t-test. No evidence of synergistically positive reactions was found in any of the experiments, which may reflect the lack of sensitivity of the naked eye immunocytochemistry grading system.

1. Differentiating Agent + Differentiating Agent

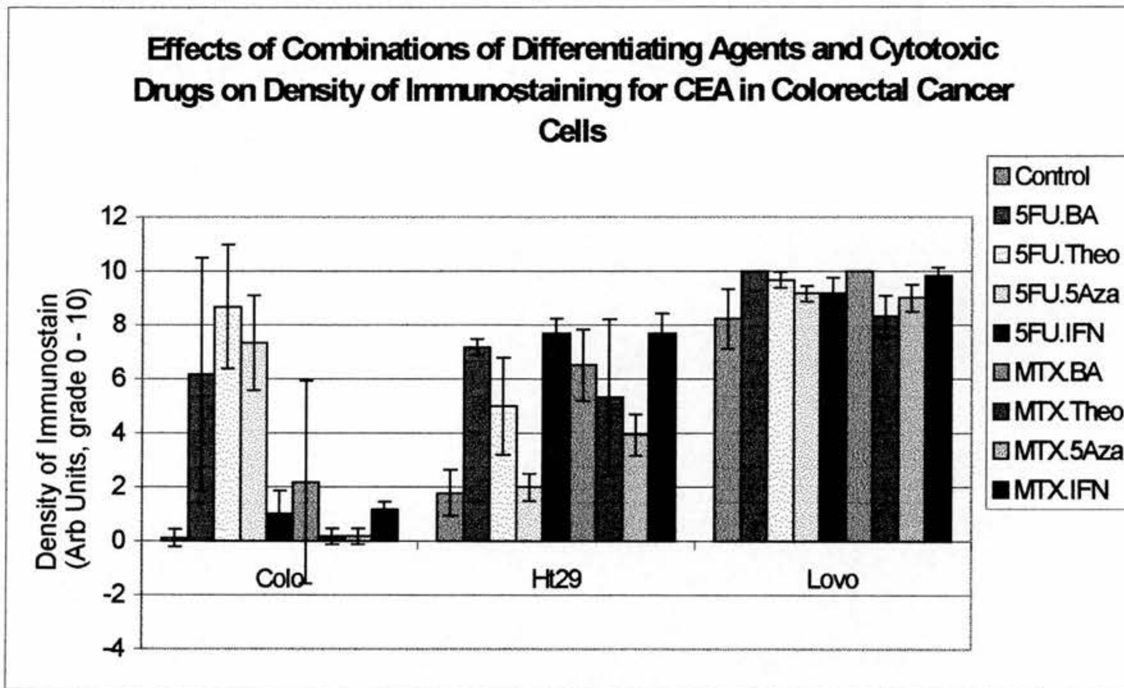


Analysis using the t-test ($p \leq 0.05$) revealed no change in staining for CEA in the Lovo cells, although used alone, Butyric acid had previously shown increased staining.

The Colo cells showed increased density of staining for CEA with Butyric Acid plus Theophylline or 5-Azacytidine and with Interferon plus 5-Azacytidine. Only 5-Azacytine was effective when the agents were tried singly.

Ht29 cells were found to have increased staining compared to controls with Butyric Acid plus Theophylline, 5-Azacytidine or Interferon – little different to the situation with single agents, where Butyric acid, Interferon and Theophylline were all effective inducers of increased CEA staining.

2. Differentiating Agent + Cytotoxic Agent



All four combinations involving 5-Fluorouracil induced increased staining for CEA in the Colo cells, notable in that 5-Azacytidine only was effective as a single agent.

In the Ht29 cells, significant increases were seen with 5-Fluoruracil plus Interferon, Butyric acid or Theophylline, the three differentiating agents seen to be effective singly.

The Lovo cells showed increased CEA staining with 5-Fluorouracil plus Butyric acid or Theophylline, although used singly only Butyric acid was effective.

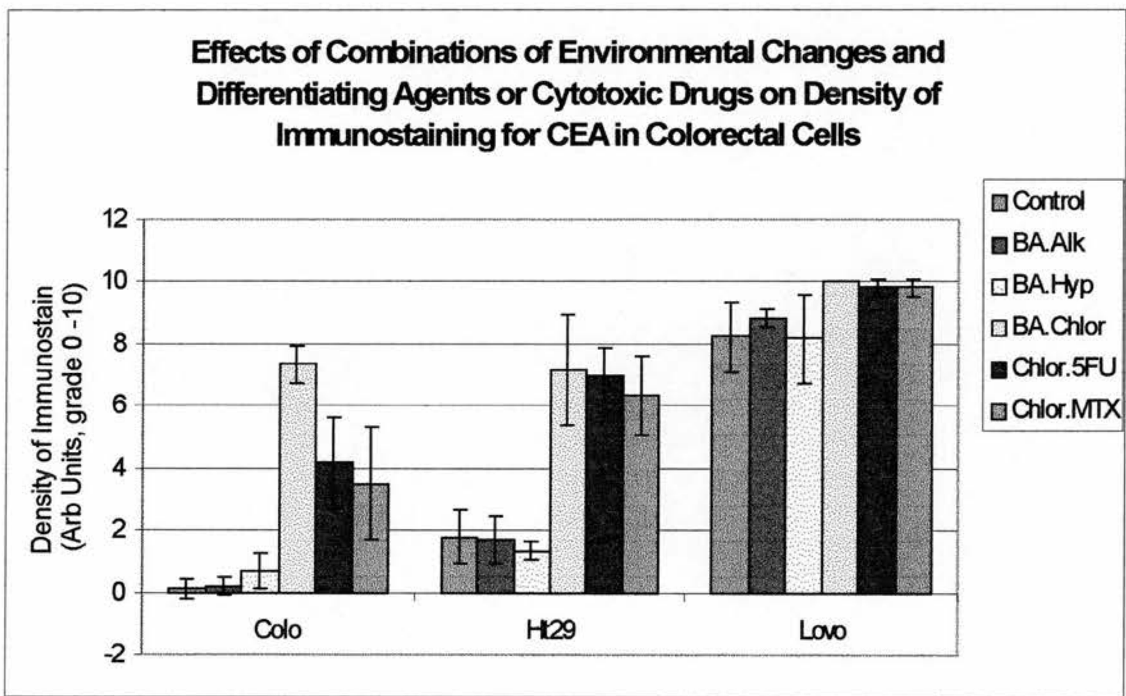
Methotrexate plus Interferon or Butyric acid was effective in inducing increased CEA staining in the Colo cells, although none of these agents used singly had any significant effect. 5-Azacytidine which

was effective used singly in the Colo cells, induced no CEA augmentation when combined with Methotrexate.

Methotrexate with any of the differentiating agents induced increased staining for CEA in the Ht29 cells, which showed increased staining with all of the agents except 5-Azacytidine used singly.

Butyric acid alone and with Methotrexate induced CEA augmentation in the Lovo cells. Interferon and Methotrexate were also effective as a combination, but not alone.

3.Environmental Factor+ Differentiating or Cytotoxic Agent

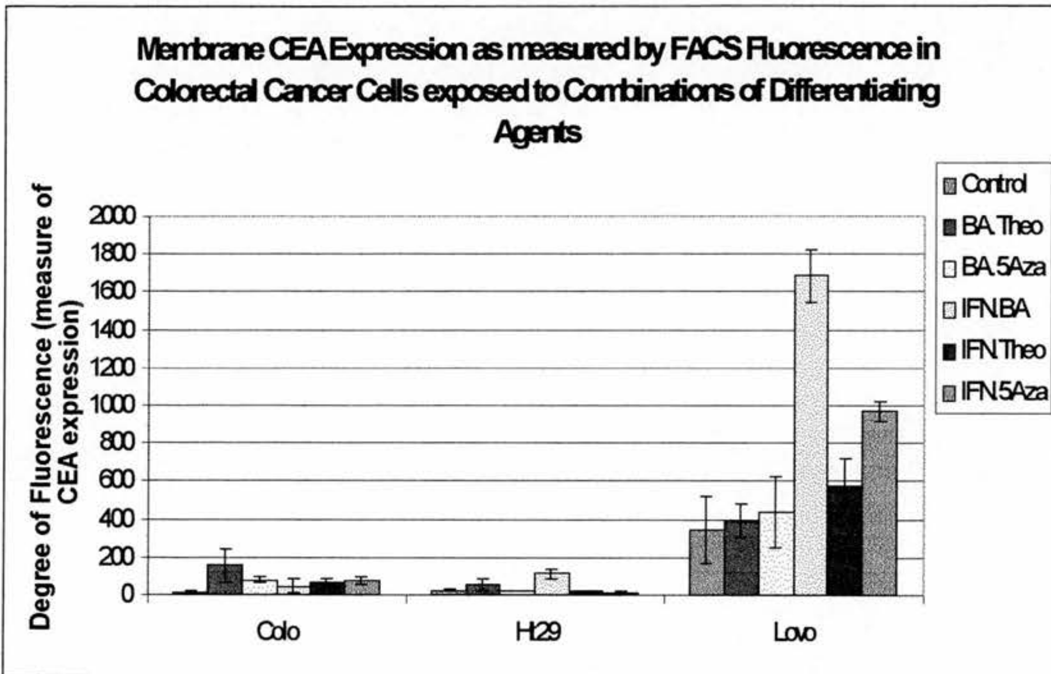


All of the combination involving Chloroquine resulted in increased staining for CEA in all of the cell lines, and it is noted that Chloroquine used alone was similarly effective in all cell lines. Butyric acid plus hypoxia induced increased CEA in the Colo cells only, and Butyric acid plus alkaline medium in none of the cells. This compares to significant augmentation of CEA seen in Ht29 and Lovo cells exposed to Butyric acid alone.

FACS FLUORESCENCE: MEMBRANE CEA

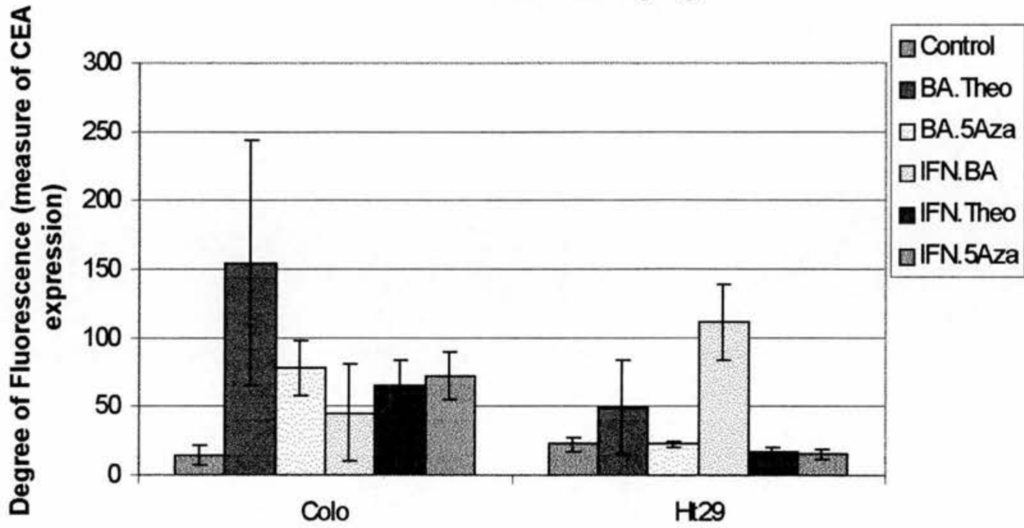
All statistical analysis was performed using the Mann Whitney-U test, at a p value of ≤ 0.05.

1. Differentiating Agent + Differentiating Agent



In the Lovo cells, only the combinations involving Interferon were effective inducers of CEA, although both Butyric acid and Theophylline when used singly, induced CEA.

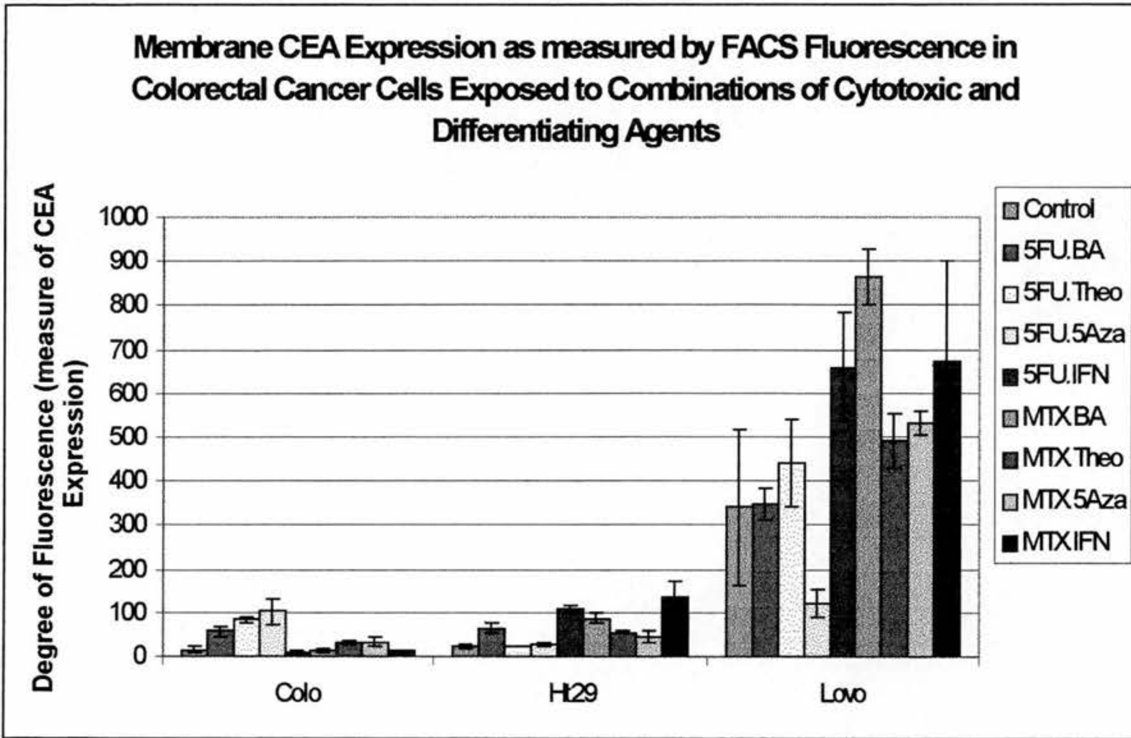
The changes in the Colo and Ht29 cells are more clearly demonstrated in the following graph:



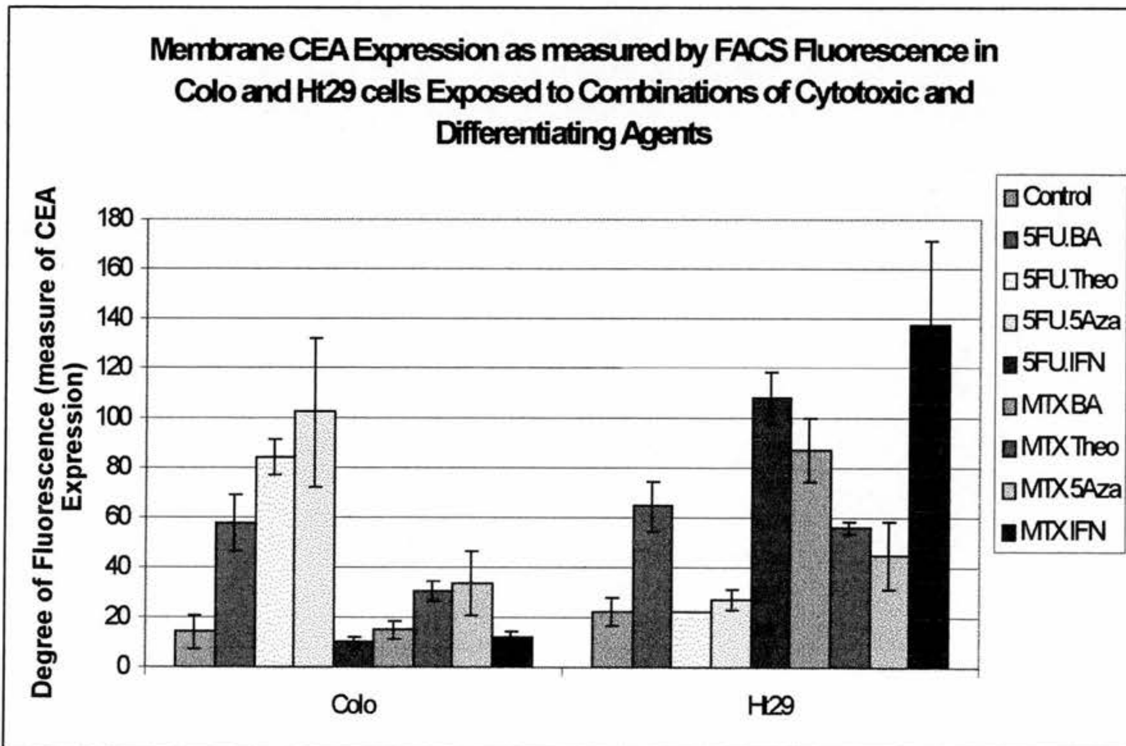
Significant augmentation of CEA was seen with all combinations in Colo cells, despite the fact that only Theophylline and 5-Azacytidine were effective as single agents.

Ht29 cells showed increased staining for membrane CEA with Butyric acid plus Theophylline or Interferon. All three of these agents were effective singly, but combinations other than the two mentioned, or involving 5-Azacytidine did not induce increased CEA expression.

2. Differentiating Agent + Cytotoxic Agent



The changes in the Colo and Ht29 cells are more clearly shown in the following graph:

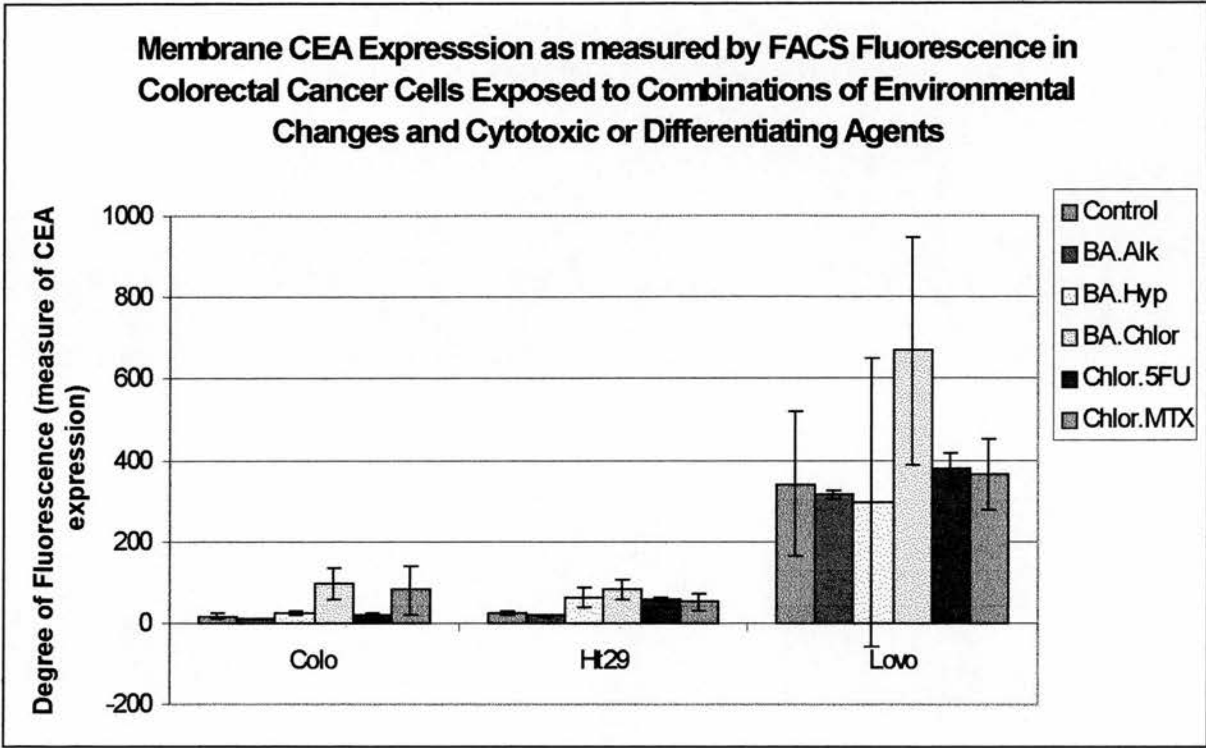


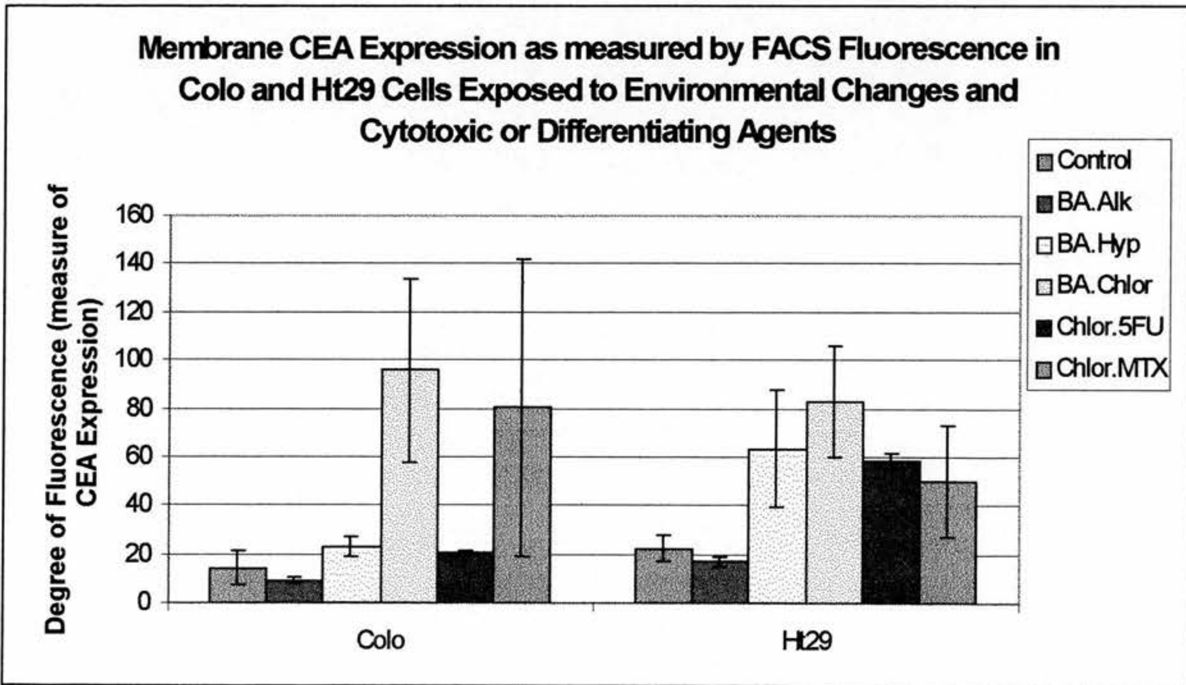
In the Colo cells, 5-Fluorouracil plus Butyric acid, Theophylline or 5-Azacytidine, but not Interferon, induced increased CEA. Used alone, neither 5-Fluorouracil nor Butyric acid were effective in Colo cells. Methotrexate, which was successful used alone, produced increased CEA in combination with Theophylline and 5-Azacytidine only.

In the Ht29 cells, Theophylline and 5-Fluorouracil lost their CEA-induction abilities when in combination. 5-Fluorouracil was also ineffective when used with 5-Azacytidine, but otherwise, all the combinations of successful single agents continued to produce increased CEA staining. No evidence of a synergistic increase in CEA was seen.

Combinations of Interferon with 5-Fluorouracil or Methotrexate and of Butyric acid with Methotrexate showed increased CEA augmentation in the Lovo cells. This compares to increased augmentation seen with Methotrexate, Butyric acid, Interferon and Theophylline used as single agents. Additive or synergistically increased levels of CEA were not seen.

3. Environmental Factor+ Differentiating or Cytotoxic Agent





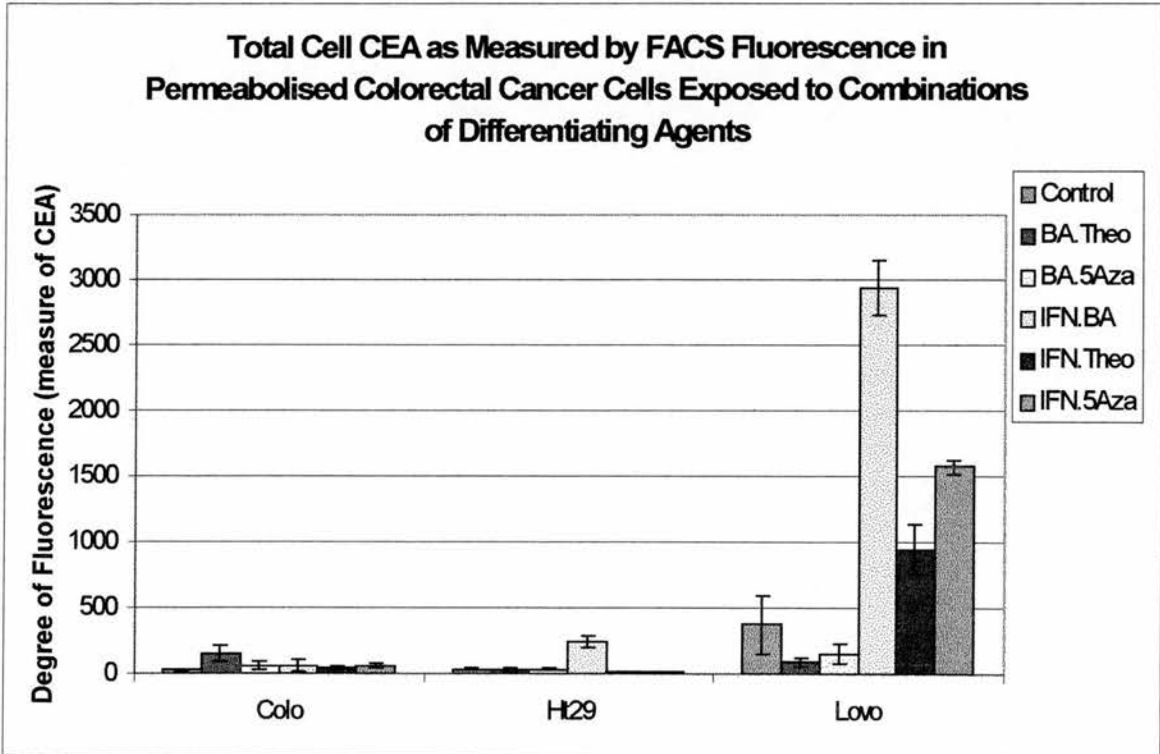
In the Colo cells, there was a small increase in CEA with Butyric acid and hypoxia, (smaller than with hypoxia alone). There was a more substantial increase in CEA with Chloroquine plus Butyric acid or Methotrexate, but not significantly different to the results produced by Chloroquine or Methotrexate alone.

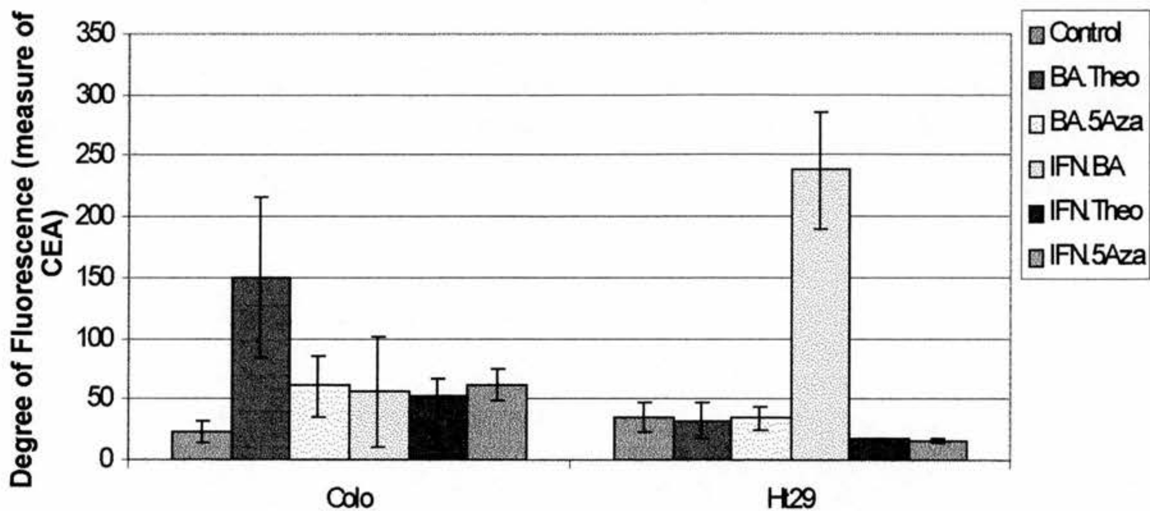
All of the combination except Butyric acid and alkaline medium produced increased CEA in the Ht29 cells, and all of the agents used alone except the alkaline medium had a similar effect.

In the Lovo cells, although Butyric acid, Chloroquine, 5-Fluorouracil and Methotrexate were all effective as single agents, only the Chloroquine and Butyric acid in combination produced increased CEA.

FACS FLUORESCENCE: TOTAL CELL CEA CONTENT

1. Differentiating Agent + Differentiating Agent



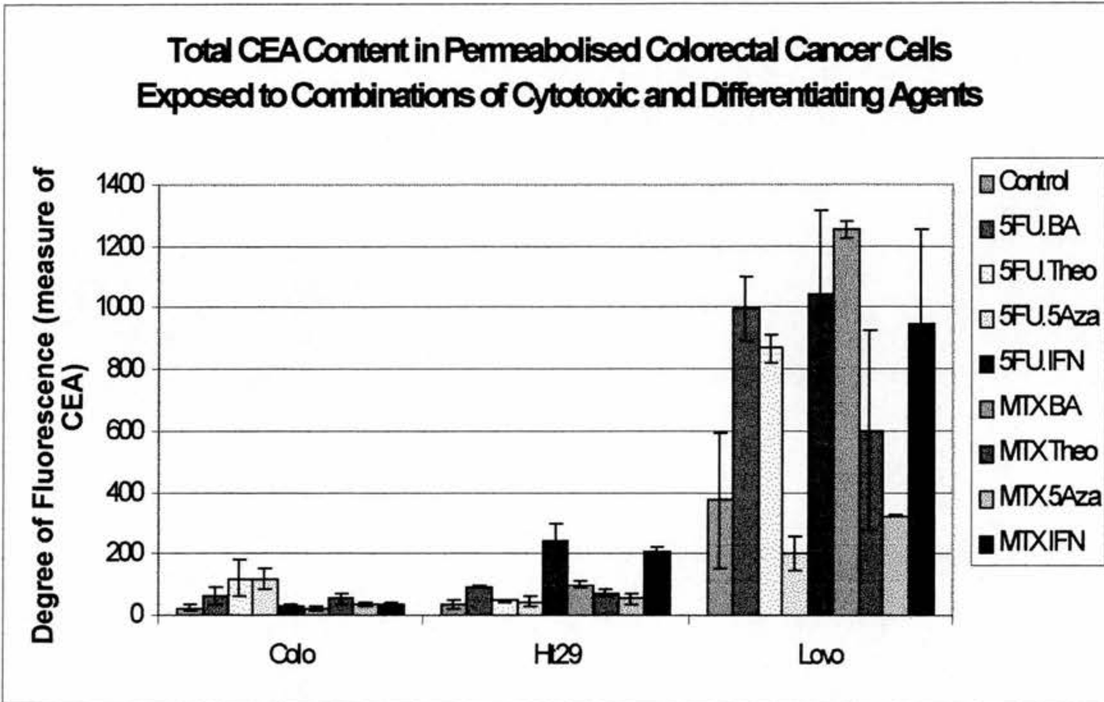


In the Colo cells, only combinations including Theophylline and 5-Azacytidine (the two successful single agents) were effective inducers of CEA.

A quite different result was obtained in the Ht29 cells, where despite the effectiveness of all of the agents except 5-Azacytidine when used alone, only the combination of Interferon and Butyric acid induced augmentation of CEA.

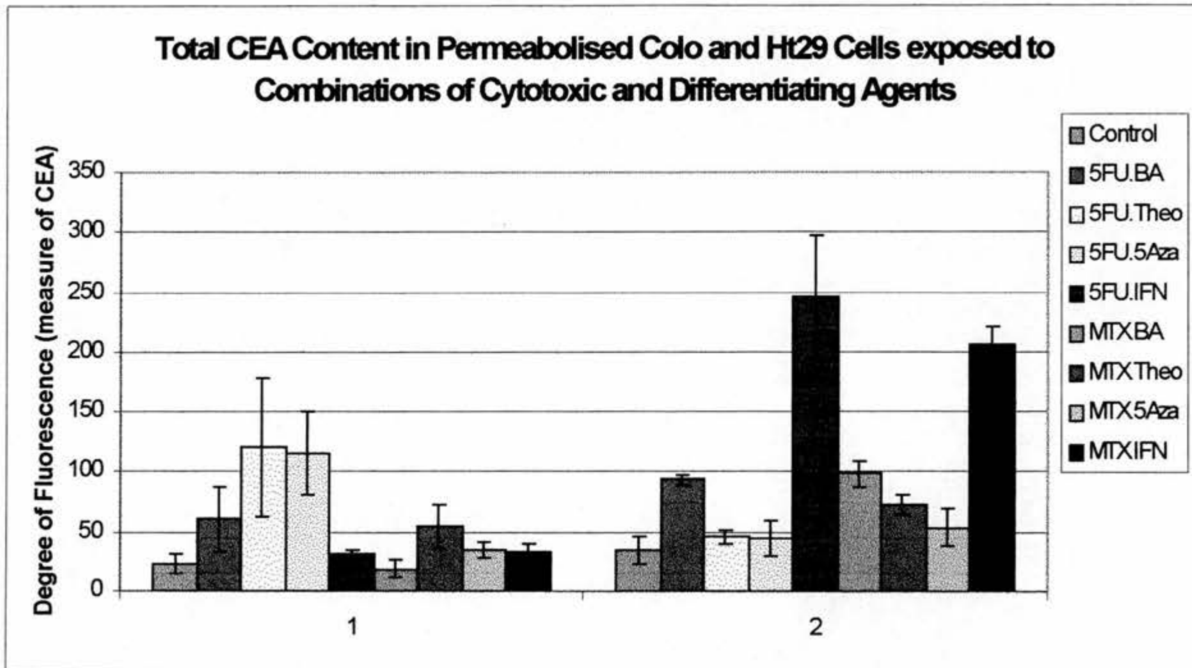
In the Lovo cells, very impressive increases in CEA were seen in all of the Interferon combinations – additive, if not synergistic increases, despite the ineffectiveness of Theophylline and 5-Azacytidine used as single agents. The same effect was not seen with Butyric acid, where single success was lost in combination with Theophylline or 5-Azacytidine.

2. Differentiating Agent + Cytotoxic Agent



Only Butyric acid and Interferon induced total CEA content increases in the Lovo cells when used alone; combinations of these two agents with 5-Fluorouracil or Methotrexate were also effective CEA inducers. Additionally, 5-Fluorouracil plus Theophylline produced significant augmentation of CEA, despite the lack of results from these agents used singly.

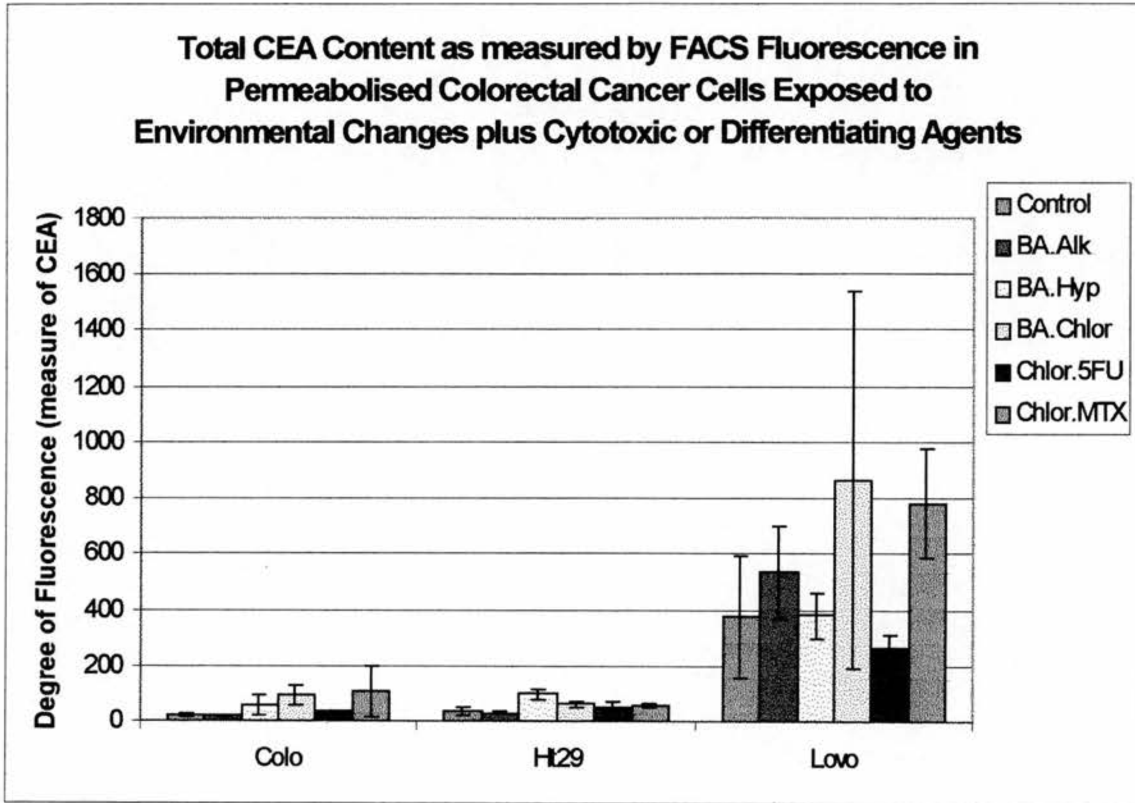
The changes in the Colo and Ht29 cells are more clearly shown in the following graph:



In the Colo cells, 5-Fluorouracil and Butyric acid, which were not effective as single agents, did produce increased CEA in combination. Additionally, 5-Fluorouracil was effective in combination with Theophylline and 5-Azacytidine, also successful single CEA-inducers. Methotrexate, however, which was effective as a single agent, only produced increased CEA in combination with Theophylline and 5-Azacytidine.

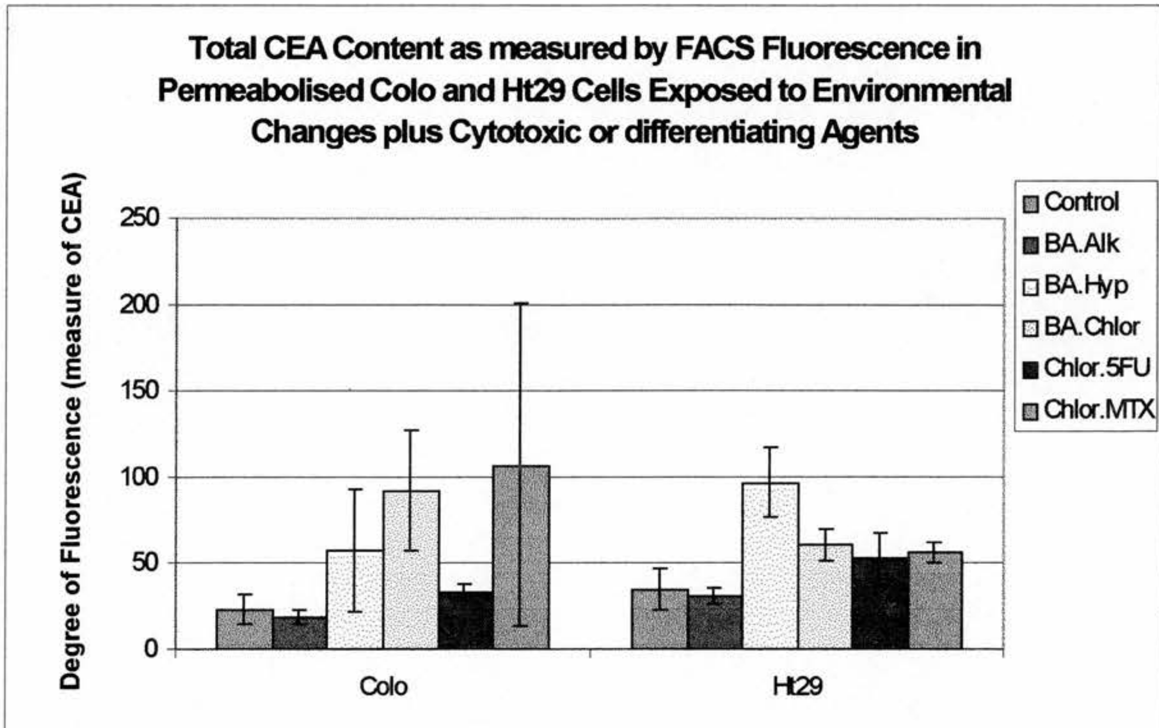
With the exception of 5-Fluorouracil plus 5-Azacytidine, all of the combinations of 5-Fluorouracil or Methotrexate plus a differentiating agent produced augmentation of CEA in the Ht29 cells. No sign of a synergistic response in CEA expression was seen. It is noted that 5-Azacytidine is the only agent which did not induce increased CEA when used alone.

3. Environmental Factor+ Differentiating or Cytotoxic Agent



Only Chloroquine plus Methotrexate maintained the CEA-inducing effects of the agents used singly in the Lovo cells. The positive effects of Butyric acid were lost in combination with Chloroquine, hypoxia or alkaline medium, as were the effects of Chloroquine with 5-Fluorouracil.

The changes in the Colo and Ht29 cells are more clearly shown below:



Butyric acid plus hypoxia, and Chloroquine plus Butyric acid or Methotrexate produced increases in total Colo cell CEA. Chloroquine was effective when used singly, but not in combination with 5-Fluorouracil.

In the Ht29 cells, all of the combinations except Butyric acid plus alkaline medium produced augmentation of CEA. Apart from that loss of action of Butyric acid, there was no significant difference between the combination results and the results from the agents used singly.

Overall Summary of Combination Experiments

Most of the combinations of agents tested produced growth inhibition and CEA augmentation in the three cell lines to a similar degree to that seen with the agents used separately. There were some alterations, however, with some combinations being less effective at inducing CEA or inhibiting cell growth , and some more effective. These changes were as follows:

Growth Inhibition

No increases in growth inhibition were measured. Impaired growth inhibition was seen in :

Colo cells:

- Ifn + 5FU
- Ifn+ Methotrexate
- Butyric Acid + Alkaline medium

Ht29 cells:

- 5FU + Butyric Acid
- 5FU + Theophylline
- 5FU + 5-Azacytidine
- Butyric Acid + Alkaline medium

Lovo cells:

- Butyric Acid + Alkaline medium
- Chloroquine + 5FU
- Chloroquine + Methotrexate

CEA Augmentation (membrane and total unless otherwise stated)

Cells	Impaired CEA Augmentation	Improved CEA Augmentation
Colo	Interferon +Theophylline	5FU + Butyric Acid
	Interferon + 5-Azacytidine	5FU + Theophylline
	Interferon + 5FU	5FU + 5-Azacytidine
	Interferon + Methotrexate	
	Chloroquine + 5FU (memb)	
	Chloroquine + Methotrexate	
Ht29	Interferon +Theophylline	Butyric Acid + Interferon
	Interferon + 5-Azacytidine	Interferon + 5FU
	5FU +Theophylline	Interferon + Methotrexate
	5FU + 5-Azacytidine	
	Butyric Acid + Alkaline medium (total)	
	Chloroquine + Methotrexate (memb)	
	Chloroquine + 5FU (total)	
Lovo	5FU + Butyric Acid	Butyric Acid + Interferon
	Butyric Acid + Alkaline medium	
	Butyric Acid + Hypoxia	
	Chloroquine + 5FU	
	Chloroquine + Methotrexate (memb)	

DISCUSSION

1. Differentiating Agent + Differentiating Agent

In the previous experiments described in Chapter two, exposing the colorectal cancer cells to Butyric Acid, Theophylline or 5-Azacytidine resulted in quite different observed effects from one cell line to another. It was postulated that more than one mechanism of action was involved in the induction of CEA expression. The results of this first experiment combining differentiating agents again showed a diversity of reaction from one cell line to another, and also showed some diversity of interaction between the combined agents. Colo cells did not respond to Butyric Acid used alone, but did respond to Theophylline or to 5-Azacytidine. In combination, the augmenting effects of Theophylline and 5-Azactidine were not diminished by the addition of Butyric Acid. It may be that Butyric Acid cannot penetrate Colo cells, or has no biochemical means of interfering with the mechanism of CEA expression in Colo cells.

The Lovo cells did previously respond to Butyric Acid, but did not respond to Theophylline or 5-Azacytidine. However, in combination, the positive effects of Butyric Acid were apparently negated by Theophylline and by 5-Azacytidine. Similarly, the previously noted positive augmenting effects of all three agents in Ht29 cells were significantly decreased or negated by the use of the agents in combination..

If the reason behind these observations were simply a reaction between the differentiating agents rendering them inactive, one would not have expected the positive response to the two combinations seen in the Colo cells. Other possible explanations are: that the agents act separately on the cell, in different positions along the CEA expression pathway, such that CEA expression may be potentiated and/or inhibited with a possible summation of response amounting to zero change; or that the agents compete with each other for sites of reaction within the cell, with some agents setting off reactions and others merely blocking the site.

The observed additive effects of Butyric Acid and Interferon on Lovo and Ht29 cells may be understood if the two agents act on a CEA expression pathway in two entirely separate and independent but similarly potentiating ways. The positive effect in the Colo cells (total CEA content only) may be a result of a cumulated effect reaching a point of stimulation beyond the scope of one agent alone.

The actions of Interferon plus Theophylline or 5-Azacytidine on Lovo and Colo cells might have suggested that the use of these agents in combination do not affect their single actions on cells, were it not for the inhibitory effects observed in the Ht29 cells. In the Ht29 cells, each of the agents used separately did produce CEA augmentation but in combination no affect CEA was documented. As noted in the discussion on p.114, separate mechanisms of differentiation and CEA expression may operate between different cell lines and within one cell type, all or none of which may be stimulated or inhibited by various chemical agents.

It is noteworthy that the "response order" of the cells, according to their degree of differentiation and natural expression of CEA, (i.e. Lovo, followed by Ht29, followed by Colo) to differentiating agents, has been lost in this experiment, with Colo cells showing mush greater CEA induction effects than Ht29 cells.

Limited studies on CEA augmentation in the literature have so far not explored the mechanics of reactions in this area of combining differentiating agents.

2. Differentiating Agent + Cytotoxic Agent

Mixed results were seen on FACS analysis in the different cell lines using combinations of 5FU, Butyric acid, Theophylline and 5-Azacytidine: 5FU and Butyric Acid was the only combination to produce CEA augmentation in the Ht29 cells, despite the fact that all of the agents used singly were successful in inducing CEA expression in Ht29 cells. The Lovo cells were unaffected by 5FU and Butyric Acid although again, both of these agents used separately had had positive, CEA-augmenting

effects on Lovo cells. 5FU did continue to augment Lovo cell CEA in the presence of Theophylline or 5-Azacytidine, however.

Perhaps the most noteworthy result was in the Colo cells, which showed a potentiation of the CEA augmentation effects of Butyric Acid, Theophylline and 5-Azacytidine by use in combination with 5FU. The lack of response in terms of CEA expression in Colo cells exposed to Butyric Acid or 5-Fluorouracil alone, makes their success in combination surprising. It may be postulated that either the separate actions of the two agents on Colo cells act permissively on each other to allow the observed CEA augmentation,

Interference of Butyric Acid and 5-Fluorouracil was also seen in the Lovo cells and of 5-Fluorouracil plus Theophylline or 5-Azacytidine in Ht29 cells, but with a different endpoint, in that CEA expression was increased by the agents used alone, but not in combination.

The effects of 5-Fluorouracil were altered in different ways in each cell line, by each differentiating agent.

The effects of the various combinations on cell proliferation were different to their effects when used separately, with the Ht29 cells in particular, where no growth inhibition was seen. The Colo and Lovo cells continued to suffer growth inhibition with all three combinations of agents. These results follow a trend of inverse correlation between cell growth and proliferation.

The CEA-augmenting effects, (according to FACS), of Methotrexate, Butyric Acid, Theophylline and 5-Azacytidine on the three colorectal cancer cell lines were unchanged by using any of the differentiating agents in combination with Methotrexate - no negation of the effects of the agents used separately was seen; nor were there any additive or synergistic reactions.

The results from Immunocytochemistry echoed those from the FACS, but were not sensitive enough to identify the changes in the Colo cells or in all of the Ht29 cell samples.

Cell proliferation was inhibited in all the cell lines, to a similar degree as with the agents used alone.

Interferon plus 5-Fluorouracil or Interferon plus Methotrexate were successful combinations in the Lovo and Ht29 cells, showing potentiating or permissive effects of one agent on another. They show promise for further exploration of CEA augmentation in vitro and in vivo, although the inhibition of Methotrexate's previously noted positive effect on Colo cells may predict interference of the differentiating (or CEA expressing) effect of Methotrexate in some cells. The reasons why the effects of Methotrexate may be potentiated in some cell lines, but inhibited in others when combined with a differentiating agent are not clear from this work, and have not yet been investigated in general.

It is surprising that the results of differentiating agents in combination with 5- Fluorouracil differ so widely from those of Methotrexate, both being anti-metabolites from the same family. Methotrexate and 5-Fluorouracil obviously differ to some extent in their chemical composition, and likewise are affected in different ways (or not at all) by other compounds, such as the differentiating agents.

3. Environmental Factor + Differentiating or Cytotoxic Agent

The CEA expression augmentation previously observed with Butyric Acid was not improved by growing the cells in a hypoxic or alkaline environment; indeed the positive effects in Colo and Lovo cells were negated by using combinations of the agents and environmental change.
Cell growth was inhibited by Butyric Acid plus hypoxia, but not by Butyric Acid plus alkaline medium.

It might be expected that the differentiating actions of Butyric Acid would be neutralised to some degree by an alkaline culture medium, if it is assumed that the differentiating properties of Butyric Acid are related to its acidic nature.
Growing cells in a hypoxic environment should limit the proliferation rate, whatever other agents are present. It is noteworthy that the small cohort of remaining cells growing in the hypoxic environment were not more responsive to Butyric Acid than cells growing in a standard culture environment, which may be attributed to changes in the activity of Butyric Acid or to a change in the cell population. There is a valid question whether the remaining cohort of cells is made-up of resistant, unresponsive (to differentiating agents) and poorly differentiated cells, or of more differentiated, responsive, better-controlled cells. Since hypoxia alone produced increased amounts of CEA production (generally taken as a mark of increased differentiation), the latter theory is favoured. The drop in CEA expression with Butyric Acid and hypoxia may be due to decreased activity of Butyric Acid itself or to interference with the pathway of CEA induction mediated by Butyric Acid in the cells.

All three cell lines continued to show growth inhibition and augmentation when grown in the presence of Chloroquine and Butyric Acid, although no additive or synergistic reactions were documented. It is noted that the Colo cells showed a good response to the two agents, but did not respond to Butyric Acid alone in previous experiments. Chloroquine is known to cause intracellular alkalosis, but either this does not affect Butyric Acid or the CEA augmenting effects of Chloroquine were too great to be overshadowed by the Butyric Acid.

Chloroquine plus Methotrexate was effective at inducing CEA expression in all three cell lines, but the positive effects of Chloroquine alone were seemingly counteracted in some way by the 5-Fluorouracil, and vice-versa. No additive or synergistic results were seen when positive CEA augmentation did occur. Methotrexate used alone was a stronger, more universal agent than 5-Fluorouracil, and was perhaps strong enough to have some effect despite being in a potentially inhibitory environment. The apparent inhibition of the previously noted effects of Chloroquine by 5-Fluorouracil is more difficult to comprehend. The antimetabolite action of 5-Fluorouracil may simply have limited the ability of the cells to respond to Chloroquine, but this should also have occurred in the Methotrexate experiment, Methotrexate also having anti-metabolite actions. It is possible that Chloroquine and 5-Fluorouracil work in completely opposite directions in terms of differentiation, so that in combination an averaged, reduced effect is seen. The relation of CEA expression to differentiation or de-differentiation has not been fully explored in the literature, and the accepted view is that CEA expression is a marker of differentiation. The morphological changes accompanying altered CEA expression may shed some light into the overall effects perpetrated on the cells, and studies of morphological changes are documented in Chapter 8.

The mechanisms underlying these results are complex and potentially multiple. A full exploration into the chemical events producing the above reported effects is beyond the scope of this work; logical deductions apparent from the results only have been explored.

Conclusions

- Interferon and Theophylline or Interferon and 5-Azacytidine do not produce better CEA-inducing results on colorectal cancer cells than when used separately, and in fact may impede each others' actions in selected cells.

- Butyric Acid and Interferon work together to produce an additive CEA-augmenting effect in selected colorectal cancer cells.

- Combining Butyric Acid with Theophylline or 5-Azacytidine does not lead to improved CEA augmentation in colorectal cancer cells in vitro; but may indeed interfere with the CEA-inducing effects of the agents when used alone.

- Combining 5FU with one of the differentiating agents Butyric Acid, Theophylline or 5-Azacytidine does not necessarily improve the CEA-augmenting effects of the agents used singly; conversely possible synergistic reactions may occur in sporadic cell lines. It is not clear which cells may be predicted to show a positive or a negative response in terms of CEA expression with the various combinations of 5FU and differentiating agents.

- A combination of Interferon and 5-FU or Methotrexate has potential for improving CEA expression in selected colorectal cancer cells. The action on CEA expression appears to be accompanied by an inhibition of cell growth.

- Combinations of Methotrexate and the differentiating agents Butyric Acid, Theophylline or 5-Azacytidine did not add to the CEA-augmenting or growth inhibiting effects observed with any of these agents used alone.

- Using Butyric acid in conjunction with environmental changes of hypoxia or alkaline medium is not a successful way of augmenting CEA expression or growth inhibition in colorectal cancer cells in vitro.

- Using a combination of Butyric Acid and Chloroquine did not detract from or improve the CEA augmenting effect of Chloroquine or of Butyric Acid when used alone.

- Combining Chloroquine with cytotoxic agents does not improve the CEA augmentation seen with these agents used singly, and indeed may impede CEA induction in selected cell lines.

Many of the combinations of differentiating agents, cytotoxic drugs and environmental changes produced similar or diluted effects on cell proliferation and CEA augmentation to those seen with the agents used separately. Some evidence of additive or synergistic effects on CEA expression was seen, however, most notably with the combination of Butyric acid and Interferon in both Lovo and Ht29 cells. The increases in CEA expression in the Lovo cells were quantitatively very high (reaching seven times those seen in the Lovo control group), which is important in terms of potential bystander effect.

The main combination of interest in producing augmentation of CEA expression was Interferon and Butyric acid.

CHAPTER SEVEN: CEA RELEASE

INTRODUCTION

One of the theoretical problems with using tumour-bound CEA in vivo as a target for radiolabelled or cytotoxic agent-bearing antibodies is the mopping up of these antibodies by circulating CEA. In 1978 Goldenberg and his team showed that circulating CEA did not affect radiolocalisation of radiolabelled anti-CEA antibody (*122*). They advocated computer subtraction techniques to remove background bloodpool radioactivity prior to scintiscan interpretation. Since then the relationship between serum CEA and tumour expression of CEA has been further studied: Siccardi et al in 1989 (*241*) found that sero-positive patients actually had a higher rate of lesion identification with anti-CEA (Fab')2 fragments (80.3%) than sero-negative patients (63%). Boxer et al (*47*) in 1992 found no relation between tumour radioacivity after injection of radiolabelled anti-CEA antibody, and serum CEA levels. The mechanism of initiating CEA release from cells is not thought to be related to cell death.

Despite these reassuring studies, it is important to assess the likelihood of rising serum CEA in response to the CEA-expression inducing agents. The clinical relevance of potential increases in CEA release by augmented cancer cells has been less well investigated. In vitro studies of the effects of Suramin, a growth factor-binding competitor, on differentiation and CEA release by Ht29 cells have been carried out by Fantini et al in 1990 (*89*) and Pommier et al in 1992 (*215*). Both studies found that Suramin caused improved differentiation morphologically in the Ht29 cells, with increased CEA expression but no change in CEA release.

AIM

The aim of this work was to measure the amount of CEA released into the supernatant by the three colorectal cell lines, Colo, Ht29 and Lovo, after exposure to various agents known to increase their CEA expression. This should provide a model for estimating the possible changes in release of CEA by tumour cells in vivo into the circulation.

METHOD: RADIOIMMUNOASSAY FOR CEA

An acceptably accurate method for measuring the amount of free CEA in a solution (such as plasma in the clinical environment) is that of Radioimmunoassay. In this work, radioimmunoassay was used to measure the amount of CEA being released into the supernatant medium by the cultured colorectal cancer cells under investigation.

Procedure Principle

A solid phase two-site immunoradiometric assay called ELSA2-CEA was used. Monoclonal antibodies were prepared against stearically remote antigenic sites on the CEA molecule: the first one is coated on the ELSA solid phase, the second one, radiolabelled with Iodine 125, is used as a tracer.

The CEA molecules attached themselves to the solid ELSA-bound antibodies and in turn the radiolabelled tracer antibody was bound to this compound, to form an antibody-antigen-antibody sandwich. The unbound tracer was removed and the residual radioactivity measured taken as a measure of the amount of CEA present.

Method

Supernatants from Colo, Ht29 and Lovo cells, which had been cultured in monolayer for 5 days, were collected and frozen at -20° C for up to one month before being defrosted for radioimmunoassay. (Work from the Royal Free Hospital Department of Clinical Oncology and at the ELISA manufacturers CIS-Biotech has validated the unchanging nature of CEA after freezing for one month.) Supernatants were taken from three flasks of 10^6 cells per cell line grown in undoctored whole Medium, and in medium containing Butyric Acid, Theophylline, 5-Azacytidine, Interferon, 5-Fluorouracil, concentrated acid or concentrated alkali. In addition , supernatants were collected from

three flasks of 10^6 cells per cell line grown in fetal calf serum-depleted whole medium, and from three flasks of 10^6 cells per cell line grown in hypoxic conditions (Oxygen $\leq$ 1%).

All reagents were raised to room temperature, and a water bath was heated to 45°C.

300µl of ^{125}I anti-CEA monoclonal antibody was dispensed into all groups of ELSA-tubes.

100µl of the standards, control serum or the experiment samples were added to appropriately labelled ELSA-tubes.

Each ELSA-tube was gently mixed.

The tubes were incubated for 3 hours at 45°C.

The tubes were then washed: the contents were aspirated, 3ml of washing solution added and left for 2 minutes and then re-aspirated. This procedure was repeated twice.

The radioactivity bound to the ELSA was then measured with a gamma scintillation counter.

RESULTS ANALYSIS

A curve was drawn using the measured data from the standard concentration solutions supplied (ng/ml CEA v cpm x 10^3). The concentrations of CEA in the experiment tubes were read from this curve, according to their cpm values.

RESULTS:

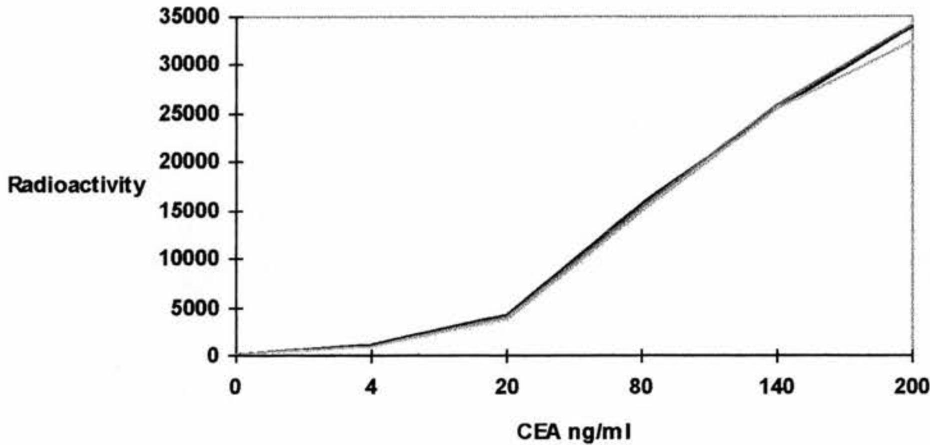
Control Values:

CEA standards ng/ml	Control Set 1	Control Set 2	Control Set 3	Control Set 4	Average
0	**203**	**150**	**196**	**191**	**185**
4	**1191**	**1067**	**1040**	**1118**	**1104.2**
20	**4271**	**4120**	**3753**	**3819**	**3990.6**
80	**15857**	**15326**	**14919**	**14880**	**15245.3**
140	**25667**	**25996**	**25509**	**25490**	**25665.6**
200	**34076**	**34276**	**32383**	**32638**	**33343.2**
SLOPES:	**171.45**	**173.37**	**165.77**	**166.43**	**169.25**

The correlations between the four sets of controls were all between 0.97 and 1.04.

The graphs of the controls against the CEA standards were as follows:

Radioimmunoassay CEA standards curves

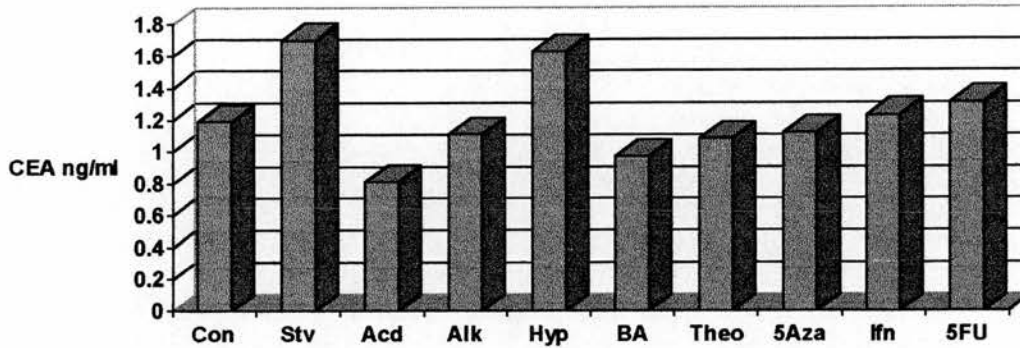


The equation for the average linear regression of the curves was: $\mathbf{X = \frac{Y - 261.11}{187.30}}$ **with r = 1.000.**

Because all of the values of measured radioactivity, i.e. the degree of CEA present in the supernatants, lay at the lower end of the standard graphs, the linear regression curve was calculated for the curves joining the points at 0, 4 and 20 ng/ml CEA standards: $\mathbf{X = \frac{Y - 260.24}{187.43}}$ **with r = 0.998.**

The measured values of radioactivity for each of the Colo, Ht29 and Lovo supernatant samples were substituted into the above equation to give X values, corresponding to the amount of CEA judged to be present in each supernatant. The results were as follows:

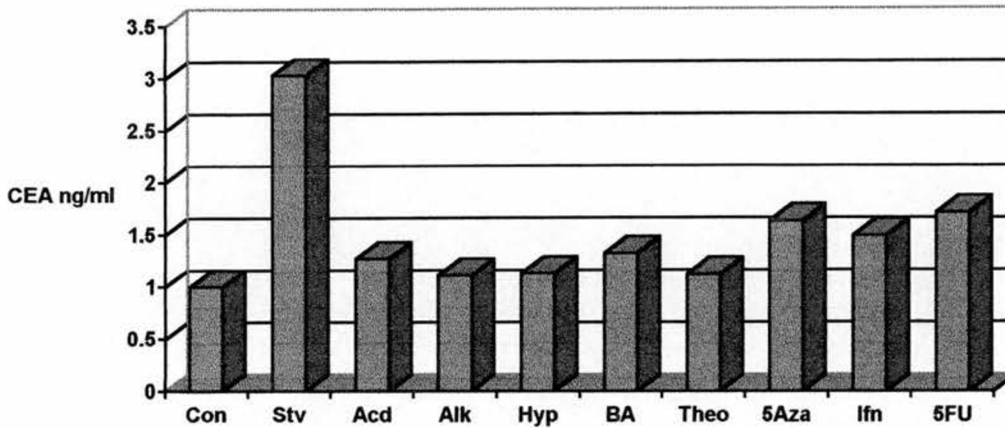
COLO CELLS:



COLO	Control	Starv	Acid	Alkali	Hypox	B.A.	Theo	5-Aza	Ifn	5-FU
	0.15	**1.92**	**0.73**	**1.1**	**1.4**	**1.32**	**1.2**	**1.07**	**1.26**	**1.38**
	0.6	**1.51**	**0.82**	**1.32**	**1.27**	**0.76**	**0.85**	**1.02**	**1.25**	**1.34**
	2.25	**1.66**	**0.89**	**0.9**	**2.21**	**0.84**	**1.2**	**1.27**	**1.19**	**1.21**
	1.72									
	1.36									
	1.07									
AVE	**1.19**	**1.70**	**0.81**	**1.11**	**1.63**	**0.97**	**1.08**	**1.12**	**1.23**	**1.31**
StDev	**0.76**	**0.21**	**0.08**	**0.21**	**0.51**	**0.30**	**0.20**	**0.13**	**0.04**	**0.09**
Ttest		**0.64**	**0.43**	**0.86**	**0.41**	**0.65**	**0.82**	**0.88**	**0.93**	**0.80**

The results show no significant change in CEA release by Colo cells exposed to various differentiating agents, 5Fu or environmental changes compared to normally grown control Colo cells, on analysis with the T-test and the Wilcoxon Rank Sum. It is particularly noted that even in conditions previously shown to significantly increase CEA expression in the Colo cells, i.e. exposure to Theophylline, 5-Azacytidine and Hypoxia, there was no reciprocal increase in CEA release.

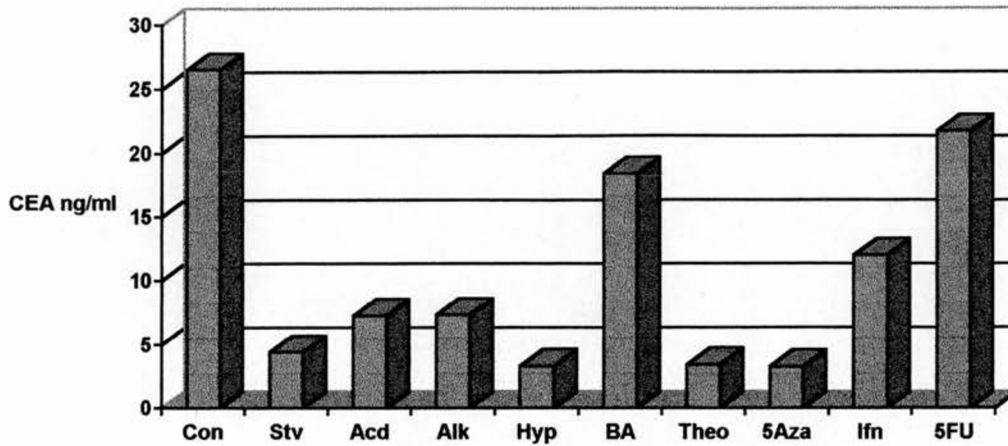
HT29 CELLS:



HT29	Control	Starv	Acid	Alkali	Hypox	B.A.	Theo	5-Aza	Ifn	5-FU
	0.71	**1.80**	**1.27**	**0.96**	**1.30**	**1.32**	**1.17**	**1.63**	**1.62**	**1.88**
	0.50	**1.94**	**1.18**	**1.07**	**1.13**	**1.40**	**0.88**	**1.09**	**1.40**	**1.56**
	0.56	**5.34**	**1.37**	**1.29**	**0.96**	**1.24**	**1.32**	**1.32**	**1.45**	**1.68**
	1.47									
	1.27									
	1.12									
AVE	**0.94**	**3.03**	**1.27**	**1.11**	**1.13**	**1.32**	**1.12**	**1.35**	**1.49**	**1.71**
StDev	**0.40**	**2.00**	**0.20**	**0.17**	**0.17**	**0.08**	**0.22**	**0.27**	**0.12**	**0.16**
Ttest		**0.03**	**0.21**	**0.26**	**0.47**	**0.16**	**0.49**	**0.16**	**0.06**	**0.02**

The results show significant increases in CEA release by Ht29 cells exposed to 5-Fluorouracil or grown in nutritionally deficient medium, compared to normally grown control Colo cells using the T-test and the non-parametric Wilcoxon Rank Sum. It is of note, however, that exposure to Butyric Acid, Interferon,Theophylline, 5-Azacytidine and Hypoxia did not result in a similar increase in CEA release. A definite relationship between increasing CEA release with increased CEA expression has therefore not been shown.

LOVO CELLS:



LOVO	Control	Starv	Acid	Alkali	Hypox	B.A.	Theo	5-Aza	Ifn	5-FU
	12.49	**5.89**	**7.93**	**7.38**	**5.69**	**25.59**	**3.49**	**2.96**	**11.82**	**10.09**
	44.26	**5.31**	**6.55**	**6.52**	**3.03**	**15.27**	**3.50**	**3.62**	**9.99**	**16.43**
	29.05	**1.88**	**7.18**	**7.97**	**	**14.21**	**3.21**	**3.15**	**14.11**	**38.57**
	27.36									
	21.45									
	24.48									
AVE	**26.52**	**4.36**	**7.22**	**7.29**	**3.31**	**18.36**	**3.40**	**3.24**	**11.97**	**21.70**
StDev	**10.47**	**2.17**	**0.69**	**0.73**	**0.40**	**6.29**	**0.16**	**0.34**	**2.06**	**14.95**
Ttest		**0.01**	**0.02**	**0.02**	**0.01**	**0.26**	**0.01**	**0.01**	**0.05**	**0.59**

The third hypoxic Lovo cell supernatant was found to have become infected; it was therefore discounted from the results.

Summary and Discussion

The results are pertinent to the living population of cells in whichever conditions were being tested. CEA measured does not refer to CEA from dead cells, the initial processing step involved discarding medium containing dead cells. Significant **decreases** were shown in CEA release by Lovo cells exposed to the differentiating agents and environmental changes which did **not** augment their CEA expression, significant with the T-test and the Wilcoxon Rank Sum. In contrast, the factors previously shown to induce increased CEA expression in Lovo cells, ie Butyric Acid, Interferon and 5-Fluorouracil had no effect, either positive or negative, on CEA release. The relevance of the inverse relationship between CEA expression and release in Lovo cells is not clear. In all three cell lines, the induced increases in membrane CEA were accompanied by no increases in CEA release into the supernatant.

The overall results confirm the absence of a direct relationship between the expression of CEA it's subsequent release. It seems feasible that a change in the characteristics of the cell, i.e. improved differentiation, may be occurring, rather than simply the stimulation of producing a cell glycoprotein. There has been much discussion and work done on the relevance of circulating CEA to tumour-associated CEA in the last 20 years. The general conclusions have been that CEA in serum does not impede the localisation of anti-CEA antibodies to tumour CEA. Limited work has been carried out on the reciprocal changes in CEA expression and release induced by exposure to the differentiating agent Suramin only *(89, 215)*. This work produces similar results to those achieved with Suramin, over the range of differentiating agents, cytotoxic drugs and environmental changes tested.

Conclusions

The augmentation of CEA expression in colorectal cancer cells in vitro does not cause a reciprocal increase in CEA released into the culture supernatant. Using this experiment as a model for the

clinical situation, it is reassuring that background circulating CEA is unlikely to be directly increased by the augmentation of CEA expression in tumour cells, according to these results.

CHAPTER EIGHT

MORPHOLOGICAL CHANGES ON ELECTRON MICROSCOPY

Introduction:

The morphological characteristics apparent on Electron Microscopy of the three human Colorectal cancer cell lines, Lovo, Ht29 and Colo were described when the cell lines were first established.

Lovo:

The cells are cuboidal or columnar and often take a glandular form. They have uniform microvilli and often a fuzzy glycocalyx at their free border, and desmosomes or junctional complexes at their cell;cell interface. The nucleus is large with a single nucleolus. There are many ribosomes and microfibril bundles, few lysosomes, intracytoplasmic vacuoles and the occasional section of Golgi (*83*).

Ht29:

These cells are less well differentiated than the Lovos. They grow in an unpolarised fashion, do not exhibit tight junctions between cells and have only sparse disorganised microvilli. There is a high nucleus to cytoplasm ratio, with relatively few organelles (*280*).

Colo:

Colo cells represent the more unusual types of Colorectal carcinoma, being related to the Amine Precursor Uptake & Decarboxylation (APUD) family. They are also undifferentiated cells, tend to be spheroid and have few desmosomes at the cell:cell junctions. They do not tend to have microvilli, but do have much Golgi apparatus, large lipid vacuoles, ribosomes and polysomes (226).

Aim:

To study the effects of a range of differentiating agents, environment changes and cytotoxic drugs, and combinations of these, on the morphology of the three cell lines Lovo, Ht29 and Colo.

Method:

Triplicate flasks containing 10^6 cells of each cell line were grown in each type of experimental condition (i.e. in medium containing Butyric Acid/ Interferon etc) alongside the flasks grown up for immunocytochemistry and FACS analysis. After the five day growth period, the cells for Electron Microscopy were fixed with Gluteraldehyde/Paraformaldehyde, scraped off the flasks and converted from monolayer culture into cell suspensions. They underwent dehyration in graded alcohols and were infiltrated with LEMIX resin to form cell pellets. The pellets were then sectioned. Three sections from each flask pellet were studied under the Electron Microscope, and photographed. (See Chapter 2 for full method)

Results

LOVO CELLS

The changes in the Lovo cell morphology, with the addition of differentiating agents ranged from evidence of cell destruction (cell swelling, blebbing, vacuolation, necrosis and the presence of cell debris) to that of improved differentiation (increased organelles, Golgi and rough Endplasmic reticulum; improved cell:cell junctions with desmosomes; increased acinar formation). The most effective agents in terms of CEA augmentation caused both cell death and/or improved differentiation - there was no monopoly on either type of effect by the successful CEA-inducers. The most striking changes are listed below, and demonstrated in the photographs overleaf:

Summary of Results: Lovo cells (compared to control samples):

1. **Butyric Acid** (photo 2): Generally an increase in the number of organelles was seen, with more Mitochondria, lysosomes and rough Endoplasmic Reticulum.

2. **Interferon:** A mixed picture of cell damage (signs of blebbing) and of increased intracellular lysosomes was seen. Additionally, several inclusion bodies, presumed to contain Interferon were noted.

3. **Theophylline**: Increased numbers of organelles, particularly mitochondria, were observed.

4. **5-Azacytidine:** Increased numbers of mitochondria were again seen, and also inclusion bodies.

5. **Hypoxia 1% O_2** (photo 3): Some evidence of necrotic cell damage was seen with disruption of the cell membrane and cytoplasmic debris. An increase in the numbers of organelles was also noted however.

6. **Chloroquine**: Signs of cell damage were the prominent features, particularly with vacuolation.

7. **Methotrexate**: The main change seen with Methotrexate was enlargement of the cells. The significance of this is not apparent.

8. **Butyric Acid + 5-Azacytidine** (photo 4): Increased numbers of organelles, particularly mitochondria and Golgi were seen. Additionally, there were some appearances suggestive of acini formation.

9. **Interferon + Theophylline:** An increased amount of rough Endoplasmic Reticulum and of Golgi was seen.

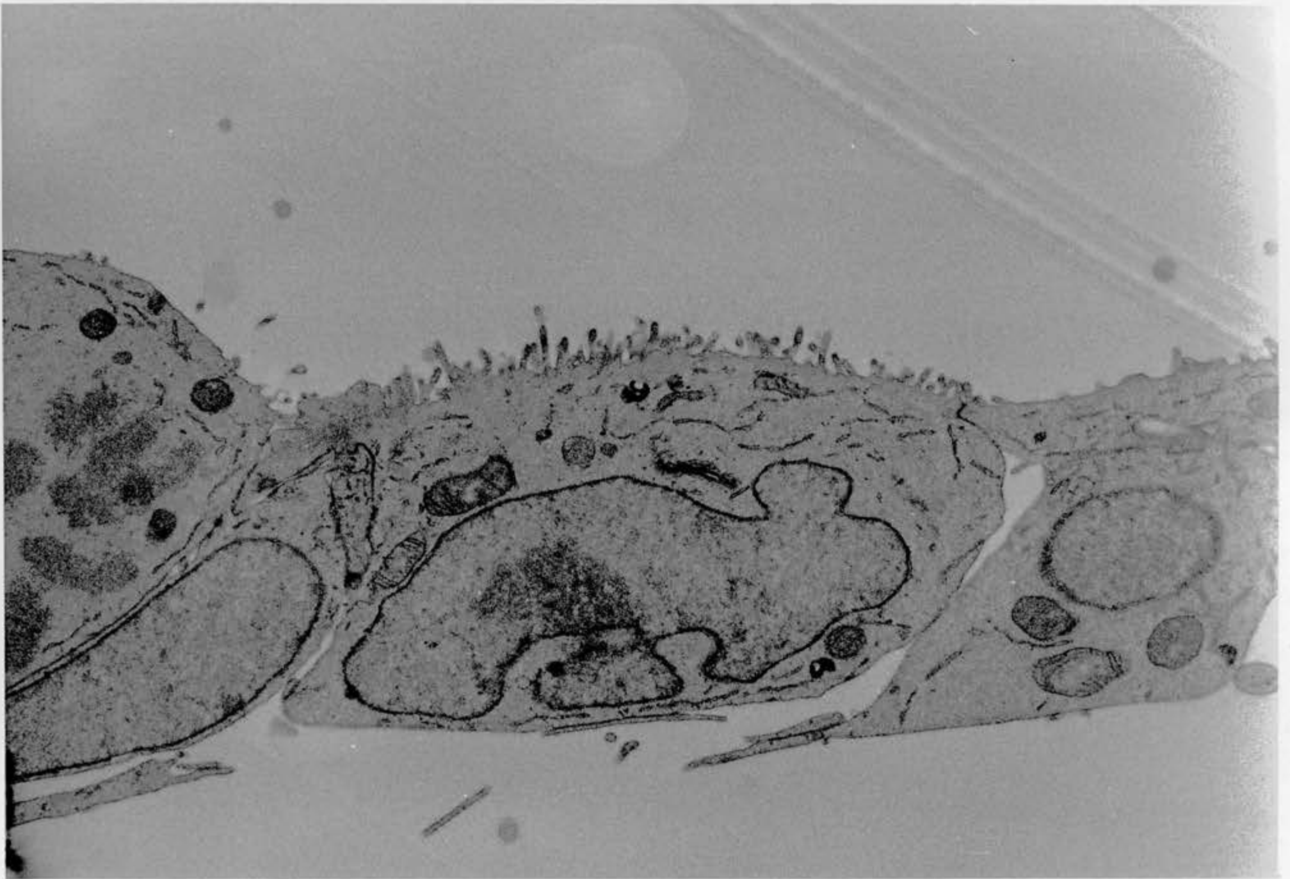
10. **Interferon + 5-Azacytidine:** Similar increases in rough Endoplasmic Reticulum and Golgi to those noted above were seen.

11. **Butyric Acid + Methotrexate:** Enlarged cells with increased numbers of organelles were seen.

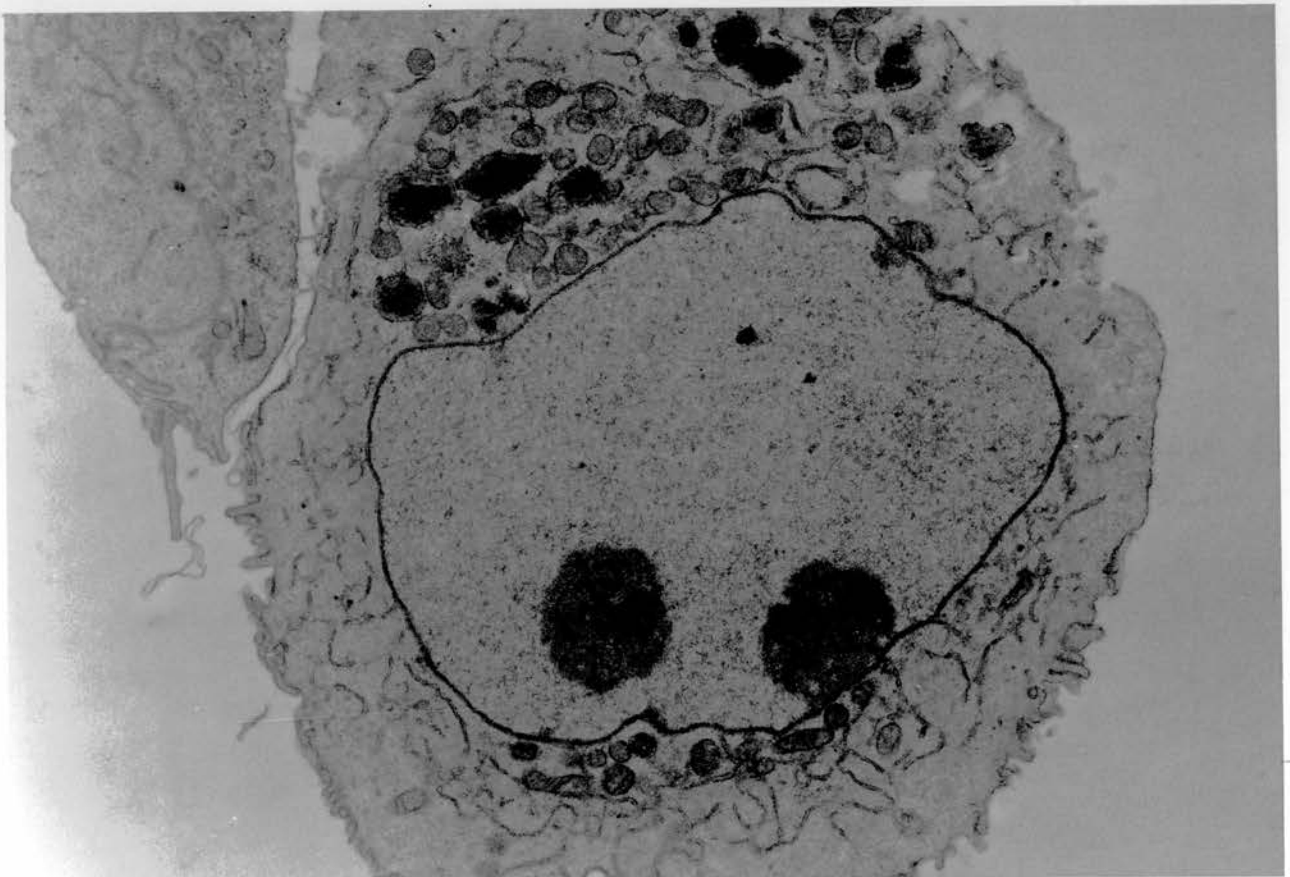
12. **Chloroquine + 5-Fluorouracil**: Increased numbers of lysosomes, desmosomes and Golgi apparatus was noted.

13. **Chloroquine + Butyric Acid**: Predominantly signs of cell damage were seen, with vacuolation and necrosis.

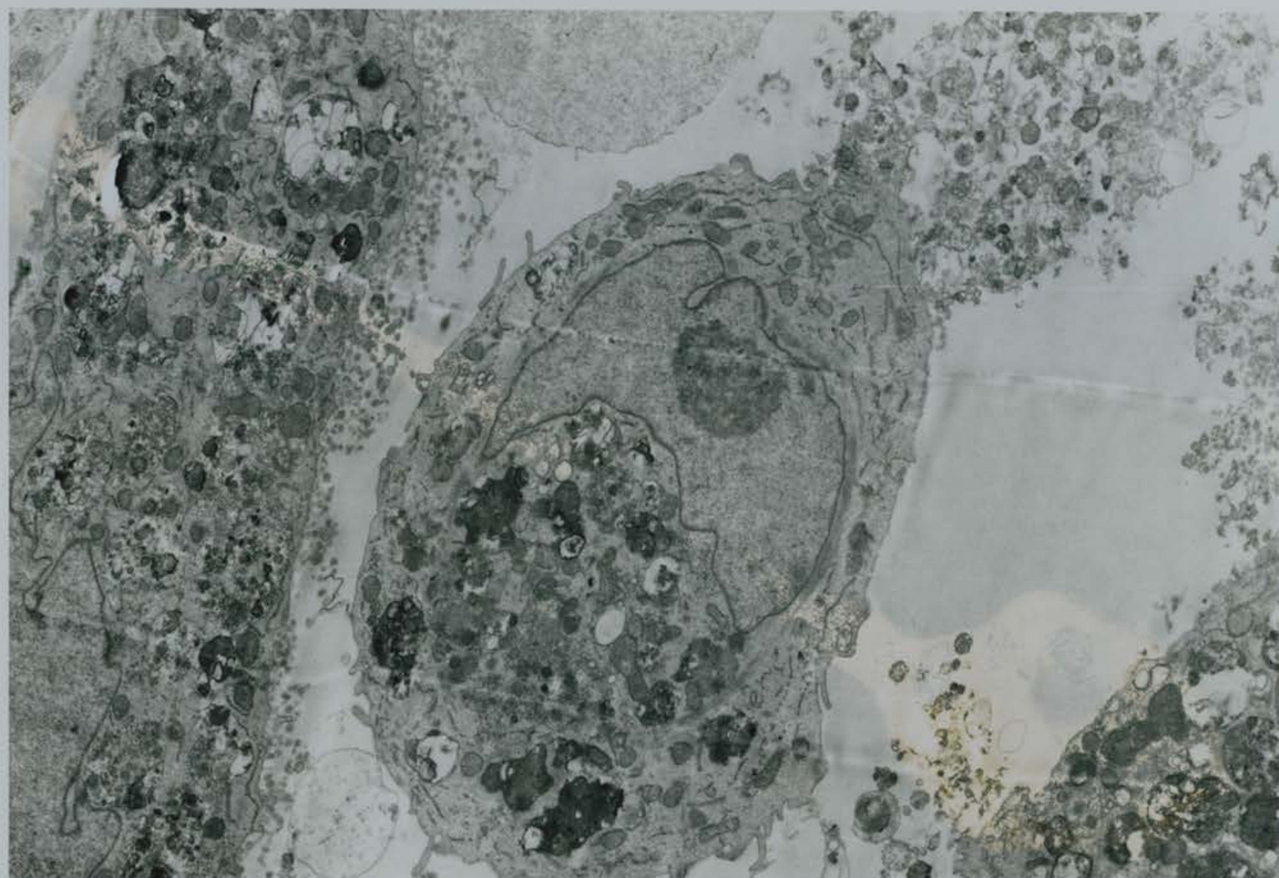
PHOTOGRAPH ONE: LOVO CONTROL CELLS



PHOTOGRAPH TWO: LOVO CELLS IN BUTYRIC ACID



PHOTOGRAPH THREE: HYPOXIC LOVO CELLS (1% O_2)



PHOTOGRAPH FOUR: LOVO CELLS WITH BUTYRIC ACID & 5-AZACYTIDINE

HT29 CELLS

As with the Lovo cells, a range of morphological changes was seen, from signs of cell damage and death to signs of increased differentiation and cell activity.

Summaryof Results: Ht29 cells (compared to control samples):

1. **Theophylline**: An increased amount of rough Endoplasmic Reticulum was noted.

2. **5-Azacytidine:** Signs of cell damage were noted, with some vacuolation, but increased numbers of ribosomes were also seen – suggesting differentiating changes in the cells.

3. **Radiation 5Gy:** Possible signs of differentiation were noted, with enlarged mitochondria and increased Golgi apparatus.

4. **Hypoxia (1% O2)**: The cells were seen to be damaged, with necrosis and cytoplasmic debris in lysosomes.

5. **Chloroquine ± But Acid/ 5FU/ Mtx:** All of the cells exposed to Chloroquine showed florid signs of cell damage, with necrosis and vacuolation (Photo 2)

6. **Methotrexate:** Increased numbers of organelles were seen, including microvilli, ribosomes and rough Endoplasmic Reticulum.

7. **Taxol:** An increased number of lipid-containing vacuoles was seen, the significance of which is unclear.

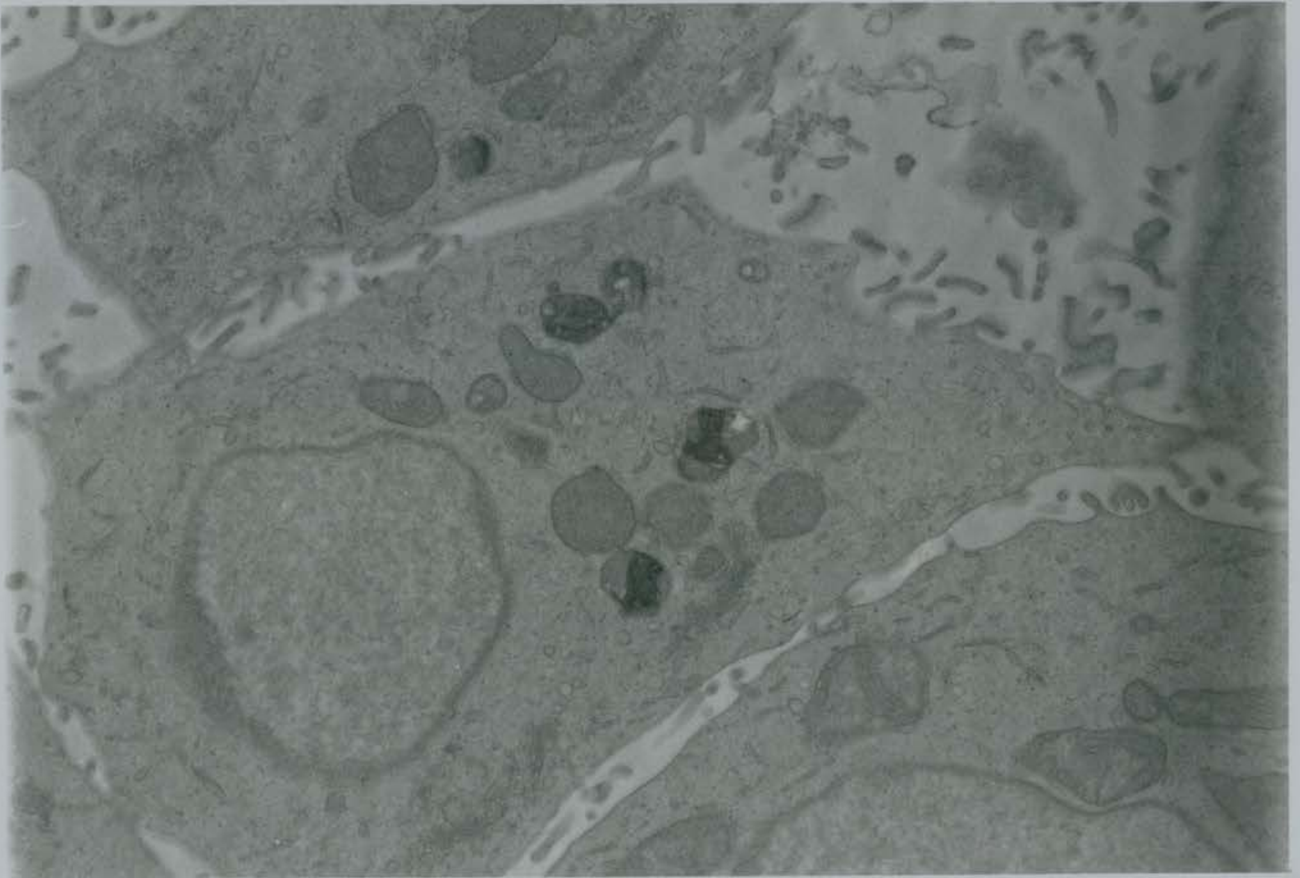
8. **Interferon + Theophylline:** Signs of improved differentiation were noted, including an increase in mitochondria and rough Endoplasmic Reticulum and the presence of tight junctions (Photo 3).

9. **5FU + IFN/ Theophylline/ 5-Azacytidine**: Cell damage with vacuolation was observed.

10. **Methotrexate + But Acid/ 5-Azacytidine**: Features seen included more mitochondria and acinus formation, consistent with improved differentiation.

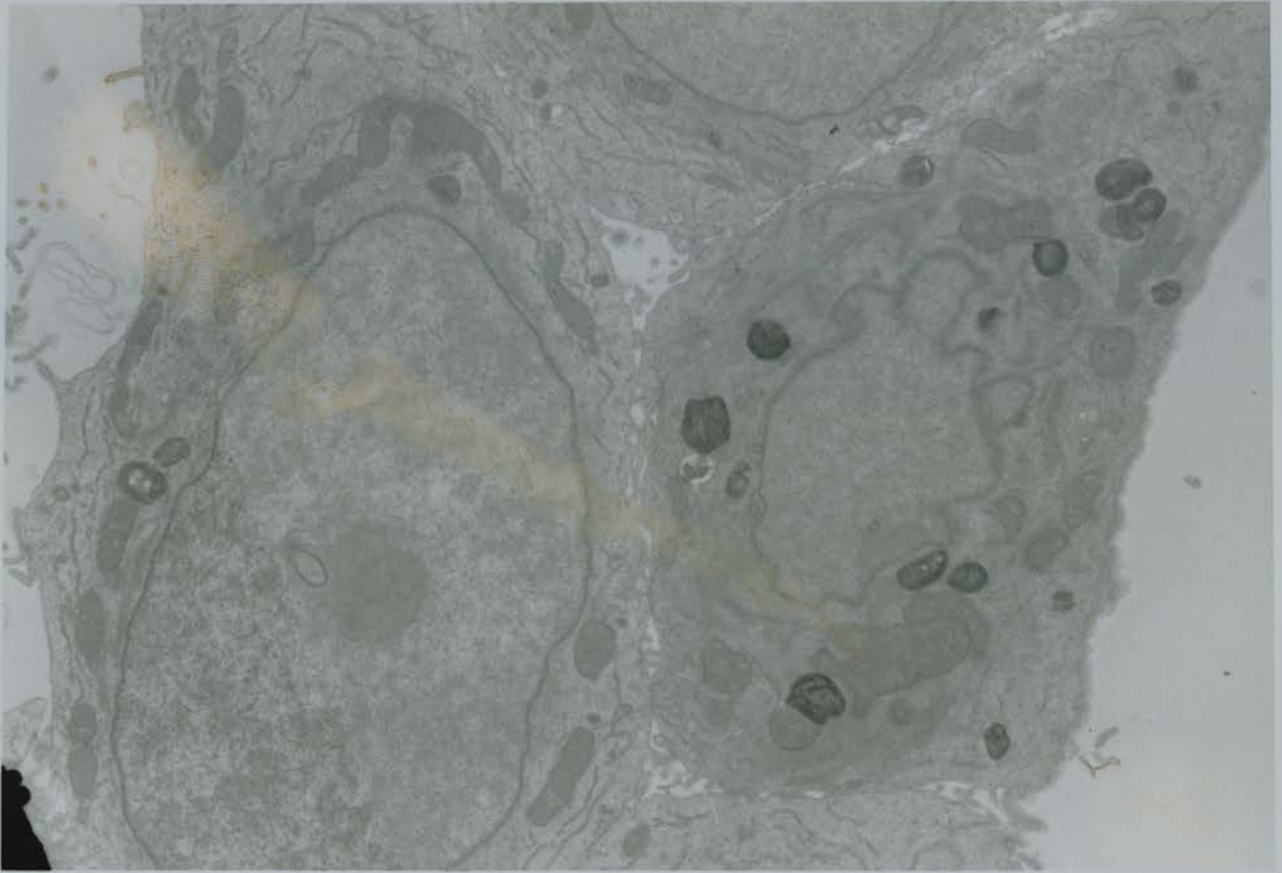
(Photo 4)

PHOTOGRAPH ONE: HT29 CONTROL CELL

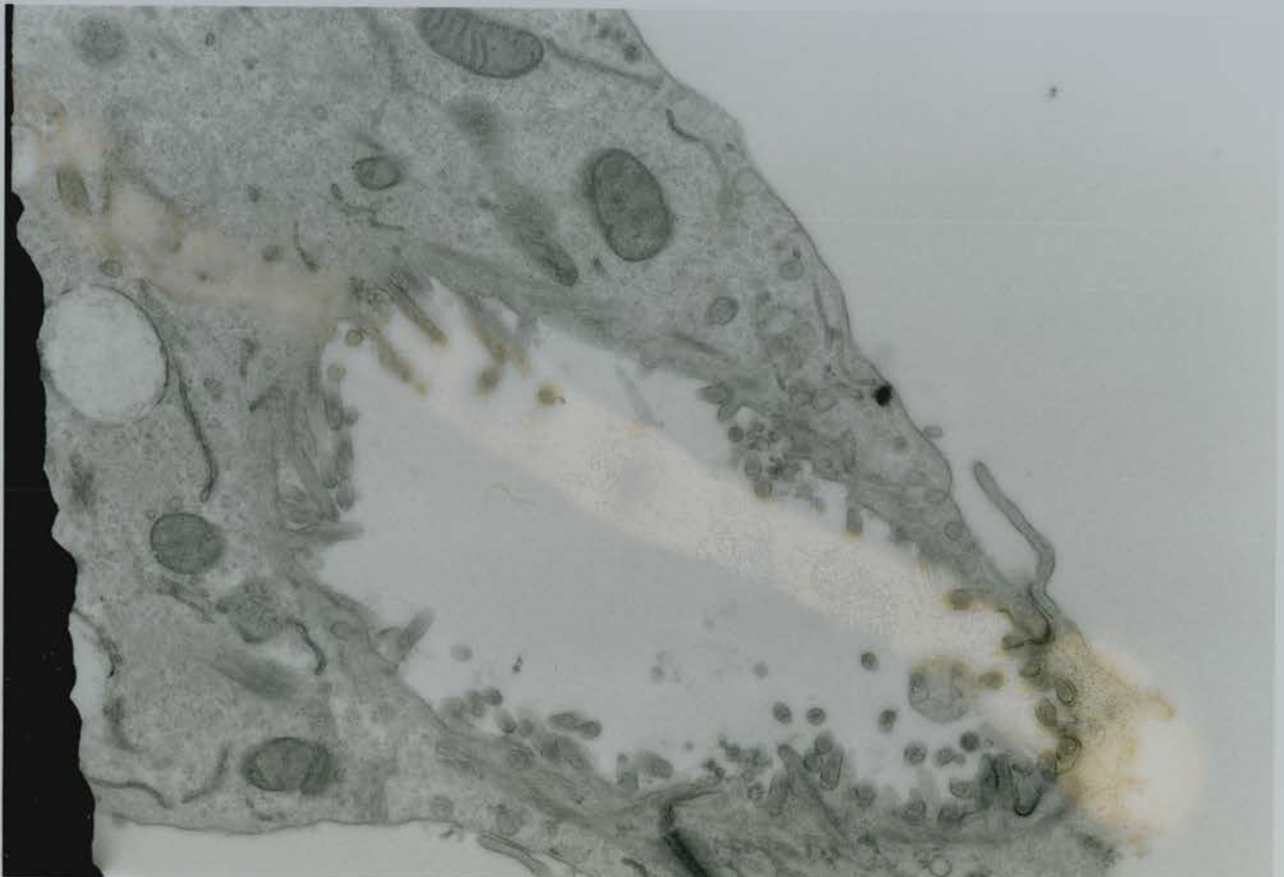


PHOTOGRAPH TWO: HT29 CELL IN CHLOROQUINE

PHOTOGRAPH THREE: HT29 CELL IN INTERFERON + THEOPHYLLINE



PHOTOGRAPH FOUR: HT29 CELL IN METHOTREXATE PLUS 5-AZACYTIDINE



COLO CELLS

Many of the effects seen in the Lovo and Ht29 cells were repeated in the Colo cells, e.g the signs of cell death associated with Chloroquine and Hypoxia in particular. The most pertinent changes to the Colo cells' morphology were as follows:

Summary of Results: Colo cells (compared to control samples):

1. **Interferon**: As noted with the Lovo cells, the presence of inclusion granules of ?Interferon was seen, along with increased Golgi apparatus.

2. **Theophylline**: Increases in rough Endoplasmic reticulum, and in numbers of lipid vacuoles were seen.

3. **5-Azacytidine:** The predominant change was that of cell damage, manifested by blebbing.

4. **Chloroquine ± ButAcid/ Methotrexate**: Features of cell damage again predominated, including necrosis; vacuolation, and an increased number of lipid vacuoles. (Photo 2)

5. **Hypoxia (1% O_2 :** Cell damage with lipid vacuolation and necrosis was noted.

6. **Radiation (5Gray)**: An increased amount of rough Endoplasmic Reticulum was seen.

7. **5-Fluorouracil**: Signs of cell damage were seen, including vacuolation and increased cytoplasmic debris.

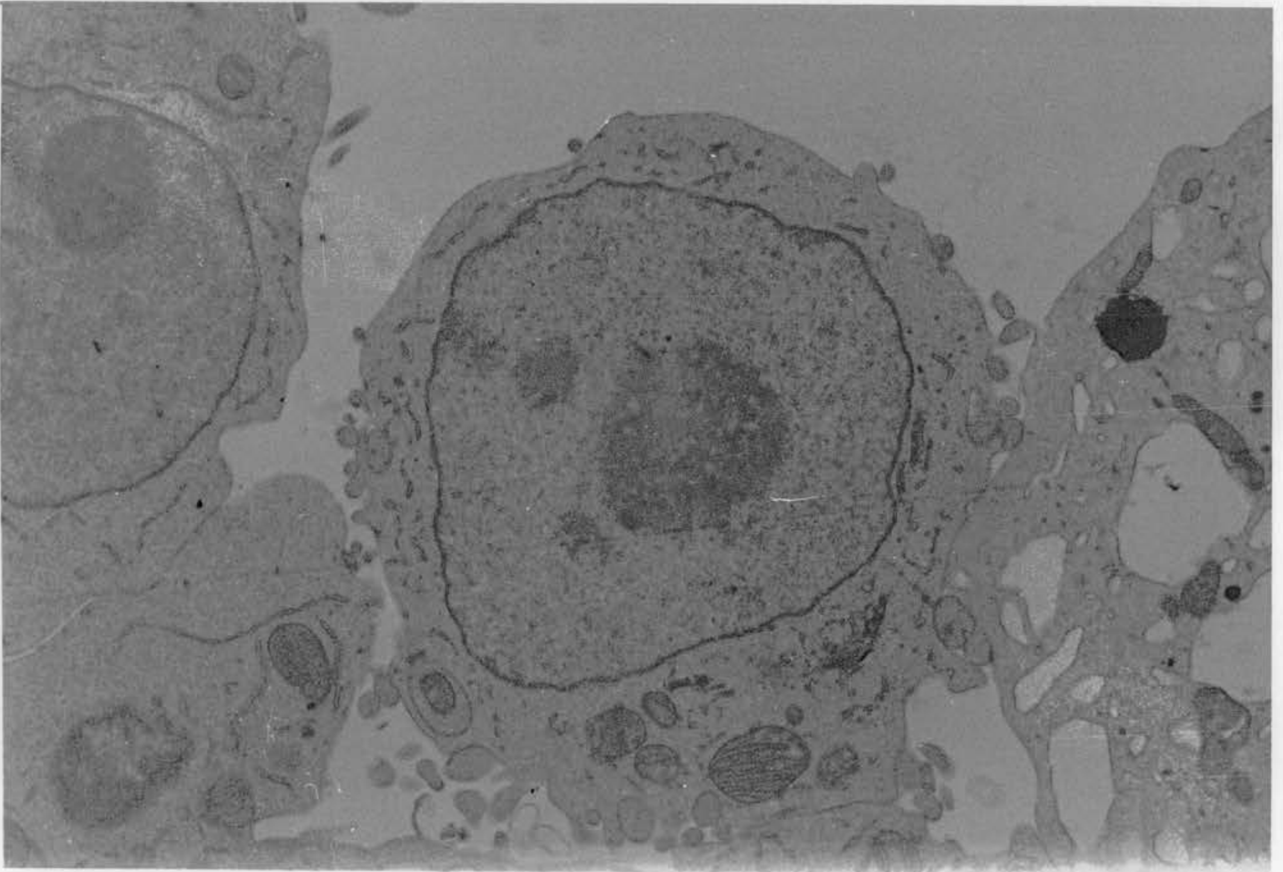
8. **Methotrexate**: The cells were enlarged, with increased amounts of mitochondria and Golgi.

9. **Interferon + Theophylline:** An increase in rough Endoplasmic Reticulum was seen.

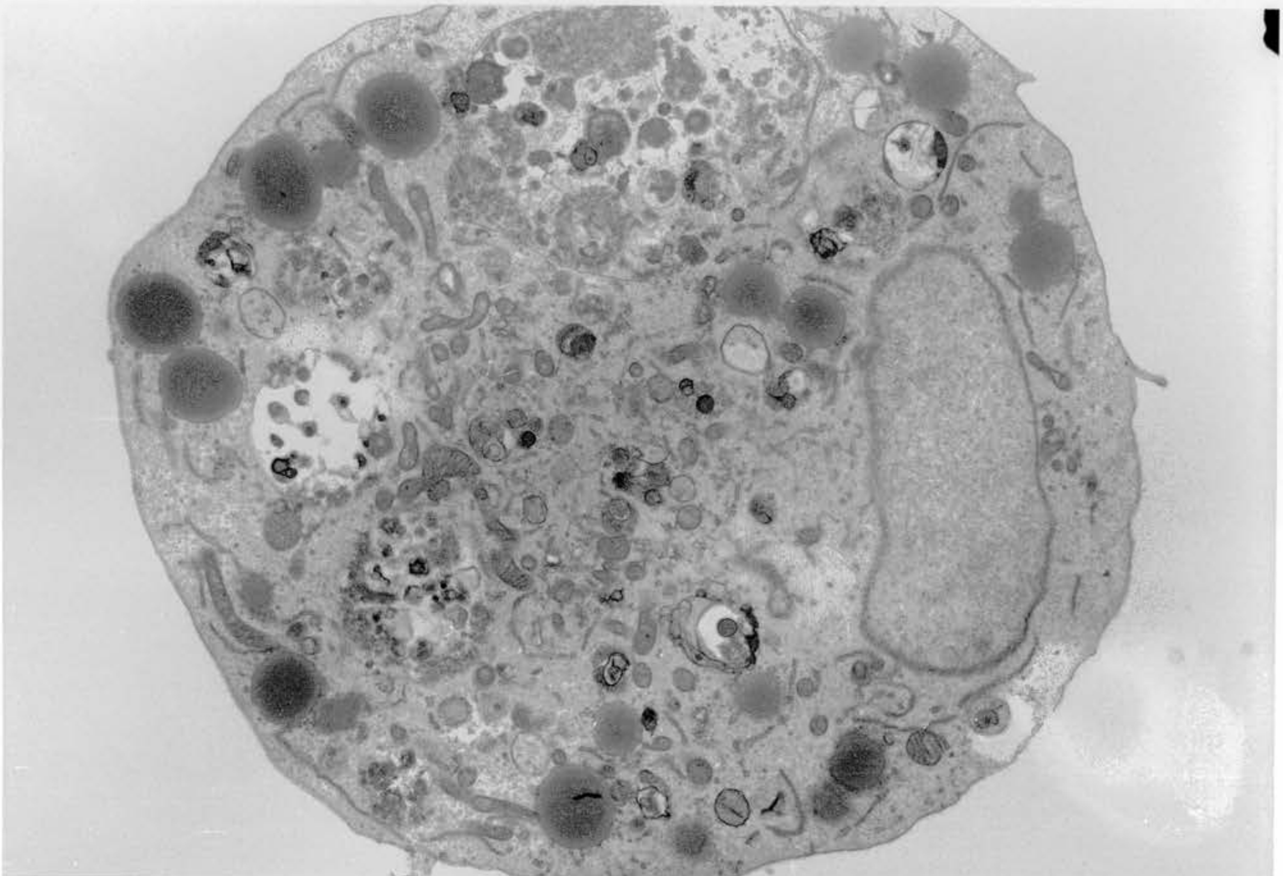
10. 5-FU + Butyric Acid/ Theophylline (Photo 3): An increase in lipid vacuoles or lysosomes was seen.

11. Methotrexate + 5-Azacytidine (Photo 4): As with Methotrexate alone, the cells were enlarged, with increased Golgi, ribosomes and rough Endoplasmic Reticulum.

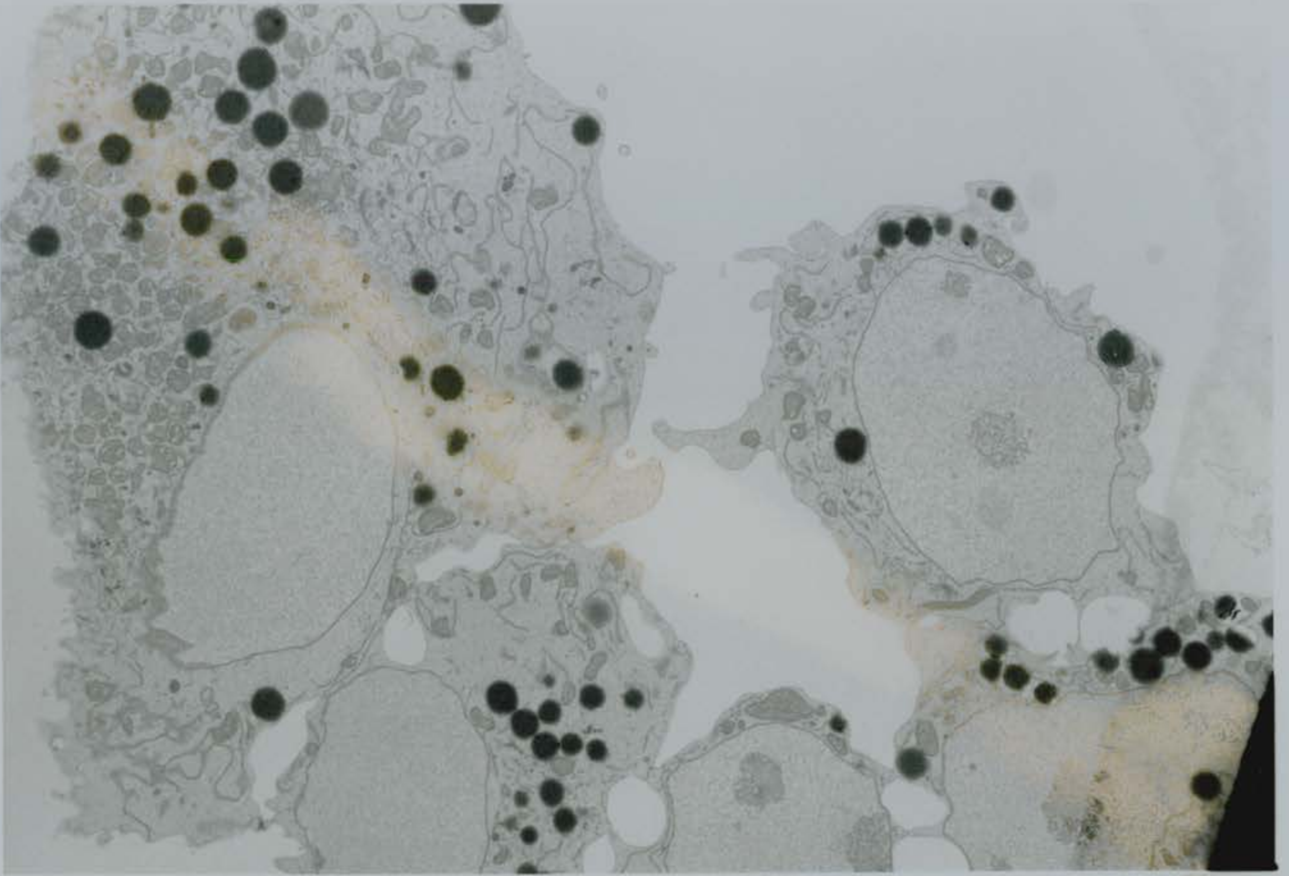
PHOTOGRAPH ONE: COLO CONTROL CELLS



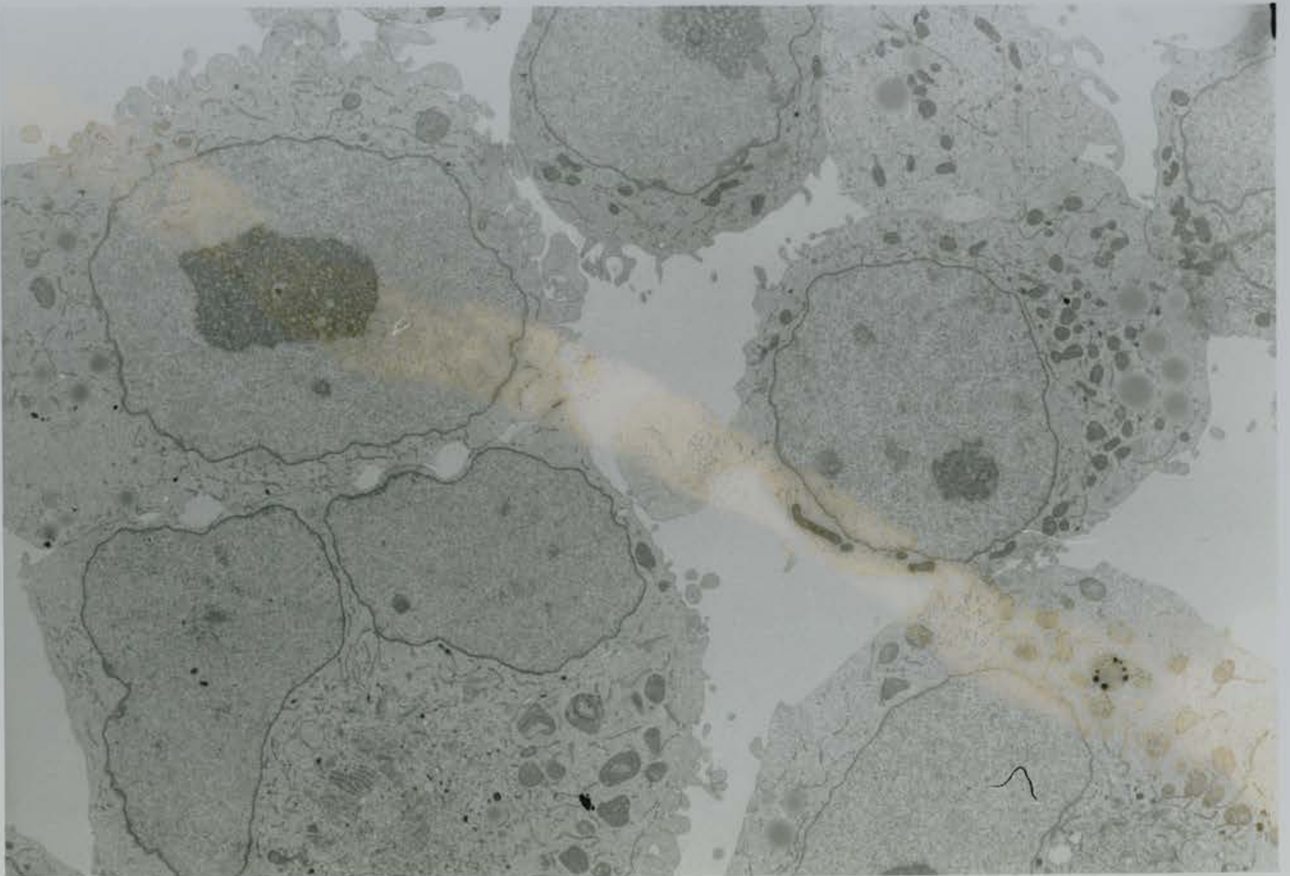
PHOTOGRAPH TWO: COLO CELLS IN CHLOROQUINE + BUTYRIC ACID



PHOTOGRAPH THREE: COLO CELLS IN 5-FLUOROURACIL PLUS BUTYRIC ACID



PHOTOGRAPH FOUR: COLO CELLS IN METHOTREXATE + 5-AZACYTIDINE



Summary

The various agents and environmental factors produced reactions in the three cell lines which tended towards either cell damage or improved cell differentiation. It can be seen from the summary table below that the different cell lines were affected in different ways by the various factors tested.

Cell Damage:

COLO	Ht29	LOVO
5-Azacytidine	5-Azacytidine	Interferon
Hypoxia 1%	Hypoxia 1%	Hypoxia 1%
Chloroquine	Chloroquine	Chloroquine
5-Fluorouracil	Chloroquine+ But Acid	Chloroquine+ But Acid
Interferon + Theophylline	Chloroquine+ Methotrexate	
Chloroquine+ But Acid	Chloroquine+ 5-Fluorouracil	
Chloroquine+ Methotrexate	5-Fluorouracil + Interferon	
	5-Fluorouracil+ Theophylline	
	5-Fluorouracil + 5-Azacytidine	

Improved Cell Differentiation:

COLO	Ht29	LOVO
Interferon	Theophylline	Butyric Acid
Theophylline	Radiotherapy	Interferon
Radiotherapy	Methotrexate	Theophylline
Methotrexate	Taxol	5-Azacytidine
5-Fluorouracil + Theophylline	Methotrexate + But Acid	Methotrexate
5-Fluorouracil + But Acid	Methotrexate + 5-Azacytidine	But Acid+ 5-Azacytidine
Methotrexate + 5-Azacytidine	Interferon + Theophylline	Interferon+ Theophylline
		Interferon+5-Azacytidine
		Methotrexate + But Acid
		Chloroquine +5 FU

Discussion:

According to these results, it appears that the Lovo cells, which express the most CEA by far of the three cell lines, show the greatest improvements in differentiation, with the greatest number of agents. However, when the factors, or combinations of factors, which produced the largest increases in CEA expression are studied, signs of cell damage are commonly seen , (particularly with Chloroquine or Chloroquine + Butyric Acid), or indeed of little morphological change (with Butyric Acid + Interferon – the most universally successful combination).

It is noted that Methotrexate caused signs of improved differentiation in all three cell lines, alone and in combination with the differentiation agents. When in combination with Chloroquine, however, the cells continued to succumb to necrosis and vacuolation. The effects of 5-Fluorouracil were dissimilar to those of Methotrexate, despite them being from the same family of anti-metabolite cytotoxic agents.

It appears therefore, that a mixed picture of improved differentiation and cell damage accompanies augmentation of CEA expression. There may be several reasons for this:

1. Cell damage involving destruction of the cell membrane may cause the release of intracellular CEA, with apparent increases in membrane CEA representing simply the glycoprotein in transit out of the leaking cell. It would be expected that increases in CEA in the supernatants of damaged cells would be seen; the results in Chapter 6 confound this theory.

2. CEA expression may be a feature of more than one intracellular event – increasing during cell differentiation and also during periods of cell damage.

3. Improved differentiation, highlighted by increases in CEA expression, lead to improvements in the cell's ability to recognise its neoplastic qualities, and switch on suicidal pathways.

Much greater understanding of the intra-cellular meaning of, and events related to, differentiation is required before the above observations may be properly explained.

Conclusions:

The Electron Microscopy studies of the morphology of the cell lines on exposure to the various differentiating agents, environmental changes, cytotoxic drugs and combinations of these suggest that there are changes in the structure of the cells, indicative both of cell damage and imminent death, and of improved differentiation associated with augmentation of CEA expression. This dual effect of cell death and differentiation may be paradoxical but useful, since the desired results are that the cells should be propelled into either differentiating or self-destructing, rather than continuing to proliferate in an undifferentiated state. The results from cell counting, immunocytochemistry and FACS confirm that the effective CEA-inducers often have significant growth inhibitory effects.

CHAPTER NINE

CHANGES IN ONCOGENE EXPRESSION IN AUGMENTED CELLS

AIM:

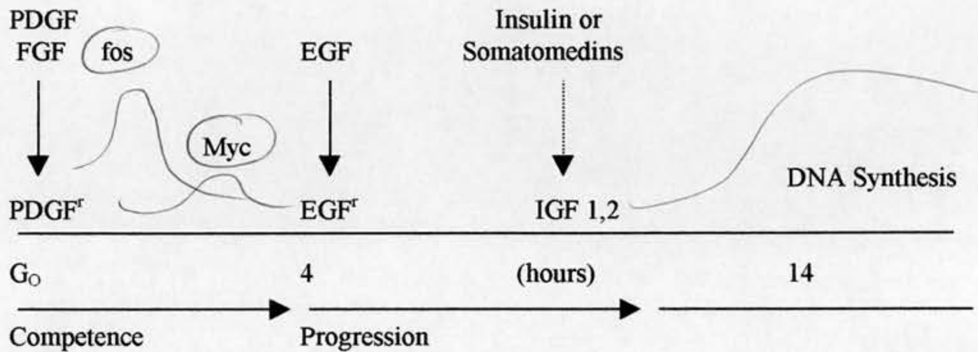
The purpose of this experiment was to investigate whether the induction of CEA expression is accompanied by changes in the expression of tumour associated genes and their product. The improved differentiation observed in CEA-augmented cells might be expected to be related to various genetically engineered changes within the cell cycle. A preliminary experiment was designed, looking for evidence of expression of 4 oncogenes - **Bcl-2**, **p53**, **K-ras** and **c-myc** in augmented and non-augmented Ht29 colorectal cancer cells.

Background:

The disregulation of physiological cell death and apoptosis is an integral part of carcinogenesis, affected by a number of oncogenes, mutated tumour suppressor genes and associated factors.). Failure of normal apoptosis mechanisms is a factor in the inappropriate survival and propagation of cancer cells. Apoptosis may be induced by many extra-and intra-cellular physiological and pathological stimuli, or by the withdrawal of inhibitory factors. The actions and interactions of the various genetic stimuli involved in apoptosis and the cell cycle are complex. A brief outline is given below.

As described in the introduction to the thesis, oncogenes are transforming genes, which may be activated by chemical or physical carcinogens to effect a phenotypic malignant transformation on their cell. The normally functioning cellular counterparts to the oncogenes are termed proto-oncogenes, and are usually important regulators of biological processes such as cell division. The products of tumour suppressor genes are negative growth regulators (*140*). The following diagram is a representation of the interaction of proto-oncogenes, tumour suppressor genes and growth factors on

the regulation of the cell cycle (*140*). The tumour suppressor genes, eg p53, are thought to act at the level of transcription of c-myc and c-fos. Several tumour suppressor genes may act in differing ways at one point, or separately at several points in the cell cycle.



In order to enter the G1 phase of the cycle, the quiescent cells need to be stimulated by Platelet-derived Growth Factor (PDGF), or Fibroblast-derived Growth Factor (FGF). This stimulation is followed by a transient rise in c-myc or fos, after which Epidermal-derived Growth Factor (EDGF) or Insulin-like Growth Factors (IGF) is needed to continue with DNA synthesis.

There are several checkpoints during the cell cycle, where regulatory genes may be activated or suppressed to proceed or delay meiosis and mitosis (*267*):

1. G1 phase

During G1, ie pre-DNA replication, p53 is crucial in delivering a veto to the cell cycle. This occurs if there are breaks in the DNA, which cause the activation of p53. In turn, p53 activates WAF1 which inhibits G1 cyclin-dependant kinases, arresting the cells in G1 (*20*). If p53 itself is mutated, a fairly common point mutation, malignant transformation may be induced rather than suppressed. C-myc is another oncogene which acts in G1 causing arrest and apoptosis (*153*The actions of both p53 and c-myc may be overridden by the product of activated Bcl-2, which inhibits apoptosis (*20,153*). Bcl-2 is found in the lower half of the colonic crypts, and has been found to be expressed by a high proportion of Colorectal adenomas, carcinomas and their metastases (211; CC4).

Overexpression of Bcl-2 prevents the apoptosis usually induced by steroids, γ-irradiation, growth factor depletion and other cell injuries (*206*).

2. G2 phase - pre mitosis.

The cell cycle may be interrupted in G2 if there are DNA breakages or mutation, which activate regulatory genes such as MEC1 and RAD9. A number of other MEC and RAD genes have been identified which respond to DNA replication errors in G2, and DNA repair errors in S phase. Mutations in MEC and RAD genes cause defective checking at G2. There is a complex interaction between the G2 checkpoint and protein kinase p34, a positive regulator of cell division, via regulatory genes such as WEE1 and CDC25. Transition from G2 to metaphase normally occurs when the relevant cyclin protein is synthesised or stabilised, when p34 combines with the cyclin, and when that combination is phosphorylated. Mutations in the regulatory genes, or interference from radiation or drugs such as hydroxyurea, may result in premature activation of p34 and inappropriate entry into mitosis.

3. Metaphase arrest.

This occurs if the microtubule assembly or function has been defective.

The degree of expression of c-myc and mutant p53 has been found to increase in a progression from normal colonic epithelium, through adenomas to carcinomas. Conversely the degree of expression of Bcl-2 has been found to fall through this same progression of normal epithelium to colonic carcinoma.

This study looked at the expression of c-myc, mutant p53 and Bcl-2 in the human colorectal cancer cell line Ht29, grown in monolayer culture with or without the addition of CEA-augmenting agents. Ht29 cells were selected for use in this experiment as they showed the greatest propensity for change on exposure to the various differentiating agents and other factors. In addition, the oncogene K-ras, which is particularly associated with colorectal carcinoma was checked. The ras family of genes are forms of proto-oncogenes with specific point mutations which induce malignant tranformation (*238*).

One previous study by La Rocca et al (*161*) has looked at the relationship between CEA expression and oncogene expression, (c-myc & K-ras among others) in 11different colorectal cell lines (not including Ht29). They found that the expression of CEA was associated with a more differentiated cell phenotye, but they did not find a specific relationship between expression of CEA and expression of the oncogenes.

Brach et al in 1993 looked at oncogene expression in myeloid leukaemia cells on exposure to cytotoxic drugs plus hamatopoetic growth factors; they found that there was a down regulation of c-myc, with features of improved differentiation in response to Leukaemia Inhibitory Factor plus Arabinofuranosylcytosine (49). A study of human Hepatoma cell lines by Saito et al in 1991 demonstrated a decrease in the expression of c-myc by cells exposed to Sodium Butyrate (234).

METHOD:

Replicate flasks containing 10^6 Ht29 cells were grown in standard monolayer tissue culture in a humidified incubator at 37°C for 5 days. Six control flasks were set up, along with 21 experiment flasks - three for each variable. The control flasks were grown in Whole Medium - Dulbecco's Modified Eagle's Medium containing 10% Fetal Calf Serum, Glutamine, Penicillin and Streptomycin. The experiment flasks were grown with the same medium but with the addition of Butyric Acid (1mM), Theophylline (10mM), 5-Azacytidine (1μM), Interferon (10ng/ml), 5-Fluorouracil (10ng/ml) or Methotrexate (10ng/ml). The last set of experiment flasks was incubated in Whole Medium, in a sealed module with a gas mixture of > 99% Nitrogen, <1% Oxygen. All of these changes to the growing conditions of the cells have been previously shown to have induced increased CEA expression.

After five days, the cells were harvested by trypsinisation, spun down, counted and resuspended at 10^6cells per ml in whole medium. Four cytospins were then made from each flask. These were fixed in Acetone at 4°C for 10 minutes, before being immunostained for Bcl-2, p53, K-ras and c-myc as follows.

The acetone was washed off with PBS x 3.

The primary antibodies were laid onto the slides, with 2 negative controls treated with PBS only. A further negative isotype-specific control using mouse IgG1 at 10μl/ml was prepared. The primary antibodies were:

1. Monoclonal Mouse Anti-Human Bcl-r Oncoprotein at 4mg/L (DAKO).
2. Monoclonal Mouse Anti-Human p53 protein at 8mg/L (DAKO).
3. Clone 234-4.2 Mouse Monoclonal antibody to K-ras protein at 10μg/ml (ONCOGENE RESEARCH PRODUCTS)
4. Mouse Monoclonal Antibody to c-myc Oncoprotein at 10mg/L (NOVOCASTRA).

The slides were incubated in a humidified chamber at room temperature for 45 minutes, before unbound antibody was washed off with PBS x 3.

Peroxidase labelled RAMP (Rabbit AntiMouse antibody) at a concentration of 13μg/ml in 10% normal human serum was then applied to every slide except one of the negative controls per set (which was treated with PBS to prevent drying out) , and the slides again incubated in a humidified chamber at room temperature for 45 minutes, then washed in PBS x 3.

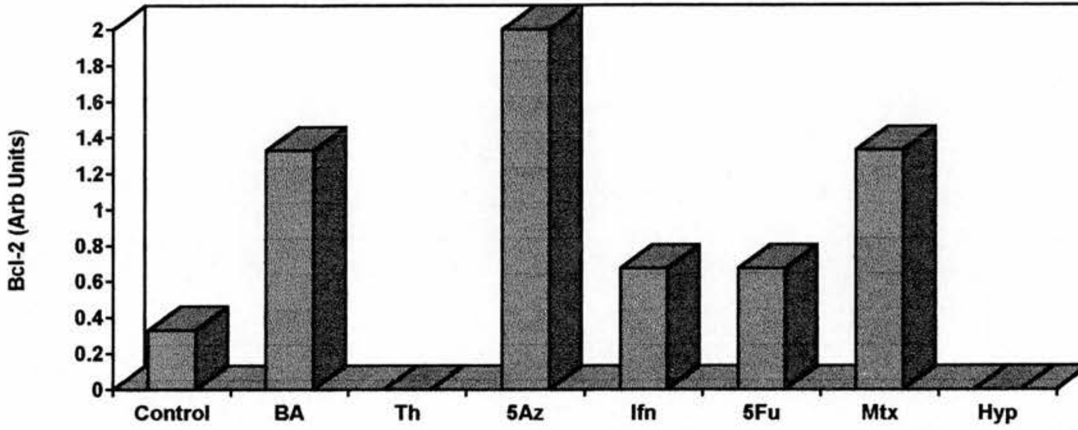
The slides were then immersed in 100ml tris/HCL buffer containing 60mg 3,3'-diaminobenzidine tetrahydrochloride, 1ml of 0.1M imidazole and 40μl 100vol H_2O_2 for 5 minutes, to develop the immunostain. The slides were then washed in distilled water x 3, and counterstained with Mayer's Haemalum for 5 minutes.

The slides were again washed in distilled water x 3, dried in methylated spirits x 3, immersed in xylene x 3 and finally mounted with DPX.

After 24 hours of drying, the cytospins were studied under light microscopy, and the scored according to the degree of staining, using the same duplicated, blind single observer-based marking system as with the CEA immunostains .

RESULTS:

BCL-2



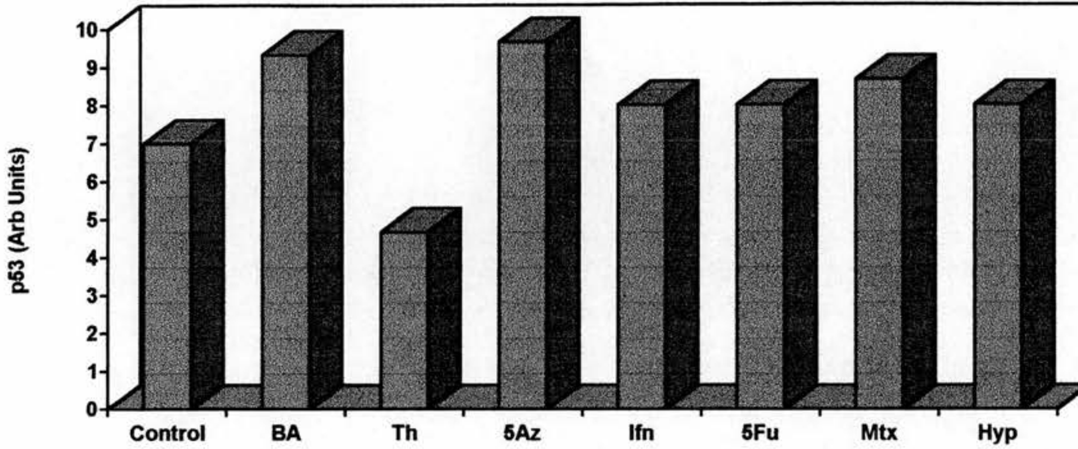
BCL-2	Control	B.A.	Theoph	5-Aza	IFN	5FU	Mtx	Hypox
Flask 1	**0**	**2**	**0**	**1**	**1**	**1**	**1**	**0**
Flask 2	**1**	**1**	**0**	**1**	**0**	**1**	**1**	**0**
Flask 3	**0**	**2**	**0**	**4**	**1**	**0**	**2**	**0**
Flask 4*	**0**							
Flask 5*	**1**							
Flask 6*	**0**							
Average	**0.33**	**1.33**	**0**	**2**	**0.67**	**0.67**	**1.33**	**0**
St. Dev	**0.52**	**0.58**	**0**	**1.73**	**0.58**	**0.58**	**0.58**	**0**
T-test		**0.03 (W)**	**0.32**	**0.05**	**0.41**	**0.42**	**0.03 (W)**	**0.32**

* Denotes the three extra control flasks

W denotes significance using the Wilcoxon Rank Sum test.

The results show a slight but significant increase in Bcl-2 expression in the Ht29 cells treated with Butyric Acid and Methotrexate, on analysis with the parametric t-test and the non-parametric Wilcoxon Rank Sum test.

p53

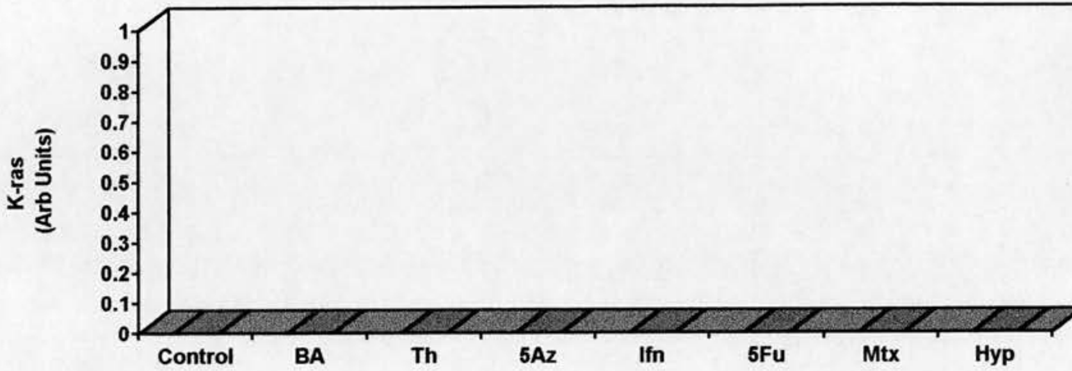


p53	Control	B.A.	Theoph	5-Aza	IFN	5FU	Mtx	Hypox
Flask 1	**4**	**10**	**7**	**9**	**6**	**10**	**9**	**8**
Flask 2	**9**	**9**	**3**	**10**	**10**	**5**	**8**	**8**
Flask 3	**9**	**9**	**4**	**10**	**8**	**9**	**9**	**8**
Flask 4*	**9**							
Flask 5*	**8**							
Flask 6*	**3**							
Average	**7.0**	**9.33**	**4.67**	**9.67**	**8.0**	**8.0**	**8.67**	**8.0**
St. Dev	**2.76**	**0.58**	**2.08**	**0.58**	**2.0**	**2.65**	**0.58**	**0**
T-test		**0.20**	**0.24**	**0.15**	**0.60**	**0.62**	**0.35**	**0.56**

* Denotes the three extra control flasks

The results show no significant differences in p53 expression in the CEA-augmented cells compared to the control cells, on analysis with the parametric t-test and the non-parametric Wilcoxon Rank Sum test. All of the cells stained strongly for p53.

K-ras

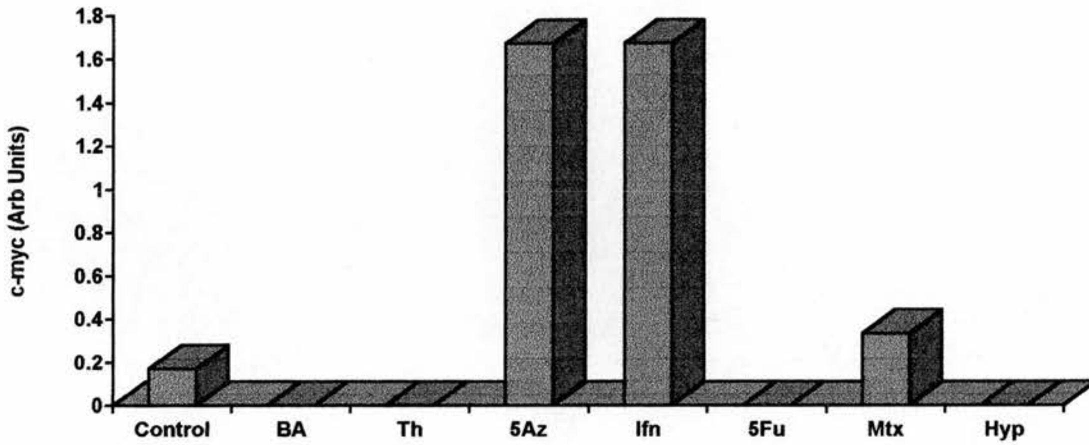


K-ras	Control	B.A.	Theoph	5-Aza	IFN	5FU	Mtx	Hypox
Flask 1	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Flask 2	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Flask 3	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Flask 4*	**0**							
Flask 5*	**0**							
Flask 6*	**0**							
Average	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
St. Dev	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
T-test		**NA**	**NA**	**NA**	**NA**	**NA**	**NA**	**NA**

* Denotes the three extra control flasks

None of the Control or the CEA-augmented cells stained positively for K-ras at the recommended concentration of 10μg/ml.

C-myc



C-myc	Control	B.A.	Theoph	5-Aza	IFN	5FU	Mtx	Hypox
Flask 1	**0**	**0**	**0**	**1**	**4**	**0**	**0**	**0**
Flask 2	**0**	**0**	**0**	**2**	**0**	**0**	**1**	**0**
Flask 3	**0**	**0**	**0**	**2**	**1**	**0**	**0**	**0**
Flask 4*	**0**							
Flask 5*	**0**							
Flask 6*	**1**							
Average	**0.17**	**0**	**0**	**1.67**	**1.67**	**0**	**0.33**	**0**
St. Dev	**0.41**	**0**	**0**	**0.58**	**2.08**	**0**	**0.58**	**0**
T-test		**0.52**	**0.52**	**0.003 (W)**	**0.11**	**0.52**	**0.63**	**0.52**

* Denotes the three extra control flasks

W denotes significance using the Wilcoxon Rank Sum test.

The results show a slight but significant increase in c-myc expression in the Ht29 cells treated with 5-Azacytidine, on analysis with the parametric t-test and the non-parametric Wilcoxon Rank Sum test.

Discussion

There was no universal change in the expression of Bcl-2, p53, K-ras or c-myc accompanying changes in CEA expression in cultured Ht29 cells. Very slight increases in Bcl-2 and c-myc expression were observed with sporadic CEA-inducing agents:

Bcl-2: Increases with Butyric Acid, 5-Azacytidine & Methotrexate

C-myc: Increase with 5-Azacytidine.

Overall there were few differences between the control and the augmented Ht29 cells, despite the highly significant changes in proliferation rates and degree of CEA expression previously noted in these cells on exposure to the various factors studied above. Bcl-2 was the only oncogene product whose expression was increased slightly by selected CEA-augmenting factors. It is interesting that Bcl-2 expression has been shown to be protective against apoptosis by previous studies (*206*)- increased expression of Bcl-2 may therefore reflect better differentiated cells protecting themselves against injury or may be a feature of ongoing cell injury, induced by the so-called differentiating factors. The previous chapter's work on the morphological features of Ht29 cells exposed to Butyric Acid, 5-Azacytidine and Methotrexate found that the latter two agents induced more a differentiated intracellular appearance, but that cells in Butyric Acid predominantly showed signs of injury and damage. It would appear that CEA-inducing agents may affect a colorectal cancer cell line in quite different ways, to produce the same end result of growth inhibition and CEA augmentation.

Conclusions:

1. There are variations in the response of a single cell line to different CEA-inducing agents and factors, which may be translated into altered Bcl-2 expression.

2. Improved differentiation of colorectal cancer cells exposed to CEA-inducing agents may involve processes other than the cell cycle regulators investigated here.

CHAPTER TEN: CONCLUSIONS and DISCUSSION

1. SINGLE CEA-INDUCERS:

Differentiating Agents:	**Butyric Acid**	**(Lovo & Ht29 augmentation)**
	Interferon	**(Lovo & Ht29 augmentation)**
	Theophylline	**(Ht29 & Colo augmentation)**
	5Azacytidine	**(Ht29 & Colo augmentation)**

The differentiating agents studied all produced significant augmentation of CEA, associated with growth inhibition in the Ht29 cells. Butyric Acid and Interferon had the same effects of CEA augmentation and growth inhibition in the Lovo cells; and Theophylline and 5-Azacytidine in the Colo cells. The fact that the effects of the agents altered from one cell line to another suggests that CEA expression may be regulated by more than one mechanism, and that these agents act via different mechanisms. This has potential clinical relevance for two reasons:

1. It may be possible to achieve additive or synergistic augmentation effects by using more than one agent simultaneously.
2. The likelihood of Colorectal cancer cells in vivo being unaffected by any of the differentiating agents is reduced if there is more than one possible mechanism to be exploited. It should be noted that in this work, it was not expected that Colo cells would express CEA, since they have been previously documented in an unaltered state as being non-expressors *(223).*

<u>Altered Environment</u>: **Chloroquine (Lovo, Ht29, Colo)**

Hypoxia 1% (Ht29,Colo)

(Hypoxia5% - Colo, less effective)

In altering the environment of the cultured cells, the most impressive changes in CEA expression and cell proliferation were seen with Chloroquine, which was active in all three cell lines, causing changes in CEA expression of a high magnitude. These results are interesting, since Chloroquine is a well-established drug in clinical use. Obviously, more work has to be done in vitro, with multi-cell spheroids, and in transfected animals before it is known whether the administration of Chloroquine to patients could improve the efficacy of tumour targetting. The Electron Microscope picture of cells exposed to Chloroquine in general showed dying cells only, rather than differentiated cells, although it is certain that some cells did survive, and that they expressed increased CEA. The serial dilution doses studied indicated a cut off point at $\geq 10^{-5}$ Molar concentration. A more graduated decline in efficacy would be expected, and further titration studies with smaller gradations in concentration may show this. Extrapolating to human dose regimens from in vitro studies is not accurate; however, dose titration figures from animal studies may be more informative.

The effects of extreme hypoxia with <1% Oxygen in the Colo and Ht29 cells were impressive, although the magnitude of Colo cell effects decreased and the Ht29 cell effects were lost as the concentration of Oxygen climbed to over 5%. The Lovo cells showed no appreciable change under hypoxic conditions. Clinically it is possible to temporarily block a tumour's arterial blood supply using injected dissolvable starch microspheres. Whether the window of time before the microspheres dissolve is long enough to induce CEA expression in the cells, and whether the degree of hypoxia attainable using this embolisation method is sufficient, is unclear. It is interesting, however that either the cells which are clearly damaged, as seen in the Electron Microscope pictures or those which are particularly resistant to a hostile external environment are seen to express significantly greater amounts of tumour-associated antigen, CEA.

<u>Cytotoxics</u>:	**5-Fluorouracil**	**(Ht29)**
	Methotrexate	**(Lovo, Ht29, Colo)**
	Taxol	**(Lovo, Ht29, Colo)**
	(Etoposide - Lovo)	

The anti-metabolite cytotoxic drugs were the only agents to cause CEA augmentation at the single dose tested of 10ng/ml. Methotrexate and Taxol induced CEA increases in all the cell lines and 5-Fluorouracil in the Ht29 cells only. Etoposide caused increased total CEA in the Lovo cells, but had no apparent effect on membrane expression. The best agent overall was Methotrexate, which interestingly was superior to 5-Fluorouracil, the most commonly used cytotoxic agent in colorectal cancer, in terms of growth inhibition and CEA augmentation.

Clinically it might be possible to give a patient a dose of Methotrexate, leave time for the disseminated tumour cells to express more CEA; then administer a bispecific antibody to CEA and to Methotrexate. This would potentially improve the penetration of Methotrexate and increase its cytotoxic effect. A bispecific antibody for CEA and Methotrexate has already been developed (*105*). More studies on Methotrexate in cell spheroids and transfected animals is needed before the true possibilities of this type of regime are known; CEA response times and dose responses to Methotrexate would have to be elucidated.

2. COMBINATIONS OF AGENTS/ ALTERED ENVIRONMENT AS CEA INDUCERS:

There are two important considerations in judging which of the combinations studied were potentially useful:

a. Those which had positive effects on all three cell lines.

This is relevant in consideration of the variability in the behaviour of colorectal cancers from one patient to the next; and as a model of any one colorectal cancer, which may be heterogeneous in terms of its CEA expression.

b. Those which resulted in the greatest degree of CEA expression in any of the cell lines.

This applies particularly to the Lovo cells, which express far more CEA than the other cell lines. It is relevant in the context of **Bystander Effect**, where anti-CEA antibody-conjugated agents, such as Methotrexate (*214*) or α-emitting Bismuth-211 (*183*), sent to CEA targets on the Lovo-type high CEA-expressor cells would be expected to have effects on the surrounding non CEA-expressing cancer cells.

a. Combinations which augment CEA in all three cell lines:

(i). Chloroquine + Butyric Acid (Best)

(ii). Interferon + Butyric Acid (Colo less marked - total CEA only)

(iii). Methotrexate + Theophylline

(iv). Methotrexate + 5-Azacytidine

There was a potentiation of the effects of Interferon by Butyric Acid, or vice versa, with a greater degree of CEA expression seen in the Lovo and Ht29 cells than with either of the agents used singly.

A similar effect was seen in the Colo cells, which were unaffected by Butyric Acid or Interferon used alone.

Methotrexate plus Theophylline or plus 5-Azacytidine did cause augmentation of all the cell lines, but the magnitude of CEA increases was not significantly greater than with Methotrexate alone.

The effects of Chloroquine alone were similarly little altered by the addition of Butyric Acid, except in Colo cells where the degree of CEA augmentation was greater than with Chloroquine alone, despite the fact that Butyric Acid alone did not induce increased CEA expression in the Colo cells.

b. Combination which produced the highest level CEA in Lovo cells: Interferon + Butyric Acid

The combination of Interferon and Butyric Acid produced very high levels of CEA expression in Lovo cells, both in the cytoplasm and on the membrane. The figures were just over double those achieved with the single agents, and between 5 and 7 times the figures in non-augmented Lovo cells. This could reflect an additive or a synergistic effect of the two agents.

3. COMPARISON OF CHANGES IN GROWTH AND CEA (BY FACS)

The relationship between growth inhibition and CEA augmentation was analysed by calculating the correlation coefficients for each cell line, which are:

	Correl Coeffic
Colo:	**-0.57**
Ht29:	**-0.54**
Lovo:	**-0.40**

A perfect inverse correlation would give a value of -1; the correlation values for each of the three cell lines begin to approach this, suggesting an inverse relationship between cell proliferation and CEA expression, which is to be expected if CEA expression is taken as a marker of differentiation. It is noteworthy that the relationship is not strong, particularly in the Lovo cells, suggesting that changes in CEA expression are not merely a function of inhibited cell growth.

4. COMPARISON OF FACS AND IMMUNOCYTOCHEMISTRY RESULTS

The sensitivity of Immunocytochemistry was found to be inferior to that of FACS from the beginning of the experiments: the Lovo cells which appeared almost maximally stained in the non-induced state with Immunocytochemistry, were clearly shown using FACS to stain much more densely when exposed to certain of the differentiating agents.

The accuracy of this initial impression was checked by plotting the Immunocytochemistry results against the FACS membrane results; this also provided a useful validation of the results obtained.

The correlation coefficients of the graphs between the Immunocytochemistry and FACS membrane results for each cell line is as follows:

Correl Coeffic

Colo: **0.67**

Ht29: **0.75**

Lovo: **0.40**

The correlation coefficients indicate a direct relationship between the Immunocytochemistry results and the FACS membrane results, strongest in the Ht29 and Colo cells, and weakly positive in the Lovo cells.

5. CHANGES IN MORPHOLOGY PERTAINING TO DIFFERENTIATION

Changes in the CEA-augmented cells were seen under Electron Microscopy. In general, the differentiating agents caused signs of improved morphological differentiation in all the cell lines; the environmental changes, signs of cell damage; and the cytotoxic drugs and combination therapies a mixture of both cell damage and improved differentiation. The most effective CEA-inducers did show associated growth-inhibiting properties, so one should expect to see signs of cell death and of improved differentiation in the CEA-expressing survivors.

6. CORRELATION BETWEEN CEA EXPRESSION & RELEASE

There was no correlation between the augmentations of CEA expression and CEA release into the cell supernatants in the Ht29 and Colo cells (Correlation coefficients –0.02 and –0.002 respectively). In the Lovo cells, there was a very weakly positive relationship, with a correlation coefficient of 0.48. It is noted, however, that the agents which caused increases in Lovo CEA expression did not cause

reciprocal rises in CEA release; but the agents which apparently decreased the Lovo CEA expression, were associated with decreases in CEA release.

These results are reassuring if the culture/supernatant set-up is taken as a model of the human tumour/ circulation relationship. If the background CEA were to dramatically rise due to the inducing agents, one might worry about a possible sink effect by circulating CEA, reducing the efficacy of targetting by administered anti-CEA antibodies. The potential for interference between circulating and cell-bound CEA in tumour targetting is a controversial point (*47,242*).

7. CHANGES IN ONCOGENE PRODUCT EXPRESSION

CEA-induced, i.e. differentiated, Ht29 cells were not shown to express greater or lesser degrees of p53 or K-ras than non-induced Ht29 cells. A slight increase in Bcl-2 expression was observed in Ht29 cells induced with Butyric Acid or with Methotrexate; and a slight increase in c-myc expression was seen in Ht29 cells induced with 5-Azacytidine. Overall, there were no striking changes observed in oncogene or tumour suppressor gene associated with augmentation of CEA. Judging by the relatively convincing inverse relationship between cell proliferation and CEA expression augmentation, some changes in the regulation of the cell cycle must be occurring. Further studies of the various controls in the cell cycle are needed before these changes are elicited, and before their relationship to the mechanisms of CEA expression may be understood.

CONTRIBUTION TO SURGICAL KNOWLEDGE

a. Behaviour of Colorectal Cancer cells

Different cell lines were seen to respond in specific and distinctive ways to differentiating agents, cytotoxic drugs, environmental changes and combinations of these factors, suggesting that more than one mechanism of cell differentiation may operate in the cells, and that these mechanisms may be vulnerable to stimulation or inhibition in a variety of ways.

"Non" CEA expressors (i.e. Colo cells, in this work) may be induced to express and be seen to express CEA with sensitive FACS imaging.

Increased CEA expression correlated with decreased cell proliferation in all the cell lines. Both tumour-associated antigen expression and growth inhibition are indicative of differentiation in cancer cells; it may be deduced that the agents and environment changes which produced increases in CEA expression and inhibition of growth may have a differentiating effect on the colorectal cancer cell populations.

Cell Morphology

Electron Microscopy studies of morphological features accompanying increased tumour-associated antigen expression showed mixed evidence for increased tumour differentiation and for tumour cell damage. Methotrexate almost universally lead to features of improved differentiation in all the cell lines; and Chloroquine conversely lead to cell damage and death.

The fact that signs of improved differentiation or of cell damage were seen to accompany increases in CEA expression suggests that the relationship between cell growth, differentiation and CEA expression is not a straightforward linear progression from an uncontrolled undifferentiated cell towards a controlled, differentiated CEA-expressing cell

c. Survey Of Potentially Useful Agents/ Combinations Of Agents To Improve Tumour Targetting Efficacy

Four groups of agents or factors were studied: differentiating agents, cytotoxic drugs, changes in environmental conditions and combinations of successful members of each of the preceding three groups.

Of the differentiating agents, all four tested (Butyric Acid, Interferon, Theophylline and 5-Azacytidine) were found to be effective in up to two of the three cell lines. The cell line which responded to all of the four agents was the Ht29 line.

The cytotoxic drugs were all tested at a single dose level only; at that dose (10ng/ml) the anti-metabolites 5-Fluorouracil, Methotrexate and Taxol were successful in inducing increased CEA expression, the latter two agents in all three cell lines.

Many of the combinations of differentiating agents, cytotoxic drugs and environmental factors interacted negatively to reduce the overall CEA augmenting effects seen, and other combinations produced no difference in effect from either one of the factors used singly. Methotrexate plus Theophylline or 5-Azacytidine produced consistent increases in membrane CEA expression and total CEA content, but these did not differ significantly from the results obtained from Methotrexate alone.. The combination of Interferon and Butyric Acid did result in an additive increase in CEA expression, most notable in the Lovo cells.

d. Relationship between CEA Expression & Release

No correlation was found between the changes in CEA expression induced by the various differentiating, cytotoxic or environmental factors and the degree of CEA released into the cell culture supernatants - further evidence that augmenting CEA expression does not automatically lead to a reciprocal rise in CEA release.

FURTHER WORK

The continuation of the work in this project may be sub-categorised into in vitro and in vivo work.

In Vitro:

Further studies of colorectal cancer cells in vitro which would follow on from this work include studies of cells in monolayer and in spheroids or organelles.

In both of these set-ups, the best positive results achieved in this work should be verified as being repeatable. Additionally, the successful CEA-augmenting factors or combinations of factors should be tested on cultured normal, non-cancerous colon cells.

Successful CEA-inducing agents such as Chloroquine in particular, warrant closer study, checking a smaller range of serial dilutions.

One further study to be considered is to test whether the factors which produced significant increases in CEA also induce increases in other membrane or tumour- associated antigens.

In Vivo:

Before any clinical application of CEA induction can be considered, the positive CEA augmentation results should be shown to be achievable in tumour transfected animals, with the desired endpoint of increased localisation of labelled anti-CEA antibodies to the tumour cells. Dose: response curves for effective agents or combinations would be required.

As mentioned in the in Vitro section, the surrounding non-diseased colon in the transfected animals could also be studied for reciprocal changes in CEA expression.

The behaviour of resistant populations of cells, which did not respond to the differentiating agents, or which were left after treatment would also be of interest.

APPENDIX ONE: TITRATION EXPERIMENTS

TRYPAN BLUE TITRATION EXPERIMENT

Introduction

In order to count cells, a measured quantity of trypsinised cell suspension pre-Centrifuging is mixed with a measured quantity of Trypan Blue Stain (0.4%) and injected into a hemocytometer. Non-viable cells take up the Trypan Blue stain and show up dark. If cells are exposed to too much Trypan Blue for too long a time, viable cells as well as non-viable cells may begin to take up the dye.

Aim

To expose cells suspended in whole medium to serial dilutions of Trypan Blue, comparing the ratios of viable to nonviable cells with the various dilutions.

Method

A Colorectal cancer cell line, Lovo, was grown up in standard tissue culture: one million cells initially injected into a $25cm^2$ culture flask in 5mls of whole medium (Dulbecco's Modified Eagle's Medium, 10% Fetal Bovine Serum, Penicillin 50 000u, Streptomycin 50mg and Glutamine 5mls) and incubated in a humidified chamber at 37°C. On day 5, the cells were washed in PBS, incubated in 2mls Trypsin-EDTA for 10 minutes and re-suspended in whole medium. Aliquots of 100μl of the cell suspension were added to Ependorf tubes containing varying amounts of Trypan Blue, and were thoroughly mixed. Five minutes after mixing, a drop of each solution was injected into a Hemocytometer and the viable and non-viable cells counted.

Results

The cell counts were as follows:

Trypan (μl)	200	100	100	100	100
Lovo susp (μl)	100	100	200	400	800
Conc Try:Lov	2:1	1:1	1:2	1:4	1:8
Viable cells	39.94×10^6	32.25×10^6	25.69×10^6	26.02×10^6	24.96×10^6
Non-viable cells	7.13×10^6	7.13×10^6	6.66×10^6	2.73×10^6	2.88×10^6
% Non-viable	17.8%	22.1%	25.9%	10.5%	11.5%

Average % non-viable cells: 17.56%

Standard Deviation: 5.25

There was a significant reduction in non-viable cell percentage ($p \leq 0.1$) above dilutions of the Trypan Blue in cell suspension of 1:4.

Conclusion

Improved accuracy in cell counting with regard to cell viability may be achieved by diluting the Trypan Blue dye to 1:4 or more. Using a dilution of 1: 8 did not impede cell counting; this dilution was therefore adopted.

IMMUNOCYTOCHEMISRY TITRATION 1

Purposes:

1. To demonstrate the presence of CEA in HT29 Colorectal Cancer Cell lines by two stage indirect immunocytochemical staining.
2. To establish the most efficacious concentration of anti-CEA antibody by serial dilutions.
3. To compare the quality of cytospins of cells in Fetal Calf Serum with cells in Whole Medium.

Introduction:

CEA, (Carcinoembryonic Antigen), is a glycoprotein membrane adhesion molecule which is expressed to a variable degree by human colorectal cancer cells. HT29 cells are human colorectal cancer cells which may be grown in vitro and which have been demonstrated in previous research to be low expressors of CEA.

The presence of CEA may be assumed by indirect two stage immunocytochemistry. This involves staining CEA-expressing cells by tagging CEA with anti-CEA antibody and further tagging that compound with a second peroxidase-labelled antibody. The second antibody reacts with the immunostain, colouring the cells.

Materials and Methods:

A flask of growing HT29 cells was harvested by washing, trypsinising and centrifuging the cell suspension to produce a pellet of cells. The cells were counted and resuspended in either fetal calf Serum or whole medium to a dilution of 10^6 cells per ml.

Replicated 100μl aliquots of each solution were pipetted into separate Shandon Elliot Cytospin Chambers.

Glass microscope slides were prepared by spreading with Poly-L-Lysine. They were then dried, fitted with filter papers into the Shandon Elliot Cytospin and spun for 5 minutes at 800 rpm. A circle of cells adherent to the glass slide was produced. The cell circle was further encircled with a paraffin pen, and the slides left to dry overnight.

The slides were next fixed by immersion in acetone at 4°C for 10 minutes. The acetone was washed off with PBS x 3.

Serial dilutions of A5B7 anti-CEA antibody in 0.1% BSA, at 1:50, 1:150, 1:450, 1:1350 and 1:4050 were laid onto separate slides, with 2 negative controls per set (cells in medium and cells in FCS). The slides were incubated in a humidified chamber at room temperature for 45 minutes, before unbound antibody was washed off with PBS x 3.

Peroxidase labelled RAMP (Rabbit AntiMouse antibody) at a concentration of 13μg/ml in 10% normal human serum was then applied to every slide except one of the negative controls per set, and the slides again incubated in a humidified chamber at room temperature for 45 minutes, before being washed in PBS x 3.

The slides were then immersed in 100ml tris/HCL buffer containing 60mg 3,3'-diaminobenzidine tetrahydrochloride, 1ml of 0.1M imidazole and 40μl 100vol H_2O_2 for 5 minutes, to develop the immunostain. The slides were then washed in distilled water, and counterstained with Mayer's Haemalum for 5 minutes.

The slides were again washed in distilled water, dried in methylated spirits, immersed in xylene and finally mounted with DPX.

Results:

Cells in Medium:

Slide	Fixn	A5B7	Diln	RAMP	DAB	CEA grade (arbitrary units)
1	+	+	1:50	+	+	**1**
2	+	+	1:150	+	+	**1**
3	+	+	1:450	+	+	**1.5**
4	+	+	1:1350	+	+	**0.5**
5	+	+	1:4050	+	+	**0.25**
6	+	-	-	+	+	**0**
7	+	-	-	-	+	**0**

Cells in FCS:

Slide	Fixn	A5B7	Diln	RAMP	DAB	CEA
1	+	+	1:50	+	+	**2**
2	+	+	1:150	+	+	**2**
3	+	+	1:450	+	+	**0.5**
4	+	+	1:1350	+	+	**1**
5	+	+	1:4050	+	+	**1**
6	+	-	-	+	+	**0**
7	+	-	-	-	+	**0**

1. CEA was demonstrated in both sets of HT29 cells, although the cells spun in medium were slightly fainter, with poorer morphology compared to those spun in FCS.
2. All dilutions of anti-CEA antibody positively stained the cells. The colour was beginning to fade by dilution to 1:4050 in the cells spun in medium.

Discussion:

These results confirm that HT29 colorectal cancer cell lines produce CEA, indirectly visualised by immunocytochemistry. It is suggested that diluting anti-CEA antibody up to 1:2000 should provide acceptable results with immunostains. It is also suggested that spinning cells in fetal calf serum is superior to spinning them in whole medium, although acceptable results may be achieved using whole medium. Whole medium is cheaper, readily available and more convenient to use in the quantities required, and was therefore chosen for use in cytospins.

IMMUNOCYTOCHEMISTRY TITRATIONS 2

Purpose:

1. To explore the accepted recipe for indirect two stage immunocytochemistry in visualising CEA, checking the effects of altering time of exposure to anti-CEA antibody, time of exposure to DAB or of omitting fixation in Acetone.
2. To demonstrate the presence of CEA on Lovo cells by indirect two stage immunocytochemistry using fluorescein-conjugated or Rhodamine-conjugated Rabbit anti-Mouse Immunoglobulin.

Introduction:

CEA (Carcinoembryonic Antigen) is a glycoprotein membrane adhesion molecule, which is expressed to a variable degree by human colorectal cancer cells. Lovo cells are human colorectal cancer cells which may be grown in vitro and which have been demonstrated in previous research to be high expressors of CEA.

The presence of CEA may be assumed by indirect two stage immunocytochemistry. This involves staining CEA-expressing cells by tagging CEA with anti-CEA antibody and further tagging that compound with a second peroxidase-labelled antibody. The second antibody reacts with the immunostain, colouring the cells. A standard recipe involves fixing cytospins in Acetone, incubating with A5B7 anti-CEA antibody for 45 minutes, further incubating with peroxidase-labelled Rabbit anti-Mouse immunoglobulin and then developing the immunostain in DAB (3,3-diaminobenzidine tetrahydrochloride) buffer for 5 minutes. The need for stringent adherence to these timed steps was explored by altering various components of the recipe and recording the resultant degrees of CEA demonstrated.

In addition, 2 slides were treated with fluorescein labelled second antibody instead of RAMP, as a comparison with the technique of light microscopic immunocytochemistry.

Materials and Methods:

A flask of growing Lovo cells was harvested by washing, trypsinising and centrifuging the cell suspension to produce a pellet of cells. The cells were counted and resuspended in whole medium to a dilution of 10^6 cells per ml.

Replicated 100μl aliquots of solution were pipetted into separate Shandon Elliot Cytospin chambers.

Glass microscope slides were prepared by spreading with Poly-L-Lysine. They were then dried, fitted with filter papers into the Shandon Elliot Cytospin and spun for 5 minutes at 800 rpm. A circle of cells adherent to the glass slide was produced. The cell circle was further encircled with a paraffin pen, and the slides left to dry overnight.

The slides were next fixed by immersion in acetone at 4°C for 10 minutes, with the exception of one negative control slide. The acetone was wshed off with PBS x 3.

A dilution of A5B7 anti-CEA antibody in 0.1% BSA at 1:2000 was applied to the separate slides, with 2 negative controls. The slides were incubated in a humidified chamber at room temperature for 15, 45 or 75 minutes, before unbound antibody was washed off with PBS x 3.

Peroxidase labelled RAMP (Rabbit AntiMouse antibody) at a concentration of 13μg/ml in 10% normal human serum was then applied to 7 slides, leaving one of the negative controls and 2 others. These latter 2 slides were overlaid with 1:20 dilutions of Rhodamine-conjugated Rabbit anti-Mouse immunoglobulin and Fluorescein-conjugated Rabbit anti-Mouse immunoglobulin respectively. The slides were again incubated in a humidified chamber at room temperature for 45 minutes, then washed in PBS x 3.

All slides except the TRITC and FITC treated cytospins were then immersed in 100ml tris/HCL buffer containing 60mg 3,3'-diaminobenzidine tetrahydrochloride, 1ml of 0.1M imidazole and 40µl 100vol H_2O_2 for one, 5 or 20 minutes, to develop the immunostain. The slides were then washed in distilled water, and counterstained with Mayer's Haemalum for 5 minutes.

The TRITC and FITC slides were overlaid with 50:50 Glycerol/PBS and coverslips.

The remaining slides were again washed in distilled water, dried in methylated spirits, immersed in xylene and finally mounted with DPX and coverslips.

Results:

Slide	Acetone	A5B7 Time	2nd Ab	DAB Time	CEA	Morphol.
1	+	+ 45 mins	RAMP	+ 5 mins	+/-	**Poor**
2	0	+ 45 mins	RAMP	+ 5 mins	+	**Poor**
3	+	0	RAMP	+ 5 mins	-	**Good**
4	+	0	RAMP	+ 5 mins	-	**Good**
5	+	+ 15 mins	RAMP	+ 5 mins	++/+++	**Good**
6	+	+ 75 mins	RAMP	+ 5 mins	+++	**Good**
7	+	+ 45 mins	RAMP	+ 1 mins	+/-	**Poor**
8	+	+ 45 mins	RAMP	+ 20 mins	+	**Poor**
9	+	+ 45 mins	TRITC	0	++	**Good**
10	+	+ 45 mins	FITC	0	++	**Good**

The above results indicate that omitting the fixation step in Acetone in the traditional recipe for immunocytochemistry, and altering the time of immersion in DAB both adversely affect the degree of CEA staining and the quality of the morphology of the cells in the finished slide. Altering the time of exposure to A5B7 in the humidifier did not adversely affect CEA staining or morphology.

CEA was successfully visualised on the Lovo cells by two-stage indirect immunocytochemistry using fluorescein and rhodamine conjugated rabbit anti-mouse immunoglobulin.

FLUORESCEIN ACTIVATED CELL SORTING EXPERIMENT 1

ANTIBODY TITRATION

Introduction

Fluorescein Activated Cell Sorting (FACS) involves directing a laser beam of light through a suspension of cells. The pattern of scattering of the light beam allows the number, size and fluorescence of the cells to be accurately calculated. Human colorectal cancer cells have a residual low fluoresence. It is possible to highlight surface or cytoplasmic cell associated antigens, such as CEA, by binding them to fluorescein-conjugated antibodies. The degree of expression of these cell associated antigens can then be measured according to the degree of fluoresence seen with FACS.

The highlighting of the cell associated antigens is acheived by two stage immunostaining. A primary anti-CEA antibody is first applied to the cells, followed by a fluorescein-conjugated secondary antibody which binds to the primary antibody. Commercially available antibodies come in very concentrated form; it is necessary to establish the most efficacious dilution of antibody by performing serial dilutions.

Aim

To find the optimum concentration of primary anti-CEA antibody (A5B7 Mouse IgG type antibody) and of the negative control (Mouse IgG_1) for immunostaining and FACS.

Method

Three human colorectal cell lines, (HT29, Colo and Lovo) which are low, high and non-expressors of CEA respectively, were grown in standard tissue culture for 7 days. The cells were then harvested by trypsinising, counted and resuspended in whole medium at a concentration of 10^6 cells per ml. Fourteen Aliquots of 100μl of each cell suspension were pipetted into separate pyrex tubes, 7 for surface antigen measurement and 7 for cytoplasm antigen measurement.

The samples for cytoplasmic antigen measurement were then permeabolised: 2 mls of Ortho Permeafix at 0.5 concentration with distilled water was added to the tubes which were incubated for 30 minutes at room temperature. The samples were then centrifuged at 2000rpm for 5 minutes, the supernatants discarded and the pellets flicked. The cells were then washed twice in PBS.A (PBS, Albumin 0.2%, Azide 0.2%) and respun at 2000rpm for 5 minutes after each wash.

All the samples were then immunstained - the permeabolised and the non-permeabolised cells. Varying amounts of A5B7 (1,2,5 and 10 μl) in 100μl aliquots in PBS.A were applied. Two concentrations of the negative control mouse IgG_1 (10 and 20μl of 100μg/ml) were applied to negative control tubes. The positive control used was W6/32 MHC Class 1 antibody, which was used at standard FACS-recommended concentration.

The samples were incubated for 30 minutes at room temperature, then washed twice in PBS.A, being spun at 2000rpm for 5 minutes in between washes. The second, fluorescein conjugated antibody (Rabbit anti-mouse Fluorescein Isothionate) was then applied in 5μl aliquots at a concentration of 1:100.

The samples were again incubated at room temperature for 30 minutes, then washed twice with PBS.A and spun at 2000rpm for 5 minutes. They were then fixed in Paraformaldehyde (1%), and stored at 4°C prior to being processed throught the FACS scanner.

Results

5000 cells from each sample were put through the FACS cytometer, and the mean fluorescence measured was as follows:

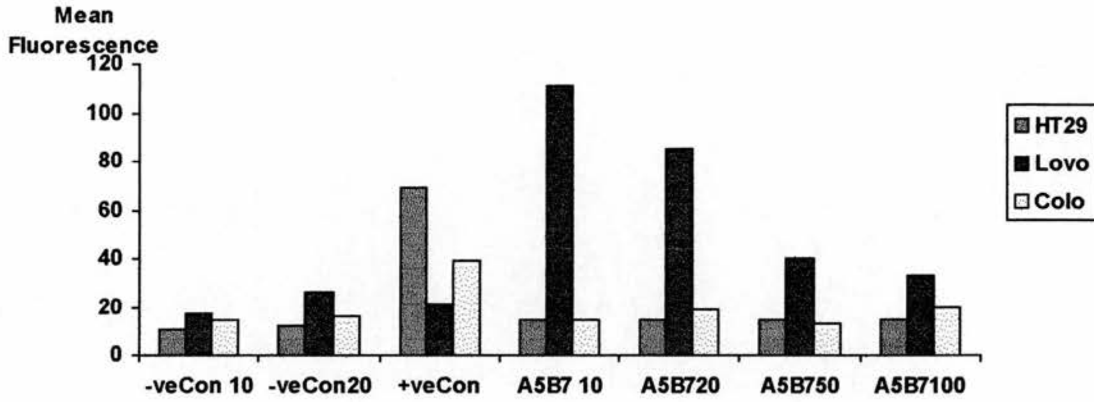
Non-permeabolised cells (ie membrane bound fluoresceinated antibody-antigen complex)

	–ve Control 10µl/ml	–ve Control 20µl/ml	+ve Control W6/32	A5B7 10µg/ml	A5B7 20µg/ml	A5B7 50µg/ml	A5B7 100µg/ml
HT29	10.88	12.35	69.42	14.83	14.76	14.74	14.29
Lovo	16.75	25.93	21.59	111.02	84.97	40.21	32.90
Colo	15.01	15.96	39.37	14.68	18.54	13.43	19.68

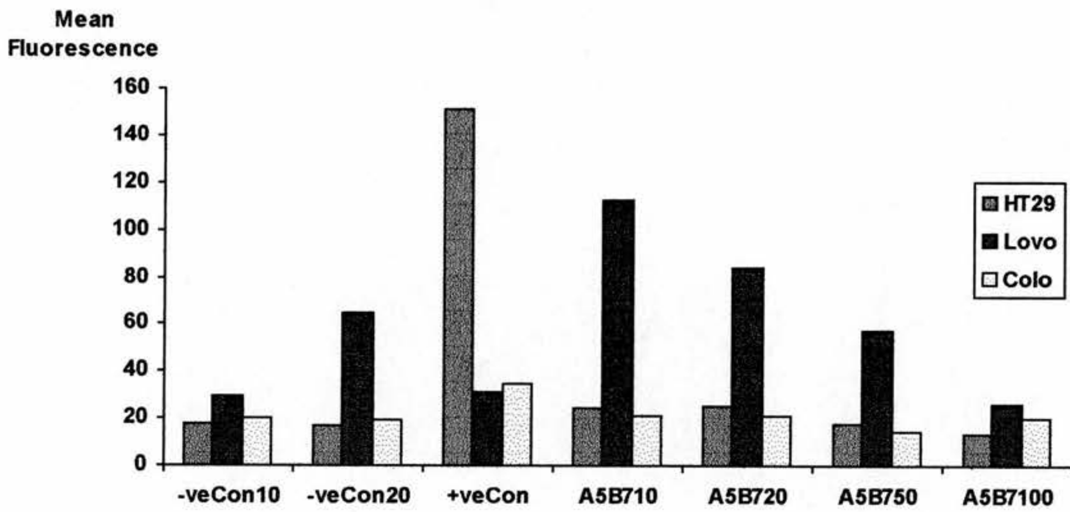
Permeabolised cells (ie cytoplasmic and membrane bound fluoresceinated antibody-antigen complex)

	–ve Control 10µl/ml	–ve Control 20µl/ml	+ve Control W6/32	A5B7 10µg/ml	A5B7 20µg/ml	A5B7 50µg/ml	A5B7 100µg/ml
HT29	17.65	17.05	150.61	24.41	25.02	17.70	13.36
Lovo	29.20	64.11	30.90	112.10	83.49	56.84	25.91
Colo	19.69	19.11	34.72	21.03	20.93	14.22	20.33

Non-permeabolised Cells



Permeabolised Cells



The results suggest that the concentrations of A5B7 used are too high - the best results were achieved with A5B7 concentration of 10μg/ml. There was no significant difference in the results with negative control antibody at 10 or 20μl/ml.

Conclusions

1. Negative control antibody may be used efficaciously at a concentration of 10μl/ml.
2. Further dilutions of A5B7 up to a maximum of 10μg/ml are recommended, in order to establish the minimum acceptable concentration of primary antibody for FACS measurement of Fluorescein-conjugated CEA

FLUORESCEIN ACTIVATED CELL SORTING EXPERIMENT 2

ANTIBODY TITRATION

Aims

1. To find the optimum concentration of primary anti-CEA antibody (A5B7 Mouse IgG type antibody) and of the negative control (Mouse IgG_1) for immunostaining and FACS, using a range of dilutions antibody extending beyond those used in Fluorescein Activated Cell Sorting Antibody Titration Experiment 1.

2. To test the suitability of RFDR.2 MHC Class 11 antibody as a positive control against anti-CEA antibody.

Method

The human colorectal cell lines, Lovo, which is a high expressor of CEA was grown in standard tissue culture for 7 days. The cells were then harvested by trypsinising, counted and resuspended in whole medium at a concentration of 10^6 cells per ml. Thirty-eight Aliquots of 100μl of each cell suspension were pipetted into separate pyrex tubes, 19 for surface antigen measurement and 19 for cytoplasm antigen measurement. In each category, 9 serial dilutions of anti-CEA antibody, 9 serial dilutions of negative control mouse IgG1 and a standard positive control, RFDR.2, were tested.

The samples for cytoplasmic antigen measurement were then permeabolised: 2 mls of Ortho Permeafix at 0.5 concentration with distilled water was added to the tubes which were incubated for 30 minutes at room temperature. The samples were then centrifuged at 2000rpm for 5 minutes, the supernatants discarded and the pellets flicked. The cells were then washed twice in PBS.A (PBS, Albumin 0.2%, Azide 0.2%) and respun at 2000rpm for 5 minutes after each wash.

All the samples were then immunstained - the permeabolised and the non-permeabolised cells. Varying amounts of A5B7 (100, 33, 10, 3.3, 1, 0.33, 0.1, 0.03 and 0.01μg/ml) in 100μl aliquots in PBS.A were applied. An identical range of concentrations of the negative control mouse IgG_1 was applied to negative control tubes. The positive control used was RFDR.2 MHC Class 11 antibody, which was used at standard FACS-recommended concentration.

The samples were incubated for 30 minutes at room temperature, then washed twice in PBS.A, being spun at 2000rpm for 5 minutes in between washes. The second, fluorescein conjugated antibody (Rabbit anti-mouse Fluorescein Isothionate) was then applied in 5μl aliquots at a concentration of 1:100.

The samples were again incubated at room temperature for 30 minutes, then washed twice with PBS.A and spun at 2000rpm for 5 minutes. They were then fixed in Paraformaldehyde (1%), and stored at 4°C prior to being run through the FACS scanner. The average fluorescence of 3000 cells in from each sample was demonstrated.

Results

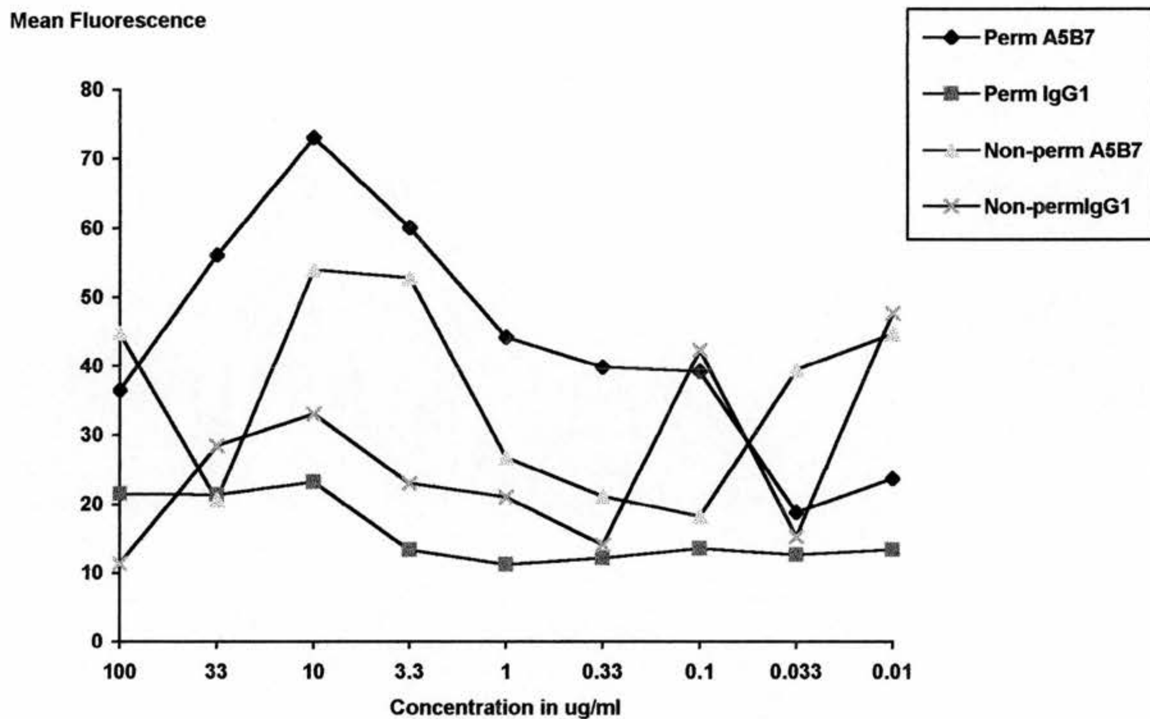
The fluorescence of permeabolised cells is a measure of the total cytoplasmic and membrane-bound CEA; that of non-permeabolised cells reflects membrane-bound CEA only.

Mean Fluorescence of Lovo cells

Concentration	Perm A5B7	Perm IgG1	Nonperm A5B7	Non-perm IgG1
100 μg/ml	**36.53**	**21.48**	**44.77**	**11.37**
33 μg/ml	**56.14**	**21.32**	**20.55**	**28.45**
10 μg/ml	**73.04**	**23.12**	**53.87**	**33.07**
3.3 μg/ml	**59.91**	**13.25**	**52.68**	**22.94**
1 μg/ml	**44.08**	**11.21**	**26.77**	**20.96**
0.3μg/ml	**39.71**	**12.09**	**21.05**	**13.98**
0.1 μg/ml	**39.11**	**13.46**	**18.16**	**42.24**
0.03 μg/ml	**18.65**	**12.52**	**39.26**	**15.17**
0.01 μg/ml	**23.53**	**13.29**	**44.49**	**47.54**

RFDR.2 control: Perm : **13.86**

Non-perm: **18.41**



Conclusions

1. Concentrations above 33 and below 0.33 μg/ml do not show significant differences between the anti-CEA antibody and the negative control, IgG1. The optimum range of concentration falls between 10 and 3.3μg/ml.

2. The proposed positive control RFDR.2 is expressed by Lovo cells to a lesser degree than CEA, and so is an inappropriate positive control.

CHLOROQUINE TITRATION EXPERIMENT:

Aim:

To identify a concentration of Chloroquine able to inhibit cell growth and induce CEA expression by serial dilutions

Method:

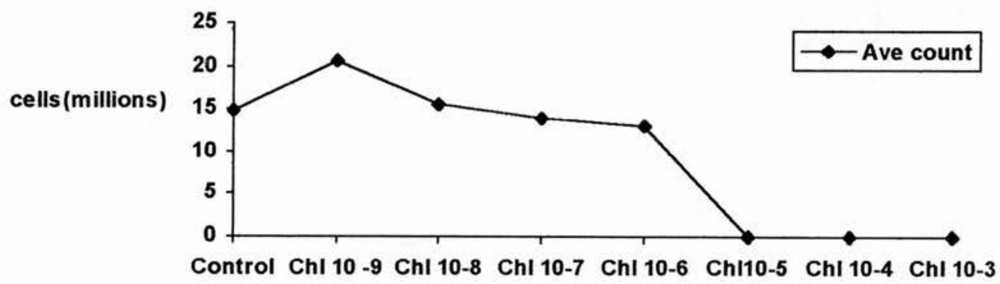
16 flasks each containing one million HT29 cells were grown in standard monolayer culure for 5 days. Duplicate control flasks were grown in normal culture medium - Dulbecco's Modified Eagle's Medium with 10% Fetal Calf Serum, Glutamine and Penicillin-Streptomycin. Chloroquine was added to this culture medium for the remaining 14 flasks, with two flasks for each serial x10 dilution from 10^{-3} M to 10^{-9} M Chloroquine.

After 5 days' growth, the cells were trypsinised, counted and stained with A5B7 anti-CEA mouse monoclonal antibody and Fluorescein-conjugated goat anti-mouse antibody. The average fluorescence of the cells was then measured using the Fluorescein Activated Cell Sorting Scanner.

Results

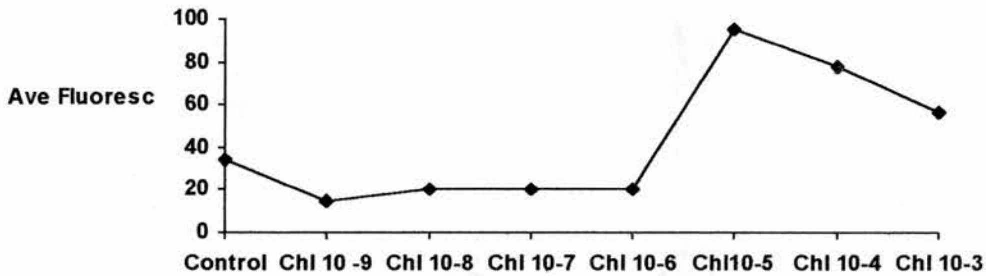
Cell Counts

	Control	Chlor 10^{-9} M	Chlor 10^{-8} M	Chlor 10^{-7} M	Chlor 10^{-6} M	Chlor 10^{-5} M	Chlor 10^{-4} M	Chlor 10^{-3} M
Sample 1	12.7	22.9	17.9	13.6	10.6	<0.1	<0.1	<0.1
Sample 2	16.7	18.1	12.8	14.2	15.4	<0.1	<0.1	<0.1
Ave	14.7	20.5	15.4	13.9	13.0	<0.1	<0.1	<0.1



FACS Fluorescence (ieMembrane CEA)

	Control	Chlor 10^{-9} M	Chlor 10^{-8} M	Chlor 10^{-7} M	Chlor 10^{-6} M	Chlor 10^{-5} M	Chlor 10^{-4} M	Chlor 10^{-3} M
Sample 1	31.3	15.9	24.2	18.5	19.7	121.2	50.7	45.2
Sample 2	38.3	13.5	17.2	23.1	20.9	67.6	108.7	67.4
Ave	34.5	14.7	20.7	20.8	20.3	95.8	77.7	56.3



A marked cut off was seen between 10^{-5} and 10^{-6} M Chloroquine: concentrations more dilute the 10^{-5} Molar Chloroquine did not inhibit cell growth or augment CEA expression, whereas concentrations of 10^{-5} M, 10^{-4} M and 10^{-3} M severely impeded cell growth and induced augmented membrane CEA expression.

Conclusion

A concentration of 10^{-5} M of Chloroquine was therefore chosen for use in future studies of all 3 cell lines.

RADIATION TITRATION EXPERIMENT

Aim

The purpose of this experiment was to identify a) a dose of radiation causing growth inhibition of HT29 cells in vitro; and b) the time after radiation that the cells exhibited growth inhibition and any changes in CEA expression

Methods

Triplicate samples of HT29 cells (one million cells approximately per sample) were irradiated with 2Grays or 5Grays of X-radiation. The cells were harvested at 3, 24, 48 and 96 hours after the radiation for the 2 Gray samples, and at 3, 48 and 72 hours after the radiation for the 5 Gray samples. All the cell samples were counted and stained for membrane CEA using two-stage fluorescein conjugated immunostains for FACS scanning, as described in Chapter 2.

Results

2 Gray dose:

GROWTH

	CONTROL Cell Counts x 10^6 cells	IRRADIATED Cell Counts x 10^6 cells
PLUS 3 HOURS	**6.3**	**5.4**
	5.8	**7.4**
	6.9	**7.5**
Average:	**6.33**	**6.77**
PLUS 24 HOURS	**4.8**	**2.7**
	5.6	**3.8**
	5.6	**3.4**
Average:	**5.33**	**3.3**
PLUS 48 HOURS	**7.7**	**6.1**
	9.4	**6.0**
	20.2	**6.6**
Average	**12.4**	**6.23**
PLUS 96 HOURS	**7.5**	**6.3**
	8.1	**7.4**
	10.8	**8.7**
Average	**8.8**	**7.47**

The cell counts showed a growth inhibition measurable 24 hours after irradiation (t-test, $p=0.008$).

CEA EXPRESSION

The corresponding measurements of CEA expression (in arbitrary units) by fluorescein-stained FACS scanning were as follows :

	PLUS 3 HOURS	PLUS 24 HOURS	PLUS 48 HOURS	PLUS 96 HOURS
Control 1	**15.36**	**13.80**	**18.62**	**27.34**
Control 2	**16.67**	**14.52**	**18.39**	**23.35**
Control 3	**25.39**	**15.24**	**21.27**	**23.48**
X-rayed 1	**14.14**	**13.62**	**21.58**	**24.74**
X-rayed 2	**34.06**	**14.13**	**21.37**	**26.32**
X-rayed 3	**19.61**	**13.06**	**22.63**	**25.38**
Negative	**20.85**	**15.23**	**20.58**	**22.33**

Analysis of these results by the t-test showed no significant rise in CEA expression at $p \leq 0.05$.

5 Gray Dose

GROWTH

	CONTROL Cell Counts x 10^6 cells	IRRADIATED Cell Counts x 10^6 cells
PLUS 3 HOURS	**1.0**	**0.3**
	1.0	**0.2**
	1.2	**0.4**
Average:	**1.67**	**0.3**
PLUS 48 HOURS	**2.4**	**2.2**
	3.1	**1.9**
	3.7	**1.3**
Average:	**3.67**	**1.8**
PLUS 72 HOURS	**6.4**	**3.2**
	5.9	**3.6**
	6.3	**3.1**
Average	**6.20**	**3.3**

The cell counts showed significant growth inhibition measurable 4 , 48 and 72 hours after irradiation (t-test, $p \leq 0.05$. The corresponding measurements of CEA expression by fluorescein-stained FACS scanning were as follows:

CEA EXPRESSION (arbitrary units)

	PLUS 3 HOURS	PLUS 48 HOURS	PLUS 72 HOURS
Control 1	**65.38**	**42.92**	**24.30**
Control 2	**67.93**	**47.90**	**30.08**
Control 3	**63.23**	**44.85**	**33.09**
X-rayed 1	**64.32**	**43.33**	**43.44**
X-rayed 2	**66.06**	**54.22**	**49.50**
X-rayed 3	**67.39**	**53.80**	**45.23**
Negative	**67.00**	**25.55**	**21.77**

Analysis of these results by the student's t-test showed a significant rise in CEA expression measurable 72 hours after irradiation ($p=0.006$).

Discussion

The above results show growth inhibitory effects of fairly high dose radiation (5Gray) observable 48 hours after irradiation, and a small but significant increase in CEA expression measurable 72 hours after irradiation. The smaller 2 Gray dose of radiation had no effect on cell growth or CEA expression.

Conclusion

Treating colorectal cancer cells with a single 5 Gray dose of irradiation may result in transient growth inhibition, and a rise in CEA expression observable after 72 hours.

APPENDIX 2

The data for the positive growth inhibitory and CEA augmentation (Immunocytochemistry and FACS) results from Chapters 2 to 5 are presented below.

Contents:	**Page**
a. Growth Results	192
b. Immunocytochemistry Results	205
c. FACS Results	211

a. Growth Results

Differentiating Agents:

Butyric Acid (BA)

	COLO *control* 10^6cells	**COLO** *BA* 10^6cells	**COLO** *BA* %con	**HT29** *control* 10^6cells	**HT29** *BA* 10^6cells	**HT29** *BA* %con	**LOVO** *control* 10^6cells	**LOVO** *BA* 10^6cells	**LOVO** *BA* %con
	5.5	**1.9**	**30.9**	**5.6**	**2.9**	**40.8**	**2.9**	**1.3**	**39.4**
	6.8	**1.2**	**19.5**	**8.6**	**5.0**	**70.4**	**3.7**	**0.5**	**15.2**
		1.1	**17.9**		**3.9**	**54.9**		**0.7**	**21.2**
Ave			**22.8**			**55.5**			**25.3**
St Dev			**7.1**			**15.0**			**12.6**
t-test			**0.004**			**0.10**			**0.01**

Theophylline (Theo):

	COLO *control* 10^6cells	**COLO** *Theo* 10^6cells	**COLO** *Theo* %con	**HT29** *control* 10^6cells	**HT29** *Theo* 10^6cells	**HT29** *Theo* %con	**LOVO** *control* 10^6cells	**LOVO** *Theo* 10^6cells	**LOVO** *Theo* %con
	5.5	**0.2**	**3.3**	**5.6**	**1.5**	**21.1**	**2.9**	**0.5**	**15.2**
	6.8	**0.2**	**3.3**	**8.6**	**1.3**	**18.3**	**3.7**	**0.1**	**3.0**
		0.5	**8.1**		**1.3**	**18.3**		**0.1**	**2.0**
Ave			**4.9**			**19.2**			**7.1**
St Dev			**2.8**			**1.6**			**7.0**
t-test			**0.001**			**0.01**			**0.002**

5-Azacytidine:

	COLO *control* 10^6cells	**COLO** *5-Aza* 10^6cells	**COLO** *5-Aza* %con	**HT29** *control* 10^6cells	**HT29** *5-Aza* 10^6cells	**HT29** *5-Aza* %con	**LOVO** *control* 10^6cells	**LOVO** *5-Aza* 10^6cells	**LOVO** *5-Aza* %con
	5.5	**0.1**	**1.6**	**5.6**	**0.4**	**5.6**	**2.9**	**0.1**	**3.0**
	6.8	**0.1**	**1.6**	**8.6**	**0.2**	**2.8**	**3.7**	**0.1**	**3.0**
		0.1	**1.6**		**0.2**	**5.6**		**0.1**	**3.0**
Ave			**1.6**			**4.7**			**3.0**
St Dev			**0**			**1.6**			**0**
t-test			**0.001**			**0.009**			**0.002**

Interferon:

	COLO *control* 10^6cells	**COLO** *IFN* 10^6cells	**COLO** *IFN* %con	**HT29** *control* 10^6cells	**HT29** *IFN* 10^6cells	**HT29** *IFN* %con	**LOVO** *control* 10^6cells	**LOVO** *IFN* 10^6cells	**LOVO** *IFN* %con
	14.3	**11.2**	**92.2**	**12.7**	**2.9**	**25.4**	**9.6**	**3.0**	**36.1**
	10.0	**6.6**	**54.3**	**10.1**	**2.6**	**22.8**	**7.0**	**0.5**	**6.0**
		8.3	**68.3**		**2.8**	**24.6**		**0.7**	**8.4**
Ave			**71.6**			**24.3**			**16.8**
St Dev			**19.2**			**1.3**			**16.7**
t-test			**0.24**			**0.003**			**0.02**

Environmental Changes

Chloroquine:

	COLO *control* 10⁶cells	**COLO** *Chlor* 10⁶cells	**COLO** *Chlor* %con	**HT29** *control* 10⁶cells	**HT29** *Chlor* 10⁶cells	**HT29** *Chlor* %con	**LOVO** *control* 10⁶cells	**LOVO** *Chlor* 10⁶cells	**LOVO** *Chlor* %con
	10.1	**0.1**	**0.9**	**12.0**	**0.1**	**0.8**	**12.6**	**0.2**	**1.9**
	12.0	**0.1**	**0.9**	**14.0**	**0.1**	**0.8**	**8.3**	**0.5**	**4.8**
		0.2	**1.8**		**0.1**	**0..8**		**0.1**	**1.0**
Ave			**1.2**			**0.8**			**2.6**
St Dev			**0.5**			**0**			**2.0**
t-test			**0.0006**			**0.0004**			**0.008**

Starvation:

	COLO *control* 10⁶cells	**COLO** Starv 10⁶cells	**COLO** *Starv* %con	**HT29** *control* 10⁶cells	**HT29** *Starv* 10⁶cells	**HT29** *Starv* %con	**LOVO** *control* 10⁶cells	**LOVO** *Starv* 10⁶cells	**LOVO** *Starv* %con
	7.0	**1.3**	**17.1**	**14.4**	**3.6**	**34.3**	**10.2**	**1.6**	**19.4**
	8.2	**2.3**	**30.3**	**6.6**	**3.5**	**33.3**	**6.3**	**0.6**	**7.3**
		2.4	**31.6**		**4.2**	**40.0**		**3.4**	**41.2**
Ave			**26.3**			**35.7**			**22.6**
St Dev			**8.0**			**3.6**			**17.2**
t-test			**0.003**			**0.10**			**0.03**

Hypoxia (1% Oxygen):

	COLO *control* 10⁶cells	**COLO** *Hyp1%* 10⁶cells	**COLO** *Hyp1%* %con	**HT29** *control* 10⁶cells	**HT29** *Hyp1%* 10⁶cells	**HT29** *Hyp1%* %con	**LOVO** *control* 10⁶cells	**LOVO** *Hyp1%* 10⁶cells	**LOVO** *Hyp1%* %con
	4.4	**0.1**	**2.5**	**3.6**	**0.1**	**2.7**	**5.4**	**0.1**	**2.1**
	3.6	**0.1**	**2.5**	**3.9**	**0.1**	**2.7**	**4.0**	**0.1**	**2.1**
		0.1	**2.5**		**0.1**	**2.7**		**0.1**	**2.1**
Ave			**2.5**			**2.7**			**2.1**
St Dev			**0**			**0**			**0**
t-test			**0.001**			**6x10-5**			**0.003**

Hypoxia (5% Oxygen):

	COLO *control* 10^6cells	COLO *Hyp5%* 10^6cells	COLO *Hyp5%* %con	HT29 *control* 10^6cells	HT29 *Hyp5%* 10^6cells	HT29 *Hyp5%* %con	LOVO *control* 10^6cells	LOVO *Hyp5%* 10^6cells	LOVO *Hyp5%* %con
	1.6	**0.6**	**42.8**	**10.9**	**9.2**	**80.7**	**1.2**	**3.3**	**103.1**
	1.2	**1.3**	**92.9**	**11.9**	**8.2**	**71.9**	**5.2**	**4.3**	**134.4**
		0.4	**28.6**		**6.0**	**52.6**		**4.7**	**146.8**
Ave			**54.8**			**68.4**			**128.1**
St Dev			**33.8**			**14.4**			**22.5**
t-test			**0.20**			**0.07**			**0.61**

Hypoxia (12% Oxygen):

	COLO *control* 10^6cells	COLO *Hyp12* 10^6cells	COLO *Hyp12* %con	HT29 *control* 10^6cells	HT29 *Hyp12* 10^6cells	HT29 *Hyp12* %con	LOVO *control* 10^6cells	LOVO *Hyp12* 10^6cells	LOVO *Hyp12* %con
	1.6	**0.7**	**50.0**	**10.9**	**7.6**	**66.7**	**1.2**	**5.3**	**165.6**
	1.2	**1.0**	**71.4**	**11.9**	**11.2**	**98.2**	**5.2**	**4.2**	**131.2**
		0.8	**57.1**		**6.1**	**53.5**		**5.4**	**168.8**
Ave			**59.5**			**72.8**			**155.2**
St Dev			**10.9**			**23.0**			**20.8**
t-test			**0.06**			**0.22**			**0.34**

Radiation:

	COLO *control* 10^6cells	COLO *DXT* 10^6cells	COLO *DXT* %con	HT29 *control* 10^6cells	HT29 *DXT* 10^6cells	HT29 *DXT* %con	LOVO *control* 10^6cells	LOVO *DXT* 10^6cells	LOVO *DXT* %con
	4.6	**5.8**	**130.3**	**8.9**	**5.6**	**61.9**	**7.2**	**4.1**	**56.2**
	4.9	**4.7**	**105.6**	**9.2**	**4.3**	**47.5**	**7.4**	**4.3**	**58.9**
		5.1	**146**		**6.5**	**71.8**		**6.6**	**90.4**
Ave			**127.3**			**60.4**			**68.5**
St Dev			**20.4**			**12.2**			**19.01**
t-test			**0.37**			**0.02**			**0.11**

Cytotoxic Drugs

5-Fluorouracil:

	COLO *control* 10^6cells	**COLO** *5FU* 10^6cells	**COLO** *5FU* %con	**HT29** *control* 10^6cells	**HT29** *5FU* 10^6cells	**HT29** *5FU* %con	**LOVO** *control* 10^6cells	**LOVO** *5FU* 10^6cells	**LOVO** *5FU* %con
	11.1	**6.2**	**60.0**	**5.0**	**0.7**	**8.4**	**2.2**	**1.0**	**54.1**
	9.6	**5.9**	**57.0**	**11.6**	**0.5**	**6.0**	**1.5**	**1.5**	**81.1**
		6.0	**58.0**		**0.3**	**3.6**		**1.4**	**75.7**
Ave			**58.3**			**6.0**			**70.3**
St Dev			**1.5**			**2.4**			**14.3**
t-test			**0.005**			**0.05**			**0.19**

Methotrexate:

	COLO *control* 10^6cells	**COLO** *Mtx* 10^6cells	**COLO** *Mtx* %con	**HT29** *control* 10^6cells	**HT29** *Mtx* 10^6cells	**HT29** *Mtx* %con	**LOVO** *control* 10^6cells	**LOVO** *Mtx* 10^6cells	**LOVO** *Mtx* %con
	13.2	**1.4**	**10.1**	**22.8**	**0.9**	**5.5**	**8.9**	**1.4**	**13.3**
	14.6	**0.8**	**5.8**	**9.8**	**0.7**	**4.3**	**12.1**	**2.2**	**21.0**
		0.8	**5.8**		**2.2**	**13.5**		**2.3**	**21.9**
Ave			**7.2**			**7.8**			**18.7**
St Dev			**2.5**			**5.0**			**4.7**
t-test			**0.0002**			**0.05**			**0.006**

Taxol:

	COLO *control* 10^6cells	**COLO** *Tax* 10^6cells	**COLO** *Tax* %con	**HT29** *control* 10^6cells	**HT29** *Tax* 10^6cells	**HT29** *Tax* %con	**LOVO** *control* 10^6cells	**LOVO** *Tax* 10^6cells	**LOVO** *Tax* %con
	8.5	**2.9**	**38.4**	**6.4**	**0.1**	**1.9**	**7.5**	**0.6**	**6.8**
	6.6	**1.4**	**18.5**	**4.1**	**0.2**	**3.8**	**11.0**	**0.6**	**6.8**
		2.3	**30.5**		**0.1**	**1.9**	**7.5**	**1.1**	**12.5**
							9.2		
Ave			**29.1**			**2.5**			**13.0**
St Dev			**10**			**1.1**			
t-test			**0.01**			**0.009**			**0.005**

Etoposide:

	COLO *control* 10^6cells	**COLO** *Etop* 10^6cells	**COLO** *Etop* %con	**HT29** *control* 10^6cells	**HT29** *Etop* 10^6cells	**HT29** *Etop* %con	**LOVO** *control* 10^6cells	**LOVO** *Etop* 10^6cells	**LOVO** *Etop* %con
	14.3	**10.5**	**86.4**	**12.7**	**6.9**	**60.5**	**9.6**	**6.0**	**72.3**
	10.0	**8.5**	**70.0**	**10.1**	**11.0**	**96.5**	**7.0**	**2.8**	**33.7**
		10.1	**83.1**		**14.3**	**125.4**		**5.1**	**61.4**
Ave			**79.8**			**94.1**			**55.8**
St Dev			**8.7**			**32.5**			**19.9**
t-test			**0.26**			**0.83**			**0.10**

Vincristine:

	COLO *control* 10^6cells	**COLO** *Vinc* 10^6cells	**COLO** *Vinc* %con	**HT29** *control* 10^6cells	**HT29** *Vinc* 10^6cells	**HT29** *Vinc* %con	**LOVO** *control* 10^6cells	**LOVO** *Vinc* 10^6cells	**LOVO** *Vinc* %con
	16.0	**10.5**	**86.4**	**29.5**	**20.1**	**88.2**	**13.7**	**11.0**	**101.4**
	21.5	**22.4**	**119.5**	**16.1**	**9.8**	**43.0**	**8.0**	**9.7**	**89.4**
		16.7	**89.1**		**16.9**	**74.1**		**4.9**	**45.2**
Ave			**88.2**			**68.4**			**78.7**
St Dev			**31.8**			**23.1**			**29.6**
t-test			**0.68**			**0.34**			**0.52**

Combinations

BUTYRIC ACID & THEOPHYLLINE:

	COLO *control* 10^6cells	**COLO** *Ba+Th* 10^6cells	**COLO** *Ba+Th* %con	**HT29** *control* 10^6cells	**HT29** *Ba+Th* 10^6cells	**HT29** *Ba+Th* %con	**LOVO** *control* 10^6cells	**LOVO** *Ba+Th* 10^6cells	**LOVO** *Ba+Th* %con
	5.6	**0.1**	**1.7**	**12.6**	**1.4**	**12.1**	**4.2**	**0.1**	**1.8**
	6.1	**0.1**	**1.7**	**10.5**	**0.8**	**6.9**	**5.0**	**0.1**	**1.8**
		0.1	**1.7**		**2.9**	**25**	**6.5**	**0.1**	**1.8**
							6.7		
Ave			**1.7**			**14.7**			**1.8**
St Dev			**0**			**9.3**			**0**
t-test			**7x10-5**			**0.003**			**0.0006**

BUTYRIC ACID & 5 AZACYTIDINE:

	COLO *control* 10^6cells	**COLO** *Ba5az* 10^6cells	**COLO** *Ba5az* %con	**HT29** *control* 10^6cells	**HT29** *Ba5az* 10^6cells	**HT29** *Ba5az* %con	**LOVO** *control* 10^6cells	**LOVO** *Ba5az* 10^6cells	**LOVO** *Ba5az* %con
	5.6	**0.1**	**1.7**	**12.6**	**3.5**	**30.2**	**4.2**	**0.2**	**3.6**
	6.1	**0.1**	**1.7**	**10.5**	**5.3**	**45.7**	**5.0**	**0.1**	**1.8**
		0.2	**3.4**		**5.2**	**44.8**	**6.5**	**0.1**	**1.8**
							6.7		
Ave			**2.3**			**40.2**			**2.4**
St Dev			**1**			**8.7**			**1**
t-test			**8x10-5**			**0.008**			**0.0006**

BUTYRIC ACID & INTERFERON:

	COLO *control* 10^6cells	**COLO** *BaIfn* 10^6cells	**COLO** *BaIfn* %con	**HT29** *control* 10^6cells	**HT29** *BaIfn* 10^6cells	**HT29** *BaIfn* %con	**LOVO** *control* 10^6cells	**LOVO** *BaIfn* 10^6cells	**LOVO** *BaIfn* %con
	7.4	**7.2**	**81.8**	**6.5**	**2.8**	**34.6**	**7.5**	**0.1**	**1.1**
	10.3	**8.7**	**98.9**	**9.7**	**1.6**	**19.8**	**11.0**	**0.1**	**1.1**
		11.0	**125.0**		**2.8**	**34.6**	**7.5**	**0.1**	**1.1**
							9.2		
Ave			**101.9**			**2.5**			**1.1**
St Dev			**21.8**			**1.1**			**0**
t-test			**0.95**			**0.02**			**0.0003**

INTERFERON & THEOPHYLLINE:

	COLO *control* 10^6cells	**COLO** *Ifn+Th* 10^6cells	**COLO** *Ifn+Th* %con	**HT29** *control* 10^6cells	**HT29** *Ifn+Th* 10^6cells	**HT29** *Ifn+Th* %con	**LOVO** *control* 10^6cells	**LOVO** *Ifn+Th* 10^6cells	**LOVO** *Ifn+Th* %con
	5.6	**0.1**	**1.9**	**3.3**	**0.6**	**17.6**	**7.5**	**0.1**	**1.1**
	4.8	**0.1**	**1.9**	**3.4**	**0.8**	**23.5**	**7.5**	**0.1**	**1.1**
		0.1	**1.9**		**0.6**	**17.6**	**11.0**	**0.1**	**1.1**
							9.2		
Ave			**1.9**			**19.6**			**1.1**
St Dev			**0**			**3.4**			**0**
t-test			**0.0001**			**9x10-5**			**0.0003**

INTERFERON & 5 AZACYTIDINE:

	COLO *control* 10^6cells	**COLO** *Ifn+5az* 10^6cells	**COLO** *Ifn+5az* %con	**HT29** *control* 10^6cells	**HT29** *Ifn+5az* 10^6cells	**HT29** *Ifn+5az* %con	**LOVO** *control* 10^6cells	**LOVO** *Ifn+5az* 10^6cells	**LOVO** *Ifn5az* %con
	5.6	**0.1**	**1.9**	**3.3**	**1.2**	**35.3**	**7.5**	**0.1**	**1.1**
	4.8	**0.1**	**1.9**	**3.4**	**1.9**	**55.9**	**7.5**	**0.1**	**1.1**
		0.1	**1.9**		**1.6**	**47.1**	**11.0**	**0.1**	**1.1**
							9.2		
Ave			**1.9**			**46.1**			**1.1**
St Dev			**0**			**10.3**			**0**
t-test			**0.0001**			**0.007**			**0.0003**

5-FLUOROURACIL & BUTYRIC ACID:

	COLO *control* 10^6cells	**COLO** *5FU.Ba* 10^6cells	**COLO** *5FU.Ba* %con	**HT29** *control* 10^6cells	**HT29** *5FU.Ba* 10^6cells	**HT29** *5FU.Ba* %con	**LOVO** *control* 10^6cells	**LOVO** *5FU.Ba* 10^6cells	**LOVO** *5FU.Ba* %con
	1.6	**0.4**	**28.6**	**15.2**	**5.3**	**43.8**	**10.4**	**5.2**	**46.4**
	1.2	**0.2**	**14.3**	**9.0**	**6.5**	**53.7**	**12.4**	**3.1**	**27.6**
		0.3	**21.4**		**8.6**	**71.1**		**5.8**	**51.8**
Ave			**21.4**			**56.2**			**41.9**
St Dev			**7.2**			**13.8**			**12.7**
t-test			**0.007**			**0.14**			**0.01**

5-FLUOROURACIL & THEOPHYLLINE:

	COLO *control* 10^6cells	**COLO** *5FU.Th* 10^6cells	**COLO** *5FU.Th* %con	**HT29** *control* 10^6cells	**HT29** *5FU.Th* 10^6cells	**HT29** *5FU.Th* %con	**LOVO** *control* 10^6cells	**LOVO** *5FU.Th* 10^6cells	**LOVO** *5FU.Th* %con
	1.6	**0.4**	**7.1**	**15.2**	**10.6**	**87.6**	**10.4**	**0.5**	**4.5**
	1.2	**0.2**	**7.1**	**9.0**	**10.2**	**84.3**	**12.4**	**0.6**	**5.4**
		0.3	**7.1**		**16.1**	**133.1**		**2.7**	**24.1**
Ave			**7.1**			**101.7**			**11.3**
St Dev			**0**			**27.3**			**11.1**
t-test			**0.003**			**0.96**			**0.003**

5-FLUOROURACIL & 5-AZACYTIDINE:

	COLO *control* 10^6cells	**COLO** *5FU.5a* 10^6cells	**COLO** *5FU.5a* %con	**HT29** *control* 10^6cells	**HT29** *5FU.5a* 10^6cells	**HT29** *5FU.5a* %con	**LOVO** *control* 10^6cells	**LOVO** *5FU.5a* 10^6cells	**LOVO** *5FU.5a* %con
	1.6	**0.1**	**7.1**	**15.2**	**7.2**	**59.5**	**10.4**	**0.1**	**0.9**
	1.2	**0.1**	**7.1**	**9.0**	**6.3**	**52.1**	**12.4**	**0.1**	**0.9**
		0.1	**7.1**		**6.2**	**51.2**		**0.1**	**0.9**
Ave			**7.1**			**54.3**			**0.9**
St Dev			**0**			**4.6**			**0**
t-test			**0.003**			**0.10**			**0.003**

METHOTREXATE & BUTYRIC ACID:

	COLO *control* 10^6cells	COLO *Mtx.Ba* 10^6cells	COLO *Mtx.Ba* %con	HT29 *control* 10^6cells	HT29 *Mtx.Ba* 10^6cells	HT29 *Mtx.Ba* %con	LOVO *control* 10^6cells	LOVO *Mtx.Ba* 10^6cells	LOVO *Mtx.Ba* %con
	6.2	3.5	51.5	4.7	1.2	24.0	4.2	0.9	16.1
	7.3	3.8	55.7	5.3	1.3	26.0	5.0	0.7	12.5
		5.3	77.9		1.3	26.0	6.5	0.7	12.5
							6.7		
Ave			61.8			25.3			13.7
St Dev			14.1			1.2			2.1
t-test			0.05			0.0005			0.001

METHOTREXATE & THEOPHYLLINE:

	COLO *control* 10^6cells	COLO *Mtx.Th* 10^6cells	COLO *Mtx.Th* %con	HT29 *control* 10^6cells	HT29 *Mtx.Th* 10^6cells	HT29 *Mtx.Th* %con	LOVO *control* 10^6cells	LOVO *Mtx.Th* 10^6cells	LOVO *Mtx.Th* %con
	6.2	0.2	2.9	4.7	0.7	14.0	4.2	0.3	5.4
	7.3	0.2	2.9	5.3	0.5	10.0	5.0	0.5	8.9
		0.2	2.9		0.5	10.0	6.5	0.5	8.9
							6.7		
Ave			2.9			11.3			7.7
St Dev			0			2.3			2.0
t-test			0.0005			0.0003			0.0008

METHOTREXATE & 5-AZACYTIDINE:

	COLO *control* 10^6cells	COLO *Mtx.5A* 10^6cells	COLO *Mtx.5a* %con	HT29 *control* 10^6cells	HT29 *Mtx.5a* 10^6cells	HT29 *Mtx.5a* %con	LOVO *control* 10^6cells	LOVO *Mtx.5a* 10^6cells	LOVO *Mtx.5a* %con
	6.2	0.4	5.9	4.7	0.6	12.0	4.2	0.1	1.8
	7.3	0.5	7.4	5.3	0.7	14.0	5.0	0.1	1.8
		0.5	7.4		1.0	20.0	6.5	0.1	1.8
							6.7		
Ave			6.9			15.3			1.8
St Dev			0.9			4.2			0
t-test			0.0006			0.0006			0.0006

INTERFERON & 5-FLUOROURACIL:

	COLO *control* 10^6cells	**COLO** *Ifn.5F U* 10^6cells	**COLO** *Ifn.5F U* %con	**HT29** *control* 10^6cells	**HT29** *Ifn.5F U* 10^6cells	**HT29** *Ifn.5F U* %con	**LOVO** *control* 10^6cells	**LOVO** *Ifn.5F U* 10^6cells	**LOVO** *Ifn.5F U* %con
	7.4	**8.8**	**100.0**	**6.5**	**1.1**	**13.6**	**7.5**	**0.1**	**1.1**
	10.3	**7.1**	**80.7**	**9.7**	**1.1**	**13.6**	**7.5**	**0.1**	**1.1**
		5.1	**58.0**		**0.8**	**9.9**	**11.0**	**0.1**	**1.1**
							9.2		
Ave			**79.6**			**12.**			**1.1**
St Dev			**21.0**			**2.1**			**0**
t-test			**0.37**			**0.01**			**0.0003**

INTERFERON & METHOTREXATE:

	COLO *control* 10^6cells	**COLO** *Ifn.Mtx* 10^6cells	**COLO** *Ifn.Mtx* %con	**HT29** *control* 10^6cells	**HT29** *Ifn.Mtx* 10^6cells	**HT29** *Ifn.Mtx* %con	**LOVO** *control* 10^6cells	**LOVO** *Ifn.Mtx* 10^6cells	**LOVO** *Ifn.Mtx* %con
	7.4	**5.6**	**63.6**	**6.5**	**1.0**	**12.3**	**7.5**	**0.1**	**1.1**
	10.3	**7.1**	**80.7**	**9.7**	**0.8**	**9.9**	**7.5**	**0.1**	**1.1**
		8.7	**98.9**		**1.6**	**19.8**	**11.0**	**0.4**	**4.5**
							9.2		
Ave			**81.1**			**14.0**			**1.1**
St Dev			**17.6**			**5.2**			
t-test			**0.36**			**0.01**			**0.0003**

BUTYRIC ACID & HYPOXIA (1%OXYGEN):

	COLO *control* 10^6cells	**COLO** *Ba.Hyp* 10^6cells	**COLO** *Ba.Hyp* %con	**HT29** *control* 10^6cells	**HT29** *Ba.Hyp* 10^6cells	**HT29** *Ba.Hyp* %con	**LOVO** *control* 10^6cells	**LOVO** *Ba.Hyp* 10^6cells	**LOVO** *Ba.Hyp* %con
	15	**1**	**6.1**	**17.5**	**0.1**	**0.5**	**11.1**	**0.9**	**10.5**
	17.7	**0.7**	**4.3**	**22.9**	**0.1**	**0.5**	**7**	**0.8**	**9.3**
		0.5	**3.0**		**0.1**	**0.5**		**0.5**	**5.8**
Ave			**4.5**			**0.5**			**8.5**
St Dev			**1.6**			**0**			**2.4**
t-test			**0.0006**			**0.002**			**0.01**

BUTYRIC ACID & ALKALINE MEDIUM:

	COLO *control* 10^6cells	**COLO** *Ba.Alk* 10^6cells	**COLO** *Ba.Alk* %con	**HT29** *control* 10^6cells	**HT29** *Ba.Alk* 10^6cells	**HT29** *Ba.Alk* %con	**LOVO** *control* 10^6cells	**LOVO** *Ba.Alk* 10^6cells	**LOVO** *Ba.Alk* %con
	15	**21.4**	**130.5**	**17.5**	**26.0**	**128.7**	**11.1**	**19.1**	**222.1**
	17.7	**15.0**	**91.5**	**22.9**	**29.0**	**143.6**	**7**	**8.5**	**98.8**
		14.2	**86.6**		**26.0**	**128.7**		**6.3**	**73.3**
Ave			**108.9**			**133.7**			**131.4**
St Dev			**24.1**			**8.6**			**79.6**
t-test			**0.88**			**0.06**			**0.70**

BUTYRIC ACID & CHLOROQUINE:

	COLO *control* 10^6cells	**COLO** *Ba.Chl* 10^6cells	**COLO** *Ba.Chl* %con	**HT29** *control* 10^6cells	**HT29** *Ba.Chl* 10^6cells	**HT29** *Ba.Chl* %con	**LOVO** *control* 10^6cells	**LOVO** *Ba.Chl* 10^6cells	**LOVO** *Ba.Chl* %con
	7.2	**0.1**	**1.1**	**14.4**	**0.1**	**0.7**	**9.6**	**3.5**	**40.7**
	10.5	**0.1**	**1.1**	**13.2**	**0.1**	**0.7**	**7.6**	**6.6**	**76.7**
		0.1	**1.1**		**0.1**	**0.7**		**2.2**	**25.6**
Ave			**1.1**			**0.7**			**47.7**
St Dev			**0**						**26.3**
t-test			**0.006**			**8x10-5**			**0.09**

CHLOROQUINE + 5-FLUOROURACIL:

	COLO *control* 10^6cells	**COLO** *Ch5FU* 10^6cells	**COLO** *Ch5FU* %con	**HT29** *control* 10^6cells	**HT29** *Ch5FU* 10^6cells	**HT29** *Ch5FU* %con	**LOVO** *control* 10^6cells	**LOVO** *Ch5FU* 10^6cells	**LOVO** *Ch5FU* %con
	7.2	**1.5**	**16.7**	**14.4**	**0.1**	**0.7**	**9.6**	**5.4**	**62.8**
	10.5	**0.6**	**6.7**	**13.2**	**0.1**	**0.7**	**7.6**	**5.6**	**65.1**
		1.1	**12.3**		**0.1**	**0.7**		**3.9**	**45.3**
Ave			**11.9**			**0.7**			**57.7**
St Dev			**5.0**			**0**			**10.8**
t-test			**0.009**			**8x10-5**			**0.04**

CHLOROQUINE +METHOTREXATE:

	COLO *control* 10^6cells	**COLO** *Ch.Mtx* 10^6cells	**COLO** *Ch.Mtx* %con	**HT29** *control* 10^6cells	**HT29** *Ch.Mtx* 10^6cells	**HT29** *Ch.Mtx* %con	**LOVO** *control* 10^6cells	**LOVO** *Ch.Mtx* 10^6cells	**LOVO** *Ch.Mtx* %con
	7.2	**0.7**	**7.8**	**14.4**	**0.2**	**1.4**	**9.6**	**7.2**	**83.7**
	10.5	**0.6**	**6.7**	**13.2**	**0.1**	**0.7**	**7.6**	**5.6**	**65.1**
		0.8	**8.9**		**0.1**	**0.7**		**4.4**	**51.2**
Ave			**7.8**			**0.9**			**66.7**
St Dev			**1.1**			**0.4**			**16.3**
t-test			**0.007**			**8x10-5**			**0.11**

b. Immunocytochemistry Results:

Differentiating Agents: Butyric Acid, Theophylline, 5-Azacytidine & Interferon

COLO	BA	Theo	5-Aza	IFN
Scores	**0**	**0.5**	**1**	**0**
	0	**0**	**3**	**0**
	0	**0**	**0.5**	**0**
AVE	**0**	**0.17**	**1.5**	**0**
St Dev	**0**	**0.29**	**1.32**	**0**
t-test	**NA**	**0.50**	**0.23**	**NA**

HT29	HT29 BA	HT29 Theo	HT29 5-Aza	HT29 IFN
Scores	**6.5**	**3.5**	**3**	**4.5**
	7	**2.5**	**2**	**2.5**
	7.5	**3.5**	**2**	**3**
AVE	**7**	**3.17**	**2.33**	**3.33**
St Dev	**0.5**	**0.58**	**0.58**	**1.04**
t-test	**0.001**	**0.06**	**0.30**	**0.05**

LOVO	LOVO BA	LOVO Theo	LOVO 5-Aza	LOVO IFN
Scores	**10**	**8**	**8**	**7.5**
	9.5	**9**	**6.5**	**8.5**
	10	**6**	**7**	**8.5**
AVE	**9.83**	**7.67**	**7.17**	**8.17**
St Dev	**0.29**	**1.53**	**0.76**	**0.58**
t-test	**0.03**	**0.79**	**0.08**	**0.72**

Environmental Changes: Chloroquine, Starvation, Radiation, Hypoxia

COLO	COLO Chlor	COLO Starv	COLO DXT	COLO Hyp<1%	COLO Hyp5%	COLO Hyp12%
	6	0	0.5	10	0.5	0
	6.5	0	2	10	1.5	0.5
	7.5	0	0.5	10	1	0
AVE	6.67	0	1	10	1	0.17
St Dev	0.76	0	0.87	0	0.5	0.29
t-test	0.003	NA	0.22	0.0001	0.07	0.50

HT29	HT29 Chlor	HT29 Starv	HT29 DXT	HT29 Hyp<1%	HT29 Hyp5%	HT29 Hyp12%
	6.5	1.5	3	6	1.5	0.5
	6.5	1.5	3.5	9	1	1
	6.5	0	3	1.5	2	1.5
AVE	6.5	1	3.17	5.5	1.5	1.0
St Dev	0	0.87	0.29	3.77	0.5	0.5
t-test	0.0003	1	0.05	0.28	0.59	0.17

LOVO	LOVO Chlor	LOVO Starv	LOVO DXT	LOVO Hyp<1%	LOVO Hyp5%	LOVO Hyp12%
	9.5	7	10	8	9	8.5
	10	9	10	8	9	8
	10	9	9	7.5	8.5	9
AVE	9.83	8.33	9.67	7.83	8.83	8.5
St Dev	0.29	1.15	0.58	0.29	0.29	0.5
t-test	0.22	0.50	0.72	0.24	0.36	0.59

Cytotoxic Drugs: 5-Fluorouracil, Methotrexate, Taxol, Etoposide, Vincristine

COLO	COLO 5FU	COLO Mtx	COLO Tax	COLO Etop	COLO Vinc
	0	**0**	**0**	**0**	**0**
	0	**0**	**1**	**0**	**0**
	0	**0**	**0**	**0**	**0**
AVE	**0**	**0**	**0.33**	**0**	**0**
St Dev	**0**	**0**	**0.58**	**0**	**0**
t-test	**NA**	**NA**	**0.50**	**NA**	**NA**

HT29	HT29 5FU	HT29 Mtx	HT29 Tax	HT29 Etop	HT29 Vinc
	1.5	**3.5**	**4**	**1**	**2**
	2.5	**4**	**4**	**1**	**1**
	3	**5.5**	**5.5**	**0.5**	**2**
AVE	**2.33**	**4.33**	**4.5**	**0.83**	**1.67**
St Dev	**0.76**	**1.04**	**0.87**	**0.29**	**0.58**
t-test	**0.10**	**0.12**	**0.05**	**0.79**	**0.50**

LOVO	LOVO 5FU	LOVO Mtx	LOVO Tax	LOVO Etop	LOVO Vinc
	8	**8**	**9**	**7**	**9.5**
	9	**8.5**	**8.5**	**7.5**	**9**
	10	**10**	**9**	**7**	**8.5**
AVE	**9**	**8.83**	**8.83**	**7.17**	**9**
St Dev	**1**	**1.04**	**0.29**	**0.29**	**0.5**
t-test	**0.04**	**0.92**	**0.03**	**0..3**	**1**

Combinations of factors:

BUTYRIC ACID & THEOPHYLLINE, 5-AZACYTIDINE OR INTERFERON:

	COLO Ba+Th	COLO Ba+5aza	COLO Ba+Ifn	HT29 Ba+Th	HT29 Ba+5aza	HT29 Ba+Ifn	LOVO Ba+Th	LOVO Ba+5aza	LOVO Ba+Ifn
	8	**7.5**	**1**	**4**	**3.5**	**6.5**	**8.5**	**8.5**	**9**
	8	**8**	**0**	**6**	**4.5**	**8.5**	**8**	**8**	**9.5**
	9	**8**	**0**	**5**	**3**	**9**	**9.5**	**7**	**10**
AVE	**8.33**	**7.83**	**0.33**	**5**	**3.67**	**8**	**8.67**	**7.83**	**9.5**
St Dev	**0.58**	**0.29**	**0.58**	**1**	**0.76**	**1.32**	**0.76**	**0.76**	**0.5**
t-test	**0.0004**	**0.0007**	**0.78**	**0.06**	**0.13**	**0.008**	**0.10**	**0.40**	**0.007**

INTERFERON & THEOPHYLLINE OR 5-AZACYTIDINE:

	COLO Ifn+Th	COLO Ifn+5az	HT29 Ifn+Th	HT29 Ifn+5aza	LOVO Ifn+Th	LOVO Ifn+5aza
	0	**2.5**	**3.5**	**4**	**9**	**9**
	1.5	**2.5**	**2**	**2**	**9.5**	**9**
	0	**0**	**2**	**1**	**9**	**9**
AVE	**0.5**	**1.67**	**2.5**	**2.33**	**9.17**	**9**
St Dev	**0.87**	**1.44**	**0.87**	**1.53**	**0.29**	**0**
t-test	**0.50**	**0.22**	**0.22**	**0.52**	**0.009**	**0.009**

5-FLUOROURACIL & BUTYRIC ACID, THEOPHYLLINE OF 5-AZACYTIDINE:

	COLO 5Fu+Ba	COLO 5Fu+Th	COLO 5Fu+5Az	HT29 5Fu+Ba	HT29 5Fu+Th	HT29 5Fu+5Az	LOVO 5Fu+Ba	LOVO 5Fu+Th	LOVO 5Fu+5Az
	7	**10**	**9**	**7**	**4.5**	**2.5**	**10**	**9.5**	**9**
	1.5	**10**	**6.5**	**7.5**	**3.5**	**1.5**	**10**	**10**	**9**
	10	**6**	**5.5**	**7**	**7**	**2**	**10**	**9**	**9.5**
AVE	**6.17**	**8.67**	**7**	**7.17**	**5**	**2.0**	**10**	**9.35**	**9.17**
St Dv	**4.31**	**2.31**	**1.8**	**0.29**	**1.8**	**0.5**	**0**	**0.5**	**0.29**
t-test	**0.15**	**0.02**	**0.01**	**0.0001**	**0.08**	**0.27**	**0.12**	**0.16**	**0.19**

METHOTREXATE & BUTYRIC ACID, THEOPHYLLINE OR 5-AZACYTIDINE:

	COLO Mtx+Ba	COLO Mtx+Th	COLO Mtx+5az	HT29 Mtx+Ba	HT29 Mtx+Th	HT29 Mtx+5az	LOVO Mtx+Ba	LOVO Mtx+Th	LOVO Mtx+5a
	0	**0**	**0**	**7.5**	**2**	**4.5**	**10**	**8.5**	**8.5**
	0	**0.5**	**0.5**	**7**	**7.5**	**4**	**10**	**7.5**	**9.5**
	6.5	**0**	**0**	**5**	**7.5**	**3**	**10**	**9**	**9**
AVE	**2.17**	**0.17**	**0.17**	**6.5**	**5.67**	**3.83**	**10**	**8.33**	**9**
St Dev	**3.75**	**0.29**	**0.29**	**1.33**	**3.17**	**0.76**	**0**	**0.76**	**0.5**
t-test	**0.60**	**0.50**	**0.50**	**0.03**	**0.27**	**0.10**	**0.0008**	**0.10**	**0.01**

INTERFERON & 5-FLUOROURACIL OR METHOTREXATE:

	COLO Ifn+5Fu	COLO Ifn+Mtx	HT29 Ifn+5Fu	HT29 Ifn+Mtx	LOVO Ifn+5Fu	LOVO Ifn+Mtx
	1.5	**1.5**	**8**	**8.5**	**8.5**	**10**
	1.5	**1**	**7**	**7.5**	**9.5**	**10**
	0	**1**	**8**	**7**	**9.5**	**9**
AVE	**1.0**	**1.17**	**7.67**	**7.67**	**9.17**	**9.67**
St Dev	**0.87**	**0.29**	**0.58**	**0.76**	**0.58**	**0.58**
t-test	**0.55**	**0.05**	**0.001**	**0.002**	**0.03**	**0.006**

BUTYRIC ACID & HYPOXIA (1%OXYGEN), ALKALINE MEDIUM OR CHLOROQUINE:

	COLO Ba+Hyp	COLO Ba+Alk	COLO Ba+Chl	HT29 Ba+Hyp	HT29 Ba+Alk	HT29 Ba+Chl	LOVO Ba+Hyp	LOVO Ba+Alk	LOVO Ba+Chl
	1	**.5**	**8**	**1.5**	**2.5**	**9**	**9**	**8.5**	**10**
	0	**0**	**7**	**1.5**	**1**	**7**	**9**	**9**	**10**
	1	**0**	**7**	**1**	**1.5**	**5.5**	**5.5**	**9**	**10**
AVE	**0.67**	**0.17**	**7.33**	**1.33**	**1.67**	**7.17**	**7.83**	**8.83**	**10**
St Dev	**0.58**	**0.29**	**0.58**	**0.29**	**0.76**	**1.76**	**2.02**	**0.29**	**0**
t-test	**0.22**	**0.50**	**0.0004**	**0.79**	**0.54**	**0.07**	**0.42**	**0.24**	**0.03**

CHLOROQUINE + 5-FLUOROURACIL OR METHOTREXATE:

	COLO Chl+5FU	COLO Chl+Mtx	HT29 Chl+5FU	HT29 Chl+Mtx	LOVO Chl+5FU	LOVO Chl+Mtx
	2.5	**1.5**	**8**	**7.5**	**9.5**	**9.5**
	5	**5**	**6.5**	**5**	**10**	**10**
	5	**4**	**6.5**	**6.5**	**10**	**10**
AVE	**4.17**	**3.5**	**7**	**6.33**	**9.83**	**9.83**
St Dev	**1.44**	**1.8**	**0.87**	**1.26**	**0.29**	**0.29**
t-test	**0.03**	**0.08**	**0.02**	**0.07**	**0.13**	**0.13**

c: FACS Results

DIFFERENTIATING AGENTS:

FACS Results:

	Lovo Surface	Lovo Total	HT29 Surface	HT29 Total	Colo Surface	Colo Total
Control 1	**435.66**	**331.71**	**14.90**	**32.35**	**6.02**	**16.34**
Control 2	**390.27**	**326.15**	**18.39**	**30.68**	**7.76**	**17.12**
But Acid 1	**754.75**	**613.04**	**46.25**	**118.80**	**9.20**	**38.64**
But Acid 2	**963.97**	**1781.94**	**49.98**	**102.95**	**13.68**	**32.50**
But Acid 3	**594.91**	**2115.88**	**39.42**	**93.93**	**11.61**	**29.61**
Neg Control	**23.52**	**35.68**	**15.89**	**26.38**	**17.12**	**21.29**
Theo 1	**686.60**	**338.47**	**30.16**	**65.88**	**91.34**	**70.93**
Theo 2	**451.47**	**878.45**	**33.14**	**79.03**	**72.57**	**67.87**
Theo 3	**587.74**	**596.76**	**28.27**	**58.59**	**21.95**	**66.11**
Neg Control	**23.52**	**35.68**	**15.89**	**26.38**	**17.12**	**21.29**
5 Aza 1	**239.27**	**143.06**	**30.88**	**41.26**	**72.26**	**66.90**
5 Aza 2	**199.30**	**202.69**	**34.59**	**40.79**	**46.96**	**72.02**
5 Aza 3	**277.13**	**219.95**	**22.84**	**40.17**	**116.62**	**62.96**
Neg Control	**23.52**	**35.68**	**15.89**	**26.38**	**17.12**	**21.29**

:

	Lovo Surface	Lovo Total	HT29 Surface	HT29 Total	Colo Surface	Colo Total
Control 1	**717.25**	**863.06**	**21.21**	**35.28**	**19.23**	**39.17**
Control 2	**632.97**	**805.54**	**18.87**	**52.26**	**6.22**	**22.27**
IFN 1	**757.46**	**1085.24**	**30.79**	**71.22**	**11.95**	**36.09**
IFN 2	**819.36**	**1206.14**	**31.77**	**51.65**	**18.51**	**24.09**
IFN 3	**845.39**	**916.15**	**36.80**	**83.44**	**9.30**	**26.87**
Neg Control	**73.35**	**59.47**	**7.74**	**10.72**	**9.30**	**7.47**

ENVIRONMENTAL FACTORS:

	Lovo Surface	Lovo Total	HT29 Surface	HT29 Total	Colo Surface	Colo Total
Control 1	**841.39**	**564.96**	**17.48**	**16.56**	**17.3**	**12.99**
Control 2	**718.95**	**390.44**	**15.64**	**16.17**	**18.74**	**25.25**
Chlor 1	**1754.34**	**1236.05**	**83.29**	**78.56**	**57.71**	**52.22**
Chlor 2	**1503.58**	**1517.69**	**53.23**	**89.67**	**62.26**	**91.58**
Chlor 3	**890.95**	**1005.39**	**53.58**	**75.42**	**60.29**	**75.59**
Neg Control	**57.07**	**22.32**	**15.83**	**12.42**	**12.49**	**35.1**
Control 1	**325.77**	**369.46**	**15.11**	**34.38**	**80.38**	**86.53**
Control 2	**248.41**	**337.19**	**16.77**	**35.76**	**11.81**	**34.19**
Starved 1	**223.05**	**364.12**	**16.14**	**53.82**	**11.98**	**20.85**
Starved 2	**308.31**	**298.64**	**15.71**	**29.53**	**8.42**	**26.54**
Starved 3	**230.19**	**275.27**	**18.58**	**27.55**	**21.45**	**35.79**
Neg Control	**18.08**	**25.13**	**18.32**	**15.05**	**107.35**	**59.79**

	Lovo Surface	Lovo Total	HT29 Surface	HT29 Total	Colo Surface	Colo Total
Control 1	**380.63**	**379.59**	**20.63**	**28.51**	**7.81**	**15.06**
Control 2	**423.93**	**160.36**	**21.61**	**22.65**	**11.57**	**12.80**
Hyp1% 1	**118.98**	**221.08**	**81.29**	**74.17**	**81.77**	**104.44**
Hyp1% 2	**228.16**	**362.16**	**73.55**	**128.43**	**67.11**	**96.74**
Hyp1% 3	**141.13**	**229.83**	**86.00**	**130.59**	**76.36**	**106.58**
Neg Control	**36.02**	**16.10**	**31.26**	**14.68**	**12.63**	**15.97**
Control 1	**73.94**	**208.01**	**22.66**	**49.01**	**21.24**	**24.30**
Control 2	**173.44**	**204.61**	**27.56**	**21.82**	**34.73**	**23.86**
Hyp5% 1	**212.65**	**222.44**	**29.36**	**33.67**	**224.41**	**94.10**
Hyp5% 2	**213.29**	**235.59**	**21.73**	**44.55**	**151.65**	**51.32**
Hyp5% 3	**221.94**	**311.87**	**24.58**	**24.01**	**29.04**	**41.40**
Hyp12% 1	**212.65**	**368.95**	**26.37**	**27.74**	**139.13**	**117.72**
Hyp12% 2	**200.40**	**270.56**	**18.02**	**32.17**	**15.90**	**18.54**
Hyp12% 3	**211.59**	**185.30**	**13.99**	**25.87**	**16.90**	**22.24**
Neg Control	**92.69**	**121.06**	**21.09**	**27.53**	**26.27**	**23.15**
Control 1	**188.33**	**308.79**	**26.89**	**19.32**	**34.96**	**53.92**
Control 2	**182.28**	**216.61**	**28.58**	**19.63**	**34.58**	**53.87**
DXT 1	**234.41**	**336.18**	**28.49**	**25.39**	**15.92**	**24.77**
DXT 2	**166.66**	**400.66**	**28.93**	**29.15**	**15.59**	**26.84**
DXT 3	**223.22**	**475.51**	**28.40**	**27.35**	**15.65**	**23.81**
Neg Control	**69.81**	**58.36**	**20.91**	**34.04**	**25.80**	**42.91**

CYTOTOXIC DRUGS:

	Lovo Surface	Lovo Total	HT29 Surface	HT29 Total	Colo Surface	Colo Total
Control 1	**291.76**	**158.34**	**18.30**	**18.90**	**14.78**	**35.27**
Control 2	**245.17**	**364.95**	**24.02**	**25.90**	**28.37**	**31.01**
5FU 1	**488.22**	**134.59**	**62.67**	**73.43**	**29.19**	**17.79**
5FU 2	**602.51**	**911.09**	**58.62**	**66.43**	**14.44**	**20.66**
5FU 3	**406.50**	**863.63**	**47.91**	**66.37**	**10.66**	**20.11**
Neg Control	**42.14**	**30.22**	**11.71**	**14.35**	**7.01**	**23.51**
Control 1	**258.22**	**142.47**	**34.35**	**61.99**	**10.53**	**17.75**
Control 2	**325.37**	**136.77**	**26.05**	**34.05**	**14.67**	**16.81**
Mtx 1	**925.87**	**297.04**	**44.84**	**44.94**	**34.45**	**34.03**
Mtx 2	**761.09**	**355.43**	**54.92**	**43.87**	**32.20**	**42.93**
Mtx 3	**662.78**	**349.59**	**37.46**	**32.16**	**28.75**	**41.02**
Neg Control	**38.74**	**36.63**	**43.23**	**44.06**	**17.35**	**25.70**
Control 1	**407.53**	**608.80**	**27.29**	**39.73**	**18.32**	**29.95**
Control 2	**424.58**	**582.57**	**25.44**	**39.85**	**21.04**	**25.81**
Control 3	**329.11**	**606.21**				
Control 4	**439.81**	**734.44**				
Taxol 1	**843.62**	**1552.97**	**72.61**	**94.34**	**21.55**	**29.51**
Taxol 2	**828.10**	**1244.46**	**80.26**	**81.88**	**22.30**	**30.34**
Taxol 3	**899.28**	**1325.72**	**69.53**	**88.51**	**21.72**	**31.02**
Neg Control	**38.36**	**42.56**	**21.07**	**34.26**	**25.51**	**25.65**

	Lovo Surface	Lovo Total	HT29 Surface	HT29 Total	Colo Surface	Colo Total
Control 1	**717.25**	**863.06**	**21.21**	**35.28**	**19.23**	**39.17**
Control 2	**632.97**	**805.54**	**18.87**	**52.26**	**6.22**	**22.27**
Etop 1	**692.45**	**756.79**	**12.39**	**36.97**	**13.91**	**29.26**
Etop 2	**594.62**	**704.88**	**15.83**	**37.58**	**7.51**	**32.90**
Etop 3	**681.61**	**796.98**	**15.94**	**71.22**	**9.58**	**26.80**
Neg Control	**73.35**	**59.47**	**7.74**	**10.72**	**9.30**	**7.47**
Control 1	**670.55**	**380.75**	**26.16**	**36.18**	**6.98**	**16.76**
Control 2	**701.38**	**340.24**	**17.59**	**26.33**	**6.42**	**31.93**
Vinc 1	**1082.17**	**364.35**	**19.31**	**26.71**	**6.53**	**17.41**
Vinc 2	**562.41**	**468.88**	**23.47**	**22.77**	**5.44**	**23.92**
Vinc 3	**565.11**	**257.06**	**22.47**	**34.41**	**9.37**	**15.62**
Neg Control	**47.11**	**49.26**	**21.93**	**28.79**	**6.81**	**17.05**

COMBINATIONS OF FACTORS:

	Lovo Surface	Lovo Total	HT29 Surface	HT29 Total	Colo Surface	Colo Total
Control 1	**209.64**	**96.73**	**22.66**	**49.01**	**21.24**	**24.3**
Control 2	**193.02**	**60.15**	**27.56**	**21.82**	**34.73**	**23.86**
Control 3	**152.31**	**168.95**				
Control 4	**224.34**	**169.75**				
Ba+Th 1	**423.34**	**100.16**	**31.81**	**17.76**	**99.62**	**167.25**
Ba+Th 2	**285.22**	**62.16**	**28.69**	**46.91**	**257.59**	**204.42**
Ba+Th 3	**458.36**	**111.31**	**88.48**	**30.65**	**105.48**	**76.85**
Ba+5aza 1	**347.32**	**118.84**	**20.47**	**33.67**	**94.90**	**68.55**
Ba+5aza 2	**316.48**	**89.63**	**25.18**	**44.55**	**55.50**	**80.51**
Ba+5aza 3	**656.45**	**235.00**	**22.11**	**24.01**	**83.47**	**32.49**
Neg Control	**28.07**	**28.56**	**21.09**	**27.53**	**26.27**	**23.15**
Control 1	**407.53**	**606.80**	**34.84**	**59.97**	**4.66**	**23.65**
Control 2	**424.58**	**582.57**	**34.74**	**59.87**	**9.92**	**27.95**
Control 3	**329.11**	**606.21**				
Control 4	**439.81**	**734.44**				
Ba+IFN 1	**1832.58**	**2701.88**	**111.07**	**183.27**	**39.88**	**29.45**
Ba+IFN 2	**1666.02**	**3051.52**	**137.61**	**269.11**	**82.93**	**108.07**
Ba+IFN 3	**1556.11**	**3075.25**	**82.67**	**262.02**	**13.31**	**30.88**
Neg Control	**38.36**	**42.56**	**44.76**	**32.64**	**17.86**	**23.83**

	Lovo Surface	Lovo Total	HT29 Surface	HT29 Total	Colo Surface	Colo Total
Control 1	**407.53**	**608.80**	**16.78**	**16.71**	**10.16**	**8.73**
Control 2	**424.58**	**582.57**	**22.87**	**32.57**	**8.56**	**13.26**
Control 3	**329.11**	**606.21**				
Control 4	**439.81**	**734.44**				
Ifn+Th 1	**422.56**	**736.99**	**13.90**	**16.35**	**49.52**	**58.66**
Ifn+Th 2	**676.89**	**1105.63**	**14.81**	**15.77**	**86.23**	**62.58**
Ifn+Th 3	**633.48**	**993.90**	**21.13**	**18.12**	**58.45**	**37.24**
Ifn+5aza 1	**1023.58**	**1636.56**	**19.84**	**15.75**	**70.27**	**76.76**
Ifn+5aza 2	**916.99**	**1537.32**	**13.36**	**14.24**	**89.64**	**52.12**
Ifn+5aza 3	**962.62**	**1533.93**	**13.13**	**17.50**	**55.98**	**55.53**
Neg Control	**38.36**	**42.56**	**21.09**	**16.43**	**8.82**	**11.95**
Control 1	**97.88**	**237.88**	**27.01**	**41.96**	**24.63**	**37.99**
Control 2	**95.28**	**299.7**	**24.72**	**37.14**	**20.55**	**39.64**
5FU+Ba 1	**339.7**	**968.24**	**60.06**	**97.29**	**52.98**	**43.35**
5FU+Ba 2	**316.53**	**911.99**	**57.88**	**89.86**	**70.41**	**92.00**
5FU+Ba 3	**387.87**	**1109.30**	**75.94**	**92.98**	**49.51**	**98.52**
5FU+Th 1	**538.21**	**847.37**	**22.44**	**43.96**	**92.31**	**101.81**
5FU+Th 2	**442.93**	**918.17**	**22.59**	**43.98**	**82.33**	**184.89**
5FU+Th 3	**343.41**	**839.97**	**22.38**	**48.46**	**78.34**	**74.33**
5FU+5aza 1	**137.33**	**139.10**	**25.88**	**45.14**	**105.85**	**153.92**
5FU+5aza 2	**195.95**	**226.83**	**31.77**	**51.19**	**129.62**	**108.30**
5FU+5aza 3	**181.90**	**242.28**	**23.67**	**38.14**	**70.69**	**85.09**
Neg Control	**20.40**	**28.16**	**35.62**	**51.23**	**29.36**	**38.58**

	Lovo Surface	Lovo Total	HT29 Surface	HT29 Total	Colo Surface	Colo Total
Control 1	**209.64**	**96.73**	**24.00**	**28.20**	**11.43**	**16.19**
Control 2	**152.31**	**60.15**	**21.52**	**32.48**	**8.29**	**12.07**
Control 3	**193.02**	**168.95**				
Control 4	**224.34**	**169.95**				
Mtx+Ba 1	**935.93**	**1232.14**	**84.10**	**87.07**	**15.31**	**15.80**
Mtx+Ba 2	**819.03**	**1240.05**	**101.17**	**100.25**	**11.00**	**13.52**
Mtx+Ba 3	**838.94**	**1285.01**	**76.54**	**107.47**	**18.58**	**26.94**
Mtx+Th 1	**526.52**	**541.72**	**53.57**	**81.21**	**34.62**	**34.19**
Mtx+Th 2	**529.57**	**949.96**	**56.82**	**64.80**	**27.25**	**59.31**
Mtx+Th 3	**416.34**	**309.08**	**58.38**	**72.81**	**29.43**	**69.50**
Mtx+5aza 1	**555.12**	**324.89**	**38.14**	**42.84**	**33.72**	**37.94**
Mtx+5aza 2	**532.50**	**317.59**	**36.36**	**45.69**	**45.76**	**39.30**
Mtx+5aza 3	**501.21**	**322.15**	**60.69**	**70.94**	**20.23**	**26.94**
Neg Control	**28.07**	**28.56**	**21.34**	**30.12**	**9.11**	**23.18**

	Lovo Surface	Lovo Total	HT29 Surface	HT29 Total	Colo Surface	Colo Total
Control 1	**407.53**	**608.80**	**34.84**	**59.97**	**4.66**	**23.65**
Control 2	**424.58**	**582.57**	**34.74**	**59.87**	**9.92**	**27.95**
Control 3	**329.11**	**606.21**				
Control 4	**439.81**	**734.44**				
Ifn+5FU 1	**529.67**	**734.86**	**119.5**	**192.51**	**9.22**	**29.14**
Ifn+5FU 2	**672.51**	**1246.89**	**106.72**	**251.26**	**9.86**	**27.92**
Ifn+5FU 3	**774.64**	**1148.94**	**98.63**	**295.26**	**12.17**	**34.64**
Ifn+Mtx 1	**890.48**	**1290.93**	**150.72**	**222.46**	**11.11**	**25.63**
Ifn+Mtx 2	**685.62**	**855.14**	**98.65**	**204.62**	**14.36**	**39.03**
Ifn+Mtx 3	**433.92**	**704.93**	**162.27**	**192.14**	**9.53**	**34.55**
Neg Control	**38.36**	**42.56**	**44.76**	**32.64**	**17.86**	**23.83**
Control 1	**243.68**	**473.84**	**23.45**	**37.55**	**12.32**	**16.28**
Control 2	**179.13**	**529.15**	**17.57**	**31.84**	**14.79**	**24.43**
Ba+Hyp 1	**698.92**	**474.08**	**89.56**	**118.71**	**19.53**	**31.92**
Ba+Hyp 2	**34.26**	**319.31**	**58.41**	**90.58**	**21.52**	**42.62**
Ba+Hyp 3	**150.37**	**345.44**	**42.06**	**80.20**	**27.63**	**98.52**
Ba+Alk 1	**305.59**	**546.96**	**18.97**	**30.23**	**10.93**	**23.11**
Ba+Alk 2	**313.53**	**361.96**	**16.80**	**26.26**	**8.41**	**14.86**
Ba+Alk 3	**325.37**	**699.04**	**15.19**	**35.69**	**8.69**	**18.36**
Neg Control	**33.25**	**49.92**	**18.56**	**32.12**	**8.86**	**16.16**

	Lovo Surface	Lovo Total	HT29 Surface	HT29 Total	Colo Surface	Colo Total
Control 1	**375.88**	**457.94**	**17.96**	**23.94**	**13.05**	**23.42**
Control 2	**560.07**	**672.20**	**22.58**	**30.33**	**15.73**	**21.38**
Ba+Chlor 1	**870.97**	**1624.31**	**88.44**	**68.25**	**102.89**	**76.64**
Ba+Chlor 2	**349.64**	**348.17**	**102.34**	**50.44**	**129.04**	**131.77**
Ba+Chlor 3	**783.22**	**625.38**	**57.50**	**62.94**	**54.68**	**68.33**
Neg Control	**26.36**	**35.02**	**16.44**	**19.20**	**9.18**	**24.76**
Control 1	**375.88**	**457.94**	**17.96**	**23.94**	**13.05**	**23.42**
Control 2	**560.07**	**672.20**	**22.58**	**30.33**	**15.73**	**21.38**
Chlr+5FU1	**425.03**	**217.22**	**61.46**	**36.37**	**19.29**	**38.20**
Chlr+5FU2	**351.03**	**245.42**	**57.88**	**63.19**	**21.46**	**30.90**
Chlr+5FU3	**356.28**	**316.74**	**54.65**	**59.48**	**20.49**	**29.90**
Chlr+Mtx1	**461.65**	**839.42**	**25.33**	**50.07**	**30.98**	**34.95**
Chlr+Mtx2	**299.19**	**568.39**	**69.97**	**57.02**	**149.26**	**212.25**
Chlr+Mtx3	**326.14**	**939.70**	**55.46**	**61.30**	**60.71**	**74.03**
Neg Control	**26.36**	**35.02**	**16.44**	**19.20**	**9.18**	**24.76**

APPENDIX 3: ERRORS

Cell Culture

The process of defrosting cancer cells and growing them on is a delicate one, with much scope for variability in the growth rates and health of the cells. Different passages of cells may be expected to behave differently from others, since effectively they should be an increasingly selected population, with only the more resistant cells surviving the rigours of repeated trypsinisation and centrifuging. Additionally, new batches of Whole Medium were being made up every 10 days, each potentially slightly different from the last.

In attempting to create as uniform a system of cell culture as possible, cells were not used beyond 10 passages; conditions in the incubator and hood were unchanging; and feeding of the cells was performed according to their confluence state or when their supernatant medium began to lighten in colour. Wherever possible, one batch of Whole Medium was used throughout each experiment. When comparing proliferation between control and experiment cells, only the flasks grown simultaneously from the same parent cells were counted.

Harvesting

Trypsin-EDTA was used for all the cells, to separate them from the tissue culture flasks. It should be explained that the live, growing cells attached themselves to the plastic base of the flasks – dead cells were unable to adhere and were sloughed off into the medium, which was subsequently removed. The cells harvested from the flasks by the Trypsin were therefore regarded as a viable growing population of cells. Trypsin has been shown to damage cells and indeed to reduce the amount of CEA activity in Lovo cells by up to 55% (*80*). A consistent method of trypsinisation was used for all the cells, and a crude measure of cell damage which occurred was provided within the Trypan Blue cell counting method, which showed dead cells clearly as bright blue on phase microscopy. The proportion of dead cells in the harvested population was less than 10% in each experiment.

Cell Counting

The accuracy of the Hemocytometer cell counting was reduced when the cell numbers fell to 0.1 x 106 cells per ml; this was the lowest number of cells which could be visualised. The results reported did not quantify the degrees of growth inhibition observed in any experiment, but merely the presence of significant growth inhibition. More sensitive methods to measure proliferation rates than cell counting could be employed if quantifying growth inhibitory effects was deemed necessary; cell counting was sufficient to demonstrate differences between control and experiment cell proliferation in this work.

Immunocytochemistry

Within the method for Immunocytochemistry are a number of measures to prevent or counteract potential errors in the staining and grading processes:

1.The antibodies were applied in solutions containing animal or human serum to provide a sink for endogenous cell peroxidase, which could otherwise cause background staining.

2. Antibodies were obtained from the same sources for each experiment.

3. Each run of immunostains for each cell line was checked against its own set of negative controls:

(a) No primary antibody

(b) No primary and no secondary antibody

(c) Isotype-specific mouse IgG1 antibody

4. Grading of the slides was all performed blind and in duplicate, by the same observer.

Fluorescein Activated Cell Sorting

As in the immunocytochemistry, mouse IgG1 isotype-specific negative controls were scanned for each cell line and each experiment.

Permeabolising the cells to measure the amount of total cell CEA is likely to cause some damage to the cells. The comparison of membrane and total CEA values in four of the experiments on Lovo cells showed significant falls in CEA levels in the permeabolised cells.

The experiment total CEA values were compared to a large number of permeabolised control cells of each cell line to isolate the effects of the tested agents from those of the Permeafix. It is recognised that the experiment cells may have been more sensitive to the Permeafix than the control cells because of the toxic effects of the experiment agents.

The number of cells analysed through the FACS scanner varied from 1000 to 3000 within each cell sample, because of the sparsity of cells in some of the experiment groups. The lowest numbers of cells still provided a normal distribution on the single dot histograms, with the Ht29 and Colo cells noted to show considerably tighter normal distributions than the Lovo cells.

Radioimmunoassay

The main potential for errors in the Radioimmunoassay occurred in the hand pipetting of the samples into the ELSA-tubes. The 4 graphs of the CEA standards which were pipetted in the same way, at the same time, by the same person, showed correlations of 0.97 - 1.04, which would indicate an acceptable level of accuracy.

Statistics

Measures to prevent errors have been described in the methodology in each chapter. It is recognised however, that Type 1 (false positive) and Type 2 (false negative) results may occur when the number of factors being tested is high. The statistical analysis used indicates positivity at $p<0.05$, implying that the result is 95% likely to be positive, with a 5% error margin. It might be expected that if more than twenty factors are studied, one result would fall into this 5% margin, but the logistical necessity of testing the various factors metachronously should combat this possible source of error.

APPENDIX FOUR: STATISTICS

COMPARISON OF TWO POPULATIONS

Replicated samples of cells which were exposed to various differentiating and cytotoxic agents and to alterations in their environment were compared to replicated control samples grown in conventional culture conditions. The parameters measured, or the **response variable**, from these two kinds of populations of cells, control and experimental, i.e. their growth rates and degree of CEA expression or release were then analysed to test for an observable differences in the populations. Significant differences between the control and experiment population would be surmised to have arisen because of the specific change in culture conditions applied.

The principle features of the response variables which were considered were:

1. Measure of Location : the means and medians.
2. Measure of Spread: the standard deviation
3. Shape of Distribution: normal or skewed.

The mean is calculated by dividing the sum of all of the sample variables by the number of samples.

The median is the mid-point sample in a range of samples variables.

The standard deviation is a measure of the spread of sample variables around the mean, and is calculated by

$$\sqrt{\frac{\mathbf{n\Sigma x^2 - (\Sigma x)^2}}{\mathbf{n(n-1)}}}$$

A distribution is taken to be "Normal" if 66% of the samples lie within one standard deviation of the mean, 95% of the variables within two standard deviations and 99% within three standard deviations. If populations of data are normally distributed, it may be analysed using parametric tests, such as the

t-test. If the data distributions are not normal, a non-parametric test such as the Wilcoxon Rank Sum test should be used for analysis.

T-test

The t-test is used to test whether there is a significant difference between the population means of two populations, using the following algebraic formula:

$$T = \frac{x_1 - x_2}{\sqrt{\frac{sp^2}{n_1} + \frac{sp^2}{n_2}}}$$

where x1 and x2 are the two sample means, n1 and n2 are the two sample sizes and s_p^2 is the pooled variance, calculated using the formula:

$$s_p^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2}$$

where s_1^2 and s_2^2 are the squares of the two standard deviations.

Populations with equal variance (taken as ± 100%) are distinguished from those with unequal variance.

Mann-Whitney-U Test

The Mann Whitney –U test describes the probability that a new experimental observation will have a rank lower than a new control observation. (The null hypothesis is that there is no difference in the ranks of experimental and control observations.) The test is performed by assigning all control and experimental observations a rank, grouping the samples from the two populations together and

ranking them upwards in numerical order, the lowest rank being one. Equal values receive equal proportions of n successive ranks.

The value U is calculated by the following equation, and then checked against tabulated values according to the sample sizes, to ascertain the p-value, or the probability of the null hypothesis being correct.

$$\mathbf{U = n_1 n_2 + 1/2 n_1 (n_1+1) - T}$$

Where n_1 is the number of observations in the experiment group, n_2 is the number of observations in the control group, and T is the sum of ranks in the smaller group.

The data from the immunocytochemstry and FACS analyses for CEA expression represent repeated examples of the same experimental process, and would therefore be expected to follow a normal distribution. The t-test is appropriate to use in looking for differences between two such populations of data; however, the small size of the populations compromises the accuracy of the t-test. The Mann Whitney-U test was therefore performed in analysing the FACS results to improve accuracy. In all tests, differences between control and experimental groups which showed significance at $p \leq 0.05$ were taken as positive results.

APPENDIX FIVE

Published work from this thesis:

Page

1. Abstract for Poster Presentation (1) **229**

   British Oncological Association Conference, St Andrews 1996

2. Abstract for Poster Presentation (2) **230**

   British Oncological Association Conference, St Andrews 1996

2. Abstract for Oral Presentation **231**

   British Association of Surgical Oncology, London RCS, 1997

DIFFERING PATTERN OF IN VITRO CEA EXPRESSION AND SUPERNATANT RELEASE BY AUGMENTED COLORECTAL CANCER CELLS

M.H.S. COLLIE; J. BHATIA; R. AUSTEN; M. C WINSLET, Dept of Surgery, Royal Free Hospital, Pond St, London NW12.

Colorectal cancer cells are known to be heterogenous expressors of the tumour associated antigen Carcinoembryonic Antigen (CEA), which may be targetted by antibodies. The efficiency of this antibody guided detection and treatment is compromised by the heterogeneity of expression of the CEA.
Certain chemical agents, cytotoxic drugs and environmental changes have been shown to increase CEA expression in colorectal cancer cells.

In this study, three colorectal cancer cell lines, Lovo,HT29 and Colo, known to be high, low and non-expressors of CEA were grown in monolayer cell culture for five days in the presence of a differentiating agent (Butyric Acid, Theophylline, 5-Azactidine or Interferon), or with altered environment (Acid medium, alkaline medium, starvation or hypoxia)or with 5Fluorouracil. The cells were then immunostained and their CEA expression measured by Fluorescein Activated Cell Sorting. Radioimmunoassay of the supernatants from the culture flasks was performed to measure CEA released.

Significant changes in CEA expression and release as analysed by the Wilcoxon Rank Sum test were as follows:

	Starve	Acid	Alkali	Hypox	ButAc	Theop	5-Azac	IFN	5FU
Colo				**Exp ↑**		**Exp ↑**	**Exp ↑**		
HT29	**Rel ↑**	**Exp ↑**		**Exp ↑**	**Exp ↑**	**Exp ↑**	**Exp ↑**	**Exp ↑**	**Exp ↑** **Rel ↑**
Lovo	**Exp ↓** **Rel ↓**	**Exp ↓** **Rel ↓**	**Exp ↓** **Rel ↓**		**Exp ↑**	**Rel ↓**	**Exp ↓** **Rel ↓**	**Exp ↑** **Rel ↓**	**Exp ↑**

The changes in CEA expression were not mirrored by similar or reciprocal changes in supernatant release, although results were influenced by natural expression levels.

In vitro, it is concluded that chemical agents or environmental changes which lead to augmented CEA expression by colorectal cancer cells do not cause matching increases in CEA supernatant release. If the same mechanism applies in vivo, augmentation may not necessarily increase serum CEA levels, with resultant high non-specific antibody binding and background noise.

GROSS VARIATION IN CEA EXPRESSION AUGMENTATION BY DIFFERENTIATING AGENTS

M.H.S.COLLIE, N.J.BRADLEY, M.C.WINSLET, Dept of Surgery, Royal Free Hospital, Pond St., London NW12.

Colorectal cancer cells are heterogenous expressors of the tumour associated antigen Carcinoembryonic Antigen (CEA). It is possible to target CEA on tumour cells in vivo with anti-CEA antibodies, conjugated to radioisotopes or cytotoxic drugs for the purposes of detection and treatment. The efficiency of this antibody guided detection is compromised by the heterogeneity of expression of the CEA.

This study compared the effects of 4 differentiating agents, (Butyric Acid, γ-Interferon, Theophylline and 5-Azacytidine) on 3 colorectal cancer cell lines, Lovo, HT29 and Colo, known to be high, low and non- expressors of CEA respectively. The cells were grown in standard tissue culture with one of the agents for 5 days, before being harvested and immunostained for CEA. The degree of CEA expression was analysed by Fluorescein Activated Cell Sorting.

Average Fluorescence of Cells:

	Control	**But Acid**	**Interferon**	**Theoph**	**5-Azacyt**
LOVO	**544.0 ± 156.3**	**768.2 ± 185.6**	**807.4 ± 45.2**	**575.07 ±117.8**	**238.6 ± 28.3**
HT29	**18.3 ± 2.6**	**45.2 ± 5.4**	**33.1 ± 3.2**	**30.9 ± 2.4**	**29.4 ± 6.0**
COLO	**9.8 ± 7.2**	**11.5 ± 2.2**	**13.2 ± 4.7**	**61.9 ± 35.9**	**78.6 ± 35.3**

Different colorectal cancer cell lines may be induced by various differentiating agents to increase their expression of CEA, but they do not appear to share common pathways to CEA expression.
Using a combination of agents may be a more efficacious way of inducing the increased CEA expression of a typical heterogenous colorectal cancer.

DIFFERENTIATING EFFECT OF CHLOROQUINE IN COLORECTAL CANCER CELLS IN VITRO

M.H.S. COLLIE; S. TOFFA; M. C WINSLET, Dept of Surgery, Royal Free Hospital, Pond St, London NW12.

Colorectal cancer cells are known to be heterogenous expressors of the tumour associated antigen Carcinoembryonic Antigen (CEA), which may be targetted by antibodies. The efficiency of this antibody guided detection and treatment is compromised by the heterogeneity of expression of the CEA, which is associated with the degree of differentiation of the cells. Altering pH has been shown to induce differentiation in lymphoblastoid cells (1).

A pH study was devised, using **Chloroquine**. Chloroquine is known to cross biological membranes and to enter lysosomes, where it becomes protonated, is no longer free to traverse the membranes and so accumulates in the lysosome, mopping up Hydrogen ions (2). The cytoplasmic pH being robbed of its hydrogen ions, thereby rises.

Ht29 colorectal cancer cells were grown in standard monolayer culture in medium containing serial dilutions of Chloroquine, from 10^{-3} M to 10^{-9} M. After 5 days, the cells were counted, immunostained for membrane CEA and scanned using Fluorescein Activated Cell Sorting. Marked growth inhibition and increased membrane CEA expression was seen in all samples exposed to Chloroquine at concentrations □ **10^{-5} Molar**.

A further study was performed, with three human colorectal cancer cell lines Lovo, HT29 and Colo (high,low and non-expressors of CEA respectively) grown in standard monolayer cell culture medium containing 10^{-5} M Chloroquine. Total cell CEA was measured in addition to membrane CEA, by permeabolising duplicates of each cell sample.

Results:

The proliferation rate of each cell line was significantly reduced (T-test, $p \leq 0.01$). Membrane and total CEA expression measured by fluorescence on FACS, was increased in all three cells according to the non-parametric Wilcoxon Rank Sum test. Using the T-test, total cell CEA was increased in all three cell lines, and membrane CEA in the Colo cells.

	Lovo Surface	Lovo Total	Ht29 Surface	HT29 Total	Colo Surface	Colo Total
Control Ave	**780.17**	**477.7**	**23.56**	**34.21**	**15.86**	**22.62**
Chlor Ave	**1382.96**	**1253.04**	**63.37**	**81.22**	**60.10**	**73.13**
T-test	**0.17**	**0.06**	**0.03**	**1x10-5**	**3x10-9**	**2x10-7**

Conclusion: Chloroquine is effective as a CEA- inducer in Colorectal cancer cells in vitro at a concentration of 10-5 M., whether they are naturally high, low or non-expressors or CEA. Further studies on cell spheroids and transfected colorectal cancer are needed, before the clinical potential of Chloroquine in tumour targetting can be realised.

References:

1. Bauer G, Hofler P, Zur Hausen H.(1982) Epstein-Barr Virus Induction by a Serum Factor. *Virology* 121,184-94.
2. Devlin TM (Ed). Textbook of Biochemistry with Clinical Correlations. Pub.John Wiley & Sons (1992). Chap 5.

SUPERVISOR: Professor M C Winslet

Royal Free Hospital & Medical School

ADVISOR: Professor A Wylie, University of Edinburgh

ACKNOWLEDGEMENTS

N J Bradley (Scientific Advice, Technique Tuition, Project Suggestions &Direction)

R Austin (Technique Tuition, Project Direction)

P Amlot FACS Tuition

J Bhatia RIA Tuition

S Toffa Technical and Project-related Suggestions

Departments of: Surgery, Electron Microscopy, Clinical Immunology & Clinical Oncology

Royal Free Hospital

Pond Street

London

1. Abrahm, J., E. C. Besa, M. Hyzinski, J. Finan, and P. Nowell. 1986. Disappearance of Cytogenetic Abnormalities and Clinical Remission During Therapy with 13-Cis-Retinoic Acid in a Patient with Myelodysplastic Syndrome: Inhibition of Growth of the Patient's Malignant Monocytoid Clone. *Blood* 67:1323-1327.

2. Ahlstrom, H., P. Enblad, A. Andersson, and L. E. Lorelius. 1989. The spatial distribution of parenterally administered monoclonal antibodies against CEA in a human colorectal tumour xenograft. *Acta Oncologica* 28:81-86.

3. Aitken, D. R., G. H. Hinkle, M. O. Thurston, S. E. Tuttle, D. T. Martin, J. Olsen, D. E. Haagensen,Jr., D. Houchens, and E. W. Martin,Jr. 1984. A gamma-detecting probe for radioimmune detection of CEA-producing tumors. Successful experimental use and clinical case report. *Diseases of the Colon & Rectum* 27:279-282.

4. Antoun, G. R., G. R. Re, N. H. A. Terry, and T. F. Zipf. 1991. Molecular Genetic Evidence for a Differentiation-Proliferation Coupling During DMSO-Induced Myeloid Maturation of HL-60 Cells: Role of the Transcription Elongation Block in the c-myc Gene. *Leukaemia Research* 15:1029-1036.

5. Aries, V., J. S. Crowther, B. S. Drasar, M. J. Hill, and R. E. O. Williams. 1969. Bacteria and the Aetiology of Cancer of the Large Bowel. *Gut* 10:334-335.

6. Arnold, M. W., S. Schneebaum, A. Berens, C. Mojzisik, G. Hinkle, and E. W. J. Martin. 1992. Radioimmunoguided Surgery Challenges Traditional Decision Making in Patients with Primary Colorectal Cancer. *Surgery* 112:624-630.

7. Arnold, M. W., S. Schneebaum, A. Berens, L. Petty, C. Mojsizik, G. Hinkle, and E. W. Martin. 1992. Intraoperative Detection of Colorectal Cancer with Radioimmunoguided Surgery and CC49, a Second-Generation Monoclonal Antibody. *Annals of Surgery* 216:627-633.

8. Attallah, A. M., C. F. Needy, P. D. Noguchi, and B. L. Elisberg. 1979. Enhancement of Carcinoembryonic Antigen Expression by Interferon. *International Journal of Cancer* 24:49-52.

9. Attiyeh, F. F. and M. W. Stearns,Jr. 1981. Second-look laparotomy based on CEA elevations in colorectal cancer. *Cancer* 47:2119-2125.

10. August, D. A., R. T. Ottow, and P. H. Sugarbaker. 1984. Clinical Perspective of Human Colorectal Cancer Metastasis. *Cancer Metastasis Reviews* 3:303-324.

11. Austin, R. C. T., N. J. Bradley, M. H. S. Collie, and M. C. Winslet. 1996. Effects of Chemotherapy and Radiotherapy on the Expresion of Carcinoembryonic Antigenby colorectal Carcinoma Cells in Vitro. *British Journal of cancer Supplement* 74:40

12. Austin, R. C. T., N. J. Bradley, M. H. S. Collie, and M. C. Winslet. 1996. The Relationship Between Internal Tumour Environment and Associated Antigen Expression; In Vitro Assessment. *British Journal of Cancer* 74:40

13. Austin, R. C. T., N. J. Bradley, and M. C. Winslet. 1995. A Comparative Study of Subjective Assessment, Image Analysis (IA) and ELISA for the Quantification of Cellular CEA Levels. *British Journal of cancer Supplement* 71:37

14. Baghdiguiian, S., B. Verrier, M. Roccabianca, G. Pommier, J. Marvaldi, and J. Fantini. 1991. Vectorial Release of Carcinoembryonic Antigen Induced by IFN-gamma in Human Colon Cancer Cells Cultured in Serum-Free Medium. *European Journal of Cancer* 27:599-604.

15. Baker, S. J., E. R. Fearon, J. M. Nigro, S. R. Hamilton, A. C. Preisinger, J. M. Jessup, P. van Tuinen, R. White, B. Vogelstein, D. H. Ledbetter, D. F. Barker, and Y. Nakamura. 1989. Chromosome 17 Deletions and p53 Gene Mutations in Colorectal Carcinomas. *Science* 244:217-221.

16. Baker, S. J., S. Markowitz, E. R. Fearon, J. K. V. Willson, and B. Vogelstein. 1990. Suppression of Human Colorectal Carcinoma Cell Growth by Wild-Type p53. *Science* 249:912-915.

17. Banjo, C., J. Shuster, and P. Gold. 1974. Intermolecular Heterogeneity of the Carcinoembryonic Antigen. *Cancer Res.* 34:2114-2121.

18. Bara, J., R. Gautier, N. Daher, H. Zaghouani, and C. Decaens. 1986. Monoclonal Antibodies against oncofetal Mucin M1 Antigens Associated with Precancerous Colonic Mucosa. *Cancer Res.* 46:3983-3989.

19. Barr, I. G., S. Miescher, V. von Fliedner, F. Bucheggar, C. Barras, A. Lanzavecchia, J. Mach, and S. Carrel. 1989. In Vivo Localisation of a Bispecific Antibody which Targets Human T Lymphocytes to Lyse Human Colon Cancer Cells. *International Journal of Cancer* 43:501-507.

20. Barr, P. J. and L. D. Tomei. 1994. Apoptosis and its Role in Human Disease. *Biotechnology* 12:487-493.

21. Bartocci, A., C. Riccardi, and E. Bonmassar. 1980. In vivo or in vitro Modulating Effect of Vincristine on the Generation of Allogeneic Cytotoxic Lymphocytes in vitro. *Journal of Immunopharmacology* 2:61-72.

22. Basham, T. Y., M. F. Bourgeade, A. A. Creasey, and T. C. Merigan. 1982. Interferon Increases HLA Synthesis in Melanoma Cells: Interferon-resistant and -sensitive Cell Lines. *proceedings of the National Academy of Sciences of the USA* 79:3265-3269.

23. Bates, P. A., J. Luo, and M. J. E. Sternberg. 1992. A Predicted Three-Dimensional Strucure for the Carcinoembryonic Antigen (CEA). *Federation of European Biochemical Societies Letters* 301:207-214.

24. Bates, S. E. and D. L. Longo. 1985. Tumour Markers: Value and Limitations in the Management of Cancer Patients. *Cancer Treatment Reviews* 12:163-207.

25. Bauer, G., P. Hofler, and H. zur Hausen. 1982. Epstain-Barr Virus Induction by a Serum Factor. *Virology* 121:184-194.

26. Beatty, J. D., D. M. Hyams, B. A. Morton, B. G. Beatty, L. E. Williams, D. Yamauchi, B. Merchant, R. J. Paxton, and J. E. Shively. 1989. Impact of radiolabeled antibody imaging on management of colon cancer. *American Journal of Surgery* 157:13-19.

27. Beatty, J. D., C. Romero, P. W. Brown, W. Lawrence, and J. J. Terz. 1979. Clinical Value of Carcinoembryonic Antigen. *Archives of Surgery* 114:563-567.

28. Beere, H. M. and J. A. Hickman. 1993. Differentiation: a Suitable Strategy for Cancer Chemotherapy? *Anti-Cancer Drug Design* 8:299-322.

29. Begent, R. H., J. A. Ledermann, A. J. Green, K. D. Bagshawe, S. J. Riggs, F. Searle, P. A. Keep, T. Adam, R. G. Dale, and M. G. Glaser. 1989. Antibody distribution and dosimetry in patients receiving radiolabelled antibody therapy for colorectal cancer. *British Journal of Cancer* 60:406-412.

30. Begent, R. H. and R. B. Pedley. 1990. Antibody targeted therapy in cancer: comparison of murine and clinical studies. [Review] [31 refs]. *Cancer Treatment Reviews* 17:373-378.

31. Begent, R. H. J., A. J. Green, K. D. Bagshawe, B. E. Jones, P. A. Keep, F. Searle, R. F. Jewkes, G. M. Barratt, and B. E. Ryman. 1982. Liposomally Entrapped Second Antibody Improves Tumour Imaging with Radiolabelled (First) Antitumor Antibody. *The Lancet* 739-742.

32. Behr, T. M., R. M. Sharkey, M. I. Juweid, R. M. Dunn, Z. Ying, C. H. Zhang, Siegel, JA, D. V. Gold, and D. M. Goldenberg. 1996. Factors influencing the pharmacokinetics, dosimetry, and diagnostic accuracy of radioimmunodetection and radioimmunotherapy of carcinoembryonic antigen-expressing tumors. *Cancer Res.* 56:1805-1816.

33. Bei, R., J. Kantor, S. V. Kashmiri, S. Abrams, and J. Schlom. 1994. Enhanced immune responses and anti-tumor activity by baculovirus recombinant carcinoembryonic antigen (CEA) in mice primed with the recombinant vaccinia CEA. *Journal of Immunotherapy with Emphasis on Tumor Immunology* 16:275-282.

34. Benchimol, S., A. Fuks, S. Jothy, N. Beauchemin, K. Shirota, and C. P. Stanners. 1989. Carcinoembryonic Antigen, a Human Tumour Marker,Functions as an Intercellular Adhesion Molecule. *Cell* 57:327-334.

35. Bennett, C., I. M. Paterson, C. M. Corbishley, and Y. A. Luqmani. 1989. Expression of growth factor and epidermal growth factor receptor encoded transcripts in human gastric tissues. *Cancer Res.* 49:2104-2111.

36. Bischof Delaloye, A. and B. Delaloye. 1992. Diagnostic Applications and Therapeutic Approaches with Different Preparations of anti-CEA antibodies. *The International Journal of Biological Markers* 7:193-197.

37. Bishop, J. M. 1991. Molecular themes in oncogenesis. [Review] [191 refs]. *Cell* 64:235-248.

38. Blumenthal, R. D., R. M. Sharkey, R. Kashi, A. M. Natale, and D. M. Goldenberg. 1992. Physiological Factors Influencing Radioantibody Uptake: A Study of Four Human Colonic Carcinomas. *International Journal of Cancer* 51:935-941.

39. Blumenthal, R. D., R. M. Sharkey, D. Snyder, and D. M. Goldenberg. 1989. Reduction by Anti-antibody Administration of the Radiotoxicity Associated with 131-Iodine-labelled Antibody to Carcinoembryonic Antigen in Cancer Radioimmunotherapy. *Journal of the National Cancer Institute* 81:194-199.

40. Bodmer, W. F., C. J. Bailey, J. Bodmer, H. J. R. Bussey, A. Ellis, P. Gorman, F. C. Lucibello, V. A. Murday, S. H. Rider, P. Scambler, D. Sheer, E. Solomon, and N. K. Spurr. 1987. Localisation of the Gene for Familial Adenomatous Polyposis on Chromosome 5. *Nature* 328:614-619.

41. Bordes, M., R. Michiels, and F. Martin. 1973. Detection by Immunofluorescence of Carcinoembryonic Antigen in Colonic Carcinoma,Other Malignant or Benign Tumours, and Non-Cancerous Tissues. *Digestion* 9:106-115.

42. Bos, J. L., E. R. Fearon, S. R. Hamilton, M. Verlaan-de Vries, J. H. van Boom, A. J. van der Erb, and B. Vogelstein. 1987. Prevalence of ras Mene Mutations in Human Colorectal Carcinomas. *Nature* 327:293-297.

43. Bosman, F. T., Y. Kikuchi, and W. N. M. Dinjens. 1997. Pathological Society of Great Britain and Ireland. (Abstr.)

44. Boucher, D., D. Cournoyer, C. P. Stanners, and A. Fuks. 1989. Studies on the Control of Gene Expression of the Carcinoembryonic Antigen Family in Human Tissue. *Cancer Res.* 49:847-852.

45. Boveri, T. 1929. The Origin of Malignant Tumours. AnonymousThe Williams and Wilkins Co. Baltimore. 26-27.

46. Bowman, J. M., J. Lawry, R. J. Landers, and J. A. Royds. 1997. Pathological Society of Great Britain and Ireland. (Abstr.)

47. Boxer, G. M., R. H. Begent, A. M. Kelly, P. J. Southall, S. B. Blair, N. A. Theodorou, P. M. Dawson, and J. A. Ledermann. 1992. Factors influencing variability of localisation of antibodies to carcinoembryonic antigen (CEA) in patients with colorectal carcinoma--implications for radioimmunotherapy. *British Journal of Cancer* 65:825-831.

48. Boyd, A. S. 1989. An overview of the retinoids. [Review] [93 refs]. *American Journal of Medicine* 86:568-574.

49. Brach, M. A., R. H. Mertelsmann, and F. Herrmann. 1993. Modulation of cytotoxicity and differentiation-inducing potential of arabinofuranosylcytosine in myeloid leukemia cells by hematopoietic cytokines. [Review] [130 refs]. *Cancer Investigation* 11:198-211.

50. Breborowicz, J., G. C. Easty, M. Birbeck, D. Robertson, R. Nery, and A. M. Neville. 1995. The Monolayer and Organ Culture of Human Colorectal Carcinomata and the Associated "Normal" Colonic Mucosa and their Production of Carcinoembryonic Antigen. *British Journal of Cancer* 31:559-569.

51. Breitz, H. B., P. L. Weiden, J. Vanderheyden, J. W. Appelbaum, M. J. Bjorn, M. F. Fer, S. B. Wolf, B. A. Ratcliff, C. A. Seiler, D. C. Foisie, D. R. Fisher, R. W. Schroff, A. R. Fritzberg, and P. G. Abrams. 1992. Clinical Experience with Rhenium-186-Labelled Monoclonal Antibodies for Radioimmunotherapy: Results of Phase I Trials. *The Journal of Nuclear Medicine* 33:1099-1109.

52. Bucheggar, F., A. Pelegrin, B. Delaloye, A. Bischof-Delaloye, and J. Mach. 1990. Iodine-131-Labelled MAb F(Ab')2 Fragments are more Efficient and ess Toxic than Intact Anti-CEA Antibodies in Radioimmunotherapy of Large Human Colon Carcinoma Grafted in Nude Mice. *The Journal of Nuclear Medicine* 31:1035-1043.

53. Buchegger, F., C. M. Haskell, M. Schreyer, B. R. Scazziga, S. Randin, S. Carrel, and J. P. Mach. 1983. Radiolabeled fragments of monoclonal antibodies against carcinoembryonic antigen for localization of human colon carcinoma grafted into nude mice. *Journal of Experimental Medicine* 158:413-427.

54. Buras, R. R., B. G. Beatty, L. E. Williams, P. M. Wanek, J. B. Harris, L. R. Hill, and J. D. Beatty. 1990. Radioimmunotherapy of human colon cancer in nude mice. *Archives of Surgery* 125:660-664.

55. Carlsson, J., E. Daniel-Szolgay, G. Frykholm, B. Glimelius, A. Hedin, and B. Larsson. 1989. Homogeneous penetration but

Heterogeneous Binding of Antibodies to Carcinoembryonic Antigen in Human Colon Carcinoma Ht29 Spheroids. *Cancer Immunology Immunotherapy* 30:269-276.

56. Cavaliere, R., E. C. Ciocatto, B. C. Giovanella, C. Heidelberger, R. O. Johnson, M. Margottini, B. Mondovi, G. Moricca, and A. Rossi-Fanelli. 1967. Selective Heat Sensitivity of Cancer Cells. *Cancer* 20:1351-1381.

57. Chakrabarty, S., J. W. Chen, X. Y. Chen, J. M. Trujillo, and P. F. Lin. 1992. Modulation of differentiation-related responses in human colon carcinoma cells by protein kinase inhibitor H-7. *Anticancer Research* 12:97-104.

58. Chakrabarty, S., Y. Jan, M. G. Brattain, A. Tobon, and J. Varani. 1989. Diverse cellular responses elicited from human colon carcinoma cells by transforming growth factor-beta. *Cancer Res.* 49:2112-2117.

59. Cobb, L. M. 1989. Intratumour factors influencing the access of antibody to tumour cells. [Review] [72 refs]. *Cancer Immunology, Immunotherapy* 28:235-240.

60. Collins, S. J., A. Bodner, R. Ting, and R. C. Gallo. 1980. Induction of Morphological and Functional Differentiation of Human Promyelocytic Leukaemia Cells (HL-60) by Compounds Which Induce Differentiation of Murine Leukemia Cells. *International Journal of Cancer* 25:213-218.

61. Collins, S., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo. 1978. Terminal Differentiation of Human Promyelocyte Leukemia Cells Induced by Dimethyl Sulfoxide and other Polar Compounds. *Proceedings of the National Academy of the USA* 75:2458-2462.

62. Costanza, M. E., V. Pinn, R. S. Schwartz, and L. Nathanson. 1973. Carcinoembryonic Antigen-Antibody Complexes in a Patient with Colonic Carcinoma and Nephrotic Syndrome. *The New England Journal of Medicine* 289:520-522.

63. Covell, D. G., J. Barbet, O. D. Holton, C. D. Black, R. J. Parker, and J. N. Weinstein. 1986. Pharmacokinetics of monoclonal immunoglobulin G1, F(ab')2, and Fab' in mice. *Cancer Res.* 46:3969-3978.

64. Creamer, B. 1967. The Turnover of the Epithelium of the Small Intestine. *British Medical Bulletin* 226-230.

65. Cutait, R., V. A. F. Alves, L. C. Lopez, D. E. Cutait, J. L. A. Borges, J. Singer, J. H. da Silva, and F. S. Goffi. 1991. Restaging of Colorectal Cancer Based on the Identification of Lymph Node Micrometastases Through Immunoperoxidase Staining of CEA and Cytokeratins. *Diseases of the Colon and Rectum* 34:917-920.

66. Daneker, G. W., Jr., A. J. Piazza, G. D. Steele,Jr., and A. M. Mercurio. 1989. Relationship between extracellular matrix interactions and degree of differentiation in human colon carcinoma cell lines. *Cancer Res.* 49:681-686.

67. Davies, T. 1981. Magic Bullets. *Nature* 289:12-13.

68. Day, E. D. and D. Pressman. 1958. Purification of Tumour-Localizing Antibodies. *Annals of the New York Academy of Sciences* 651-657.

69. De Filippi, R., S. P. Prete, A. Giuliani, E. Silvi, H. Yamaue, C. a. Nieroda, J. W. Greiner, L. de Vecchis, and E. Bonmassar. 1994. Differential Effects of Recombinant Interferon-Alpha and 5-Fluorouracil against Colon Cancer Cells or Against Peripheral Blood Mononuclear Cells. *Anticancer Research* 14:1767-1774.

70. Delattre, O., D. J. Law, Y. Remvikos, X. Sastre, A. P. Feinberg, S. Olschwang, T. Melot, R. J. Salmon, P. Validire, and G. Thomas. 1989. Multiple Genetic Defects in Distal and Proximal Colorectal Cancer. *The Lancet* 2:353-356.

71. Delwich, R., N. Zamcheck, and N. Marcon. 1973. Carcinoembryonic Antigen in Pancreatitis. *Cancer* 31:328-330.

72. Denk, H., G. Tappener, R. Eckerstorfer, and J. H. Holzner. 1972. Carcinoembryonic Antigen (CEA) in Gastrointestinal and Extragastrointestinal Tumors and its Relationship to Tumour-Cell Differentiation. *International Journal of Cancer* 10:262-272.

73. Deschner, E. E., M. Lipkin, and C. Solomon. 1966. Study of Human Rectal Epithelial Cells in vitro. II.H3-Thymidine Incorporation into Polyps adn Adjacent Mucosa. *Journal of the National Cancer Institute* 36:849-857.

74. Devlin, T.M. 1992. Textbook of Biochemistry with Clinical Correlations. AnonymousJohn Wiley and Sons,

75. Dexter, D. L., J. A. Barbarosa, and P. Calabresi. 1979. N,N-Dimethylformamide-induced Alteration of Cell Culture Characteristics and Loss of Tumorigenicity in Cultured Human Colon Carcinoma Cells. *Cancer Res.* 39:1020-1025.

76. Doerr, R. J., H. Abdel-Nabi, D. Krag, and E. Mitchell. 1991. Radiolabeled antibody imaging in the management of colorectal cancer. Results of a multicenter clinical study. *Annals of Surgery* 214:118-124.

77. Doerr, R. J., H. Abdel-Nabi, and B. Merchant. 1990. Indium 111 ZCE-025 immunoscintigraphy in occult recurrent colorectal cancer with elevated carcinoembryonic antigen level. *Archives of Surgery* 125:226-229.

78. Doll, R. 1967. Worldwide Distribution of Gastrointestinal Cancer. *National Cancer Institute Monographs* 25:173-190.

79. Donnellan, W. L. 1965. The Structure of the Colonic Mucosa. *Gastroenterology* 49:496-514.

80. Drewinko, B., M. M. Romsdahl, L. Yang, M. J. Ahearn, and J. M. Trujillo. 1976. Establishment of a Human Carcinoembryonic Antigen-producing Colon Adenocarcinoma Cell Line. *Cancer Res.* 36:467-475.

81. Drewinko, B. and L. Yang. 1976. Restriction of CEA synthesis to the Stationary phase of Growth of Cultured Human Colon Carcinoma Cells. *Experimental Cell Research* 101:414-416.

82. Drewinko, B., L. Yang, B. Barlogie, M. Romsdahl, M. Meistrich, M. A. Malahy, and B. Giovanella. 1978. Further Biologic Characteristics of a Human Carcinoembryonic Antigen-Producing Colon Carcinoma Cell Line. *Journal of the National Cancer Institute* 61:75-83.

83. Drewinko, B. and L. Yang. 1980. Observations on the Synthesis of Carcinoembryonic Antigen by an Established Human Colonic Carcinoma Cell Line. *Oncology* 37:336-342.

84. Dubos, R. 1966. The Microbiota of the Gastrointestinal Tract. *Gastroenterology* 51:868-874.

85. Dukes, C. E. 1932. The Classification of Caner of the Rectum. *Journal of Pathology* 35:323-332.

86. East, J. A., S. P. Langdon, S. Townsend, and J. A. Hickman. 1992. The Influence of Type I Collagen on the Growth and Differentiation of the Human Colonic Adenocarcinoma Cell Line Ht-29 in Vitro. *Differentiation* 50:179-188.

87. Eckert, K., T. Fuhrmann-Selter, and H. R. Maurer. 1997. Docetaxel Enhances the Expression of E-cadherin and Carcinoembryonic Antigen (CEA) on Human Colon Cancer Cell lines in Vitro. *Anticancer Research* 17:7-12.

88. Ekins, R. P. 1974. Basic Principles and Theory. *British Medical Bulletin* 30:3-11.

89. Fantini, J., J. Rognoni, J. Culouscou, G. Pommier, J. Marvaldi, and A. Tirard. 1989. Induction of Polarised Apical Expression and Vectorial Release of Carcinoembryonic Antigen (CEA) During the Process of Differentiation of Ht29-D4 Cells. *Journal of Cellular Physiology* 141:126-134.

90. Fantini, J., J. Rognoni, M. Theveniau, G. Pommier, and J. Marvaldi. 1990. Impaired Carcinoembryonic Release During the Process of Suramin-Induced Differentiation of the Human Colic Adenocarcinoma Cell clone Ht29-D4. *Journal of Cellular Physiology* 143:468-474.

91. Fearon, E. and B. Vogelstein. 1990. A Genetic Model for Colorectal Tumorigenesis. *Cell* 61:759-767.

92. Fearon, E. R., K. Cho, J. Nigro, S. Kern, J. W. Simons, J. M. Ruppert, S. R. Hamilton, A. C. Preisinger, G. Thomas, K. W. Kinzler, and B. Vogelstein. 1990. Identification of a Chromosome 18q Gene that is Altered in Colorectal Cancers. *Science* 249:49-56.

93. Fearon, E. R., S. R. Hamilton, and B. Vogelstein. 1987. Clonal Analysis of Human Colorectal Tumours. *Science* 238:193-197.

94. Fellous, M., M. Kamoun, I. Gresser, and R. Bono. 1979. Enhanced Expression of HLA Antigens and Beta2-Microglobulin on Interferon-Treated Human Lymphoid Cells. *European Journal of Immunology* 9:446-449.

95. Ferguson, P. J., J. R. Phillips, M. Selner, and C. F. Cass. 1984. Differential Activity of Vincristine and Vinblastine ageinst

Cultured Cells. *Cancer Res.* 44:3307-3312.

96. Ferrari, A. C. and S. Waxman. 1994. Differentiation agents in cancer therapy. [Review] [257 refs]. *Cancer Chemotherapy & Biological Response Modifiers* 15:337-366.

97. Fogh, J. and G. Trempe. 1975. New Human Tumour Cell Lines. In Human Tumour Cells in Vitro. J. Fogh, editor. Plenum Press, New York. 115-141.

98. Fogler, W. E., M. R. Klinger, K. G. Abraham, H. G. Gottlinger, G. Reithmuller, and E. Daddona. 1988. Enhanced Cytotoxicity against Colon Carcinoma by Combinations of noncompeting Monoclonal Antibodies to the 17-1A Antigen. *Cancer Res.* 48:6303-6308.

99. Folli, S., A. Pelegrin, Y. Chalandon, X. Yao, F. Buchegger, D. Lienard, F. Lejeune, and J. P. Mach. 1993. Tumor-necrosis factor can enhance radio-antibody uptake in human colon carcinoma xenografts by increasing vascular permeability. *International Journal of Cancer* 53:829-836.

100. Folli, S., G. Wagnieres, A. Pelegrin, J. Calmes, D. Braichotte, F. Buchegger, Y. Chalandon, N. Hardman, C. Heusser, J. Givel, G. Chapus, A. Chatelain, H. Van den Bergh, and J. Mach. 1992. Immunophotodiagnosis of Colon Carcinomas in Patients injected with Fluoresceinated Chimeric Antibodies against Carcinoembryonic Antigen. *proceedings of the National Academy of Sciences of the USA* 89:7973-7977.

101. Francis, G. E. 1994. Theoretical and practical considerations in screening strategies for differentiation agents. [Review] [14 refs]. *Differentiation* 57:63-75.

102. Friedman, J., M. Seger, H. Levinsky, and D. Allalouf. 1987. Modulation of Carcinoembryonic Antigen Release by Ht29 Colon Carcinoma Cell Line in the Presence of Different Agents. *Experientia* 43:1121-1122.

103. Friend, C., W. Scher, J. G. Holland, and T. Sato. 1971. Hemoglobin Synthesis in Murine Virus-induced Leukemia Cells in Vitro: Stimulation of Erythroid Differentiation by Dimethyl Sulfoxide. *proceedings of the National Academy of Sciences of the USA* 68:378-382.

104. Fuks, A., C. Banjo, and P. Gold. 1974. Carcinoembryonic Antigen (CEA): Molecular Biology and Clinical Applications. *Biochimica et Biophysica Acta* 417:123-152.

105. Garcia de herreros, A., M. Fabre, E. Batlle, C. Balague, and F. X. Real. 1993. The Tumour Promoter 12-O-tetradecanoylphorbol-13-acetate Blocks Differentiation of Ht29 human Colon Cancer CellH. *Journal of Cell Science* 105:1165-1172.

106. Gardner, R. C., A. E. Feinerman, P. A. Kantowitz, S. Gootblatt, M. S. Loewenstein, and N. Zamcheck. 1978. Serial Carcinoembryonic Antigen (CEA) Blood Levels in Patients with Ulcerative Colitis. *Digestive Diseases* 23:129-133.

107. Germa-Lluch, J. R., I. Alvarez, I. Carrio, F. M. Nogueras, E. Marcuello, M. Estorch, and L. Berna. 1991. Radioimmunolocalization of colorectal carcinoma. A correlation among RIL results, surgical findings, serum tumor marker levels and the presence of CEA and CA 19.9 in tumor tissue: the experience of the Hospital de la Santa Creu i Sant Pau. *Annals of Oncology* 2:409-415.

108. Giacomini, P., A. Aguzzi, S. Pestka, P. B. Fisher, and S. Ferrone. 1984. Modulation by recombinant DNA leukocyte (alpha) and fibroblast (beta) interferons of the expression and shedding of HLA- and tumor-associated antigens by human melanoma cells. *Journal of Immunology* 133:1649-1655.

109. Gibson, P., I. Moeller, O. Kagelari, M. Folino, and G. P. Young. 1992. Contrasting Effects of Butyrate on the Expression of Phenotypic Markers of Differentiation in Neoplastic and Non-neoplastic Colonic Epithelial Cells In Vitro. *Journal of Gastroenterology and Hepatology* 7:165-172.

110. Go, V. L. W. 1976. Carcinoembryonic Antigen Clinical Application. *Cancer* 37:562-566.

111. Go, V. L. W., H. Ammon, K. H. Holtermuller, E. Krag, and S. Phillips. 1975. Quantification of Carcinoembryonic Antigen-like Activities in Normal Human Gastrointestinal Secretions. *Cancer* 36:2346-2350.

112. Gold, P. 1967. Circulating Antibodies Against Carcinoembryonic Antigens of the Human Digestive System. *Cancer* 29:1663-1667.

113. Gold, P. 1978. *Cancer* 42:1399-1405.

114. Gold, P. and S. Freedman. 1965. Specific Carcinoembryonic Antigens of the human Digestive System. *Journal of Experimental Medicine* 122:467-481.

115. Gold, P. and S. O. Freedman. 1965. Demonstration of Tumour-Specific Antigens in Human Colonic Carcinomata by Immunological Tolerance and Absorption Techniques. *Journal of Experimental Medicine* 121:439-462.

116. Gold, P., M. Gold, and S. O. Freedman. 1968. Cellular Location of Carcinoembryonic Antigen of the Human Digestive System. *Cancer Res.* 28:1331-1334.

117. Gold, P., J. Krupey, and H. Ansari. 1970. Position of the Carcinoembryonic Antigen of the Human Digestive System in Ultra-structure of Tumour Cell Surface. *Journal of the National Cancer Institute* 45:219-225.

118. Goldenberg, D. M. 1981. Symposium: Carcinoembryonic Antigen: Its Role as a Marker in the Management of Cancer. A National Institutes of Health Consensus Development Conference. *Annals of internal Medicine* 94:407-409.

119. Goldenberg, D. M., F. DeLand, E. Kim, S. Bennett, F. J. Primus, j. R. van Nagell, N. Estes, P. DeSimone, and P. Rayburn. 1978. Use of Radiolabelled Anitbodies to Carcinoembryonic Antigen for the Detection and Localization of Diverse Cancers by External Photoscanning. *New England Journal of Medicine* 298:1384-1388.

120. Goldenberg, D. M., F. H. Deland, E. E. Kim, and F. J. Primus. 1980. Xenogeneic Antitumour Antibodies in Cancer Radioimmunodetection. *Transplantation Proceedings* 12:188-191.

121. Goldenberg, D. M., H. Goldenberg, R. M. Sharkey, E. Higginbotham-Ford, R. E. Lee, L. C. Swayne, K. A. Burger, D. Tsai, J. A. Horowitz, T. C. Hall, and et al. 1990. Clinical studies of cancer radioimmunodetection with carcinoembryonic antigen monoclonal antibody fragments labeled with 123I or 99mTc. *Cancer Res.* 50:909s-921s.

122. Goldenberg, D. M. and H. Hansen. 1972. Carcinoembryonic Antigen Present in Human Colonic Neoplasms Serially Propagated in Hamsters. *Science* 175:1117-1118.

123. Goldenberg, D. M., E. E. Kim, F. H. Deland, S. Bennett, and F. J. Primus. 1980. Radioimmunodetection of Cancer with Radioactive Antibodies to Carcinoembryonic Antigen. *Cancer Res.* 40:2984-2992.

124. Goligher, J.C. 1980. Surgery of the anus,rectum and colon. AnonymousBalliere Tindall, London. 467-471.

125. Goslin, R., M. J. O'Brien, G. Stel, R. Mayer, R. Wilson, J. M. Corson, and N. Zamcheck. 1981. Correlation of Plasma CEA and CEA Tissue Staining in Poorly Differentiated Colorectal Cancer. *The American Journal of Medicine* 71:246-253.

126. Greiner, J. W., P. B. Fisher, S. Pestka, and J. Schlom. 1986. Differential effects of recombinant human leukocyte interferons on cell surface antigen expression. *Cancer Res.* 46:4984-4990.

127. Greiner, J. W., F. Guadagni, P. Noguchi, S. Pestka, D. Colcher, P. B. Fisher, and J. Schlom. 1987. Recombinant Interferon Enhances Monoclonal Antibody-Targetting of Carcinoma Lesions in Vivo. *Science* 235:895-898.

128. Greiner, J. W., P. H. Hand, P. Noguchi, P. B. Fisher, S. Pestka, and J. Schlom. 1984. Enhanced expression of surface tumor-associated antigens on human breast and colon tumor cells after recombinant human leukocyte alpha-interferon treatment. *Cancer Res.* 44:3208-3214.

129. Gridley, D. S., K. L. Ewart, J. D. Cao, and D. R. Stickney. 1991. Hyperthermia enhances localization of 111In-labeled hapten to bifunctional antibody in human colon tumor xenografts. *Cancer Res.* 51:1515-1520.

130. Gridley, D. S., J. B. Slater, D. G. Mackensen, and J. M. Slater. 1996. Effect of Pre-irradiation on Radiopharmaeutical Localisation in Human Colon Tumour Xenografts Using Mono- and Bispecific Monoclonal Antibodies. *Anticancer Research* 16:3453-3458.

131. Guadagni, F., G. Tortora, M. Roselli, T. Clair, Y. S. Cho-Chung, J. Schlom, and J. W. Greiner. 1991. Carcinoembryonic Antigen Regulation in Human Colorectal Tumour Cells by a Site -Selective Cyclic AMP Analogue: A Comparison with Interferon-gamma. *International Journal of Cancer* 48:413-422.

132. Guadagni, F., P. L. Witt, P. F. Robbins, J. Schlom, and J. W. Greiner. 1990. Regulation of carcinoembryonic antigen expression in different human colorectal tumor cells by interferon-gamma. *Cancer Res.* 50:6248-6255.

133. Haenszel, H. and E. A. Dawson. 1965. A Note on Mortality form Cancer of the Colon and Rectum in the United States. *Cancer* 18:265-272.

134. Hague, A., M. Moorghen, D. Hicks, M. Chapman, and C. Paraskeva. 1994. BCL-2 expression in human colorectal adenomas and carcinomas. *Oncogene* 9:3367-3370.

135. Hareyama, M., K. Imai, K. Kubo, H. Takahashi, H. Koshiba, Y. Hinoda, Shidou, M, A. Oouchi, A. Yachi, and K. Morita. 1991. Effect of radiation on the expression of carcinoembryonic antigen of human gastric adenocarcinoma cells. *Cancer* 67:2269-2274.

136. Harwood, P. J., D. W. Britton, P. J. Southall, G. M. Boxer, G. Rawlins, and G. T. Rogers. 1986. Mapping Epitope Characteristics on Carcinoembryonic Antigen. *British Journal of Cancer* 54:75-82.

137. Herrera, M. A., T. Ming Chu, E. D. Holyoke, and A. Mittelman. 1977. CEA Monitoring of Palliative Treatment for Colorectal Carcinoma. *Annals of Surgery* 185:23-30.

138. Hill, M. J. and B. S. Drasar. 1968. Degradation of Bile Salts by human Intestinal Bacteria. *Gut* 9:22-27.

139. Hirai, H. 1977. Collabarative Clinical Study of Carcinoembryonic Antigen in Japan. *Cancer Res.* 37:2267-2274.

140. Hollywood, D., K. Sikora, and J. Ponten. 1995. The Biology of Cancer: Oncogenes. In Oxford Textbook of Pathology. AnonymousOxford University Press, Oxford. 54-73.

141. Holyoke, D., G. Reynoso, and T. M. Chu. 1972. Carcinoembryonic Antigen (CEA)in Patients With Carcinoma of the Digestive Tract. *Annals of Surgery* 176:559-563.

142. Hoosein, N., B. E. Black, D. E. Brattain, and M. G. Brattain. 1989. Promotion of Differentiation in Human Colon Carcinoma Cells by Vasoactive Intestinal Polyeptide. *Regulatory Peptides* 24:15-26.

143. Hostetter, R. B., L. B. Augustus, R. Mankarious, K. Chi, D. Fan, C. Toth, P. Thomas, and J. M. Jessup. 1990. Carcinoembryonic Antigen as a Selective Enhancer of Colorectal Cancer Metastasis. *Journal of the National Cancer Institute* 82:380-385.

144. Hsu, S. H., G. D. Luk, A. J. Krush, S. R. Hamilton, and H. H. J. Hoover. 1983. Multiclonal Origin of Polyps in Gardner Syndrome. *Science* 221:951-953.

145. Hunter, T. 1991. Cooperation Between Oncogenes. *Cell* 64:249-270.

146. Hyams, D. M., J. M. Esteban, B. G. Beatty, P. M. Wanek, and J. D. Beatty. 1989. Prolongation of survival of nude mice bearing human colon cancer. Treatment with yttrium 90-labeled anti-carcinoembryonic antigen antibody. *Archives of Surgery* 124:175-179.

147. Kalofonos, H. P., S. Stewart, and A. A. Epenetos. 1988. Antibody-Guided Diagnosis and Therapy of Malignant Lesions. *International Journal of Cancer Supplement* 2:74-80.

148. Kantor, J., R. Tran, J. Greiner, S. Pestka, P. B. Fisher, J. E. Shively, and J. Schlom. 1989. Modulation of Carcinoembryonic Antigen Messenger RNA Levels in Human Colon Carcinoma Cells by Recombinant Human gamma-Interferon. *Cancer Res.* 49:2651-2655.

149. Kelvin, F. M. and D. D. T. Maglinte. 1987. Colorectal Carcinoma: A Radiologic and Clinical Review. *Radiology* 164:1-6.

150. Kenworthy, R. and W. D. Allen. 1966. Influence of Diet and Bacteria of Small Intestinal Morphology with Special

Reference to Early Weaning and Escherichia Coli. *Journal of Comparative Pathology* 76:291-296.

151. Kim, H., J. K. Jung, J. H. Park, and C. Park. 1996. Immunohistochemical Characteristics of Colorectal Carcinoma with DNA Replication Errors. *Journal Of Korean Medical Science* 11:137-143.

152. Kim, Y. S., D. Tsao, B. Siddiqui, J. S. Whitehead, P. Arnstein, J. Bennett, and J. Hicks. 1980. Effects of Sodium Butyrate and Dimethylsulfoxide on Biochemical Properties of Human Colon Cancer Cells. *Cancer* 45:1185-1192.

153. Kirkham, N. and N.R. Lemoine. 1995. Control of Programmed Cell Death and Apoptosis. In Progress in Pathology. AnonymousChurchill Livingston, London. 5-6.

154. Kitadai, Y., R. Radinsky, C. D. Bucana, Y. Takahashi, K. Xie, E. Tahara, and I. J. Fidler. 1996. Regulation of Carcinoembryonic Antigen Expression in Human Colon Carcinoma Cells by the Organ Microenvironment. *American Journal of Pathology* 149:1157-1166.

155. Knudson, A. G. 1971. Mutation and Cancer: a Statistical study of Retinoblastoma. *Proceedings of the National Academy of Sciences of the USA* 68:820-823.

156. Kobayashi, T., M. Dohgome, and T. Kawakubo. 1990. Increase in Carcinoembryonic Antigen Release from Cancer Cells by Combined Treatment with Retinoic Acid and Low-temperature Hyperthermia. *Int. Journal of Hyperthermia* 6:785-792.

157. Koeffler, P. 1983. Induction of Differentiation of Human Acut Myelogenous Leukemia Cells: Therapeutic Implications. *Blood* 62:709-721.

158. Kohler, G. and C. Milstein. 1975. Continuos Cultures of Fused Cells Secreting Antibody of Predefine Specificity. *Nature* 256:495-497.

159. Kuhn, J. A., R. M. Corbisiero, R. R. Buras, R. G. Carroll, L. D. Wagman, L. A. Wilson, D. Yamauchi, M. M. Smith, R. Kondo, and J. D. Beatty. 1991. Intraoperative gamma detection probe with presurgical antibody imaging in colon cancer. *Archives of Surgery* 126:1398-1403.

160. Kuhn, J. A., J. Y. C. Wong, B. G. Beatty, J. M. Esteban, L. E. Williams, and J. D. Beatty. 1992. gamma-Interferon Enhancement of Carcinoembryonic Antigen Expression in Human Colon Carcinoma Xenografts. *Journal of Immunotherapy* 11:257-265.

161. La Rocca, R. V., J. Park, R. Danesi, M. Del Tacca, S. M. Steinberg, and A. F. Gazdar. 1992. Pattern of Growth Factor, Proto-oncogene and Carcinoembryonic Antigen Gene Expression in Human Colorectal Carcinoma Cell Lines. *Oncology* 49:209-214.

162. Land, H., L. F. Parada, and R. Weinberg. 1983. Cellular Oncogenes and Multistep Carcinogenesis. *Science* 222:771-778.

163. Lane, D. M., K. F. Eagle, R. H. J. Begent, L. D. Hope-Stone, A. J. Green, J. L. Casey, P. A. Keep, A. M. B. Kelly, J. A. Ledermann, M. G. Glaser, and A. J. W. Hilson. 1994. Radioimmunotherapy of Metastatic Colorectal Tumours with Iodine-131-labelled Antibody to Carcinoembryonic Antigen: PhaseI/II study with Comparative Biodistribution of Intact and F(ab')2 Antibodies. *British Journal of Cancer* 70:521-555.

164. Langmuir, V. K., J. K. McGann, F. Buchegger, and R. M. Sutherland. 1989. 131I-anticarcinoembryonic antigen therapy of LS174T human colon adenocarcinoma spheroids. *Cancer Res.* 49:3401-3406.

165. Lanza, G. J., L. Cavazzini, L. Borghi, S. Ferretti, F. Buccoliero, and M. Rubbini. 1990. Immunohistochemical Assessment of Growth Fractions in Colorectal Adenocarcinomas with Monoclonal Antibody Ki-67. *Path. Res. Pract* 186:608-618.

166. Le Doussal, J. M., A. Chetanneau, A. Gruaz-Guyon, M. Martin, E. Gautherot, P. A. Lehur, J. F. Chatal, M. Delaage, and J. Barbet. 1993. Bispecific monoclonal antibody-mediated targeting of an indium-111-labeled DTPA dimer to primary colorectal tumors: pharmacokinetics, biodistribution, scintigraphy and immune response. *Journal of Nuclear Medicine* 34:1662-1671.

167. Leavitt, J., J. C. Barrett, B. D. Crawfor, and P. O. P. Ts'o. 1978. Butyric Acid Suppression of the In Vitro Neoplastic State of Syrian Hamster Cells. *Nature* 271:262-265.

168. Leder, A. and P. Leder. 1975. Butyric Acid, A Potent Inducer of Erythroid Differentiation in Cultured Erythroleukaemia Cells. *Cell* 5:319-322.

169. Lengauer, C., K. W. Kinzler, and B. Vogelstein. 1997. Genetic instability in colorectal cancers. *Nature* 386:623-627.

170. Lesuffleur, T., A. Barbat, E. Dussaulx, and A. Zweibaum. 1990. Growth Adaption to Methotrexate of Ht29 Human Colon Carcinoma Cells Is Associated With their Ability to Differentiate into Columnar Absorptive and Mucus-secreting Cells. *Cancer Res.* 50:6334-6343.

171. Lipkin, M. 1966. Newer Measrurements for Cell Proliferation in the Colon. *Gastroenterology* 51:851-853.

172. Lipkin, M., P. Sherlock, and B. Bell. 1963. Cell Proliferation Kinetics in the Gastrointestinal Tract of Man. *Gastroenterology* 45:721-733.

173. Lo Gerfo, P., F. P. Herter, J. Braun, and H. J. Hansen. 1972. Tumour Associated Antigen with Pulmonary Neoplasms. *Annals of Surgery* 175:495-500.

174. Lo Gerfo, P., J. Krupey, and H. J. Hansen. 1971. Demonstration of an Antigen Common to Several Varieties of Neoplasia. *The New England of Journal of Medicine* 285:138-141.

175. Loewenstein, M. S. and N. Zamcheck. 1978. Carcinoembryonic Antigen (CEA) Levels in Benign Gastrointestinal Disease States. *Cancer* 42:1412-1418.

176. Lotan, R. 1980. *Biochimaca et Biophysica Acta* 605:33-43.

177. Lovqvist, A., A. Sundin, H. Ahlstrom, J. Carlsson, and H. Lundqvist. 1997. Pharmacokinetics and experimental PET imaging of a bromine-76-labeled monoclonal anti-CEA antibody. *Journal of Nuclear Medicine* 38:395-401.

178. Lumb, G. 1960. Normal Human Rectal Mucosa and its Mechanism of Repair. *Digestive Diseases* 5:836-840.

179. Maas, I. W. H. M., E. Boven, H. M. M. Schluper, and H. J. Haisma. 1991. The Effects of gamma-Interferon Combined with 5-Fluorouracil or 5-Fluoro-2'-Deoxyuridine on Proliferation and Antigen Expression in a Panel of Human Colorectal Cancer Cell Lines. *International Journal of Cancer* 48:749-756.

180. Mach, J., F. Bucheggar, M. Forni, J. Ritschard, C. Berche, J. Lumbroso, M. Schreyer, C. Girardet, R. S. Accolla, and S. Carrel. 1981. Use of Radio-labelled Monoclonal anti-CEA Antibodies fpr the Detection of Human Carcinomas by External Photoscanning and Tomoscintigraphy. *Immunology Today* 2:239-249.

181. Mach, J., S. Carrel, M. Forni, J. Ritschard, A. Donath, and P. Alberto. 1980. Tumour Localisation of Radiolabelled Antibodies against Carcinoembryonic Antigen in Patients with Carcinoma. *The New England Journal of Medicine* 303:5-10.

182. Mach, J., P. Jaeger, M. Bertholet, C.-H. Ruegsegger, R. M. Loosli, and J. Pettavel. 1974. Detection of Recurrence of Large-Bowel Carcinomata by Radioimmunoassay of Circulating Carcinoembryonic Antigen (CEA). *The Lancet* 2:535-540.

183. Mach, J. P., J. F. Chatal, J. D. Lumbroso, F. Buchegger, M. Forni, J. Ritschard, C. Berche, J. Y. Douillard, S. Carrel, M. Herlyn, and et al. 1983. Tumor localization in patients by radiolabeled monoclonal antibodies against colon carcinoma. *Cancer Res.* 43:5593-5600.

184. Mackay, A. M., S. Patel, S. Carter, U. Stevens, D. J. R. Laurence, E. H. Cooper, and A. M. Neville. 1974. Role of Serial Plasma CEA Assays in Detection of Recurrent and Metastatic Colorectal Carcinomas. *British Medical Journal* 4:382-385.

185. Macklis, R. M., B. M. Kinsey, A. I. Kassis, J. L. M. Ferrara, R. W. Atcher, J. J. Hines, C. N. Coleman, S. J. Adelstein, and W. J. Burakoff. 1988. Radioimmunotherapy with Alpha-Particle-Emitting Immunoconjugates. *Science* 240:1024-1026.

186. Mahvi, D. M., J. A. Madsen, P. L. Witt, and P. M. Sondel. 1995. Interferon alpha enhances expression of TAG-72 and carcinoembryonic antigen in patients with primary colorectal cancer *C ncer Immunology, Immunotherapy* 40:311-314.

187. Marks, P. A. and R. A. Rifkind. 1984. Differentiation Modifiers. *Cancer* 54:2766-2769.

188. Marrack, J. 1934. Nature of Antibodies. *Nature* 133:292-293.

189. Marshall, C. J. 1991. Tumor suppressor genes. [Review] [151 refs]. *Cell* 64:313-326.

190. Martin, D. T., D. R. Aitken, and M. O. Thurston. 1984. Successful Experimental Use of a Self-contained Gamma Detecting Device. *Curr Surgery* 41:193-197.

191. Martin, E.W.J., G. Hinkle, C. Mojzisik, and M.O. Thurston. 1990. Radioimmunoguided Surgery: A New Intraoperative Approach to the Detection of Tumour. In Cancer Imaging With Radiolabelled Antibodies. D.M. Goldenberg, editor. Kluwer Academic Publishers, 387-411.

192. Martin, F. and M. S. Martin. 1972. Radioimmunoassay of Carcinoembryonic Antigen in Extracts of Human Colon and Stomach. *International Journal of Cancer* 9:641-647.

193. Martin, F., M. S. Martin, M. Bordes, and C. Bourgeaux. 1972. The Specificity of Carcino-Foetal Antigens of the Human Digestive-Tract Tumours. *International Journal of Cancer* 8:315-321.

194. Mason, T. E., R. F. phifer, S. S. Spicer, R. A. Swallow, and R. B. Dreskin. 1969. An Immunoglobulin Enzyme Bridge Method for Localising Tissue Antigens. *The Journal of Histochemistry and Cytochemistry* 17:563-569.

195. Moffat, F. L., Jr., C. M. Pinsky, L. Hammershaimb, N. J. Petrelli, Y. Z. Patt, Whaley, FS, and D. M. Goldenberg. 1996. Clinical utility of external immunoscintigraphy with the IMMU-4 technetium-99m Fab' antibody fragment in patients undergoing surgery for carcinoma of the colon and rectum: results of a pivotal, phase III trial. The Immunomedics Study Group. *Journal of Clinical Oncology* 14:2295-2305.

196. Moldovsjy, P. J., H. F. Sears, C. B. Mulhern, N. D. Hammond, J. Powe, R. A. Gatenby, Z. Steplewski, and H. Koprowski. 1984. Detection of Metastatic Tumor in normal-sized Retroperitoneal Lymph Nodes by Monoclonal-antibody Imaging. *The New England Journal of Medicine* 311:106-107.

197. Moore, T. L., H. Z. Zupchik, N. Marcon, and N. Zamcheck. 1971. Carcinoembryonic Antigen Assay in Cancer of the Colon and Pancreas and Other Digestive Tract Disorders. *Digestive Diseases* 16:1-7.

198. Morikawa, K., D. Fan, Y. M. Denkins, B. Levin, J. U. Gutterman, S. M. Walker, and I. J. Fidler. 1989. Mechanisms of Combined Effect of gamma-Interferon and 5-Fluorouracil on Human Colon Cancers Implanted into Nude Mice. *Cancer Res.* 49:799-805.

199. Moses, H. L., E. Y. Yang, and J. A. Pietenpol. 1990. TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. [Review] [20 refs]. *Cell* 63:245-247.

200. Moss, A. A. 1989. Imaging of Colorectal Carcinoma. *Radiology* 170:308-310.

201. Muto, T., H. J. R. Bussey, and B. C. Morson. 1975. The Evolution of Cancer of the Colon and Rectum. *Cancer* 36:2251-2270.

202. Nabi, H. A. and R. J. Doerr. 1992. Radiolabelled monoclonal Antibody Imaging (Immunoscintigraphy) of Colorectal Cancers: Current Status and Future Perspectives. *The American Journal of Surgery* 163:448-456.

203. Nakaki, M., H. Takikawa, and M. Yamanaka. 1997. Targeting immunotherapy using the avidin-biotin system for a human colon adenocarcinoma in vitro. *Journal of International Medical Research* 25:14-23.

204. Naumovski, L. and M. Cleary. 1994. Bcl2 Inhibits Apoptosis Associated with Terminal Differentiation of HL-60 Myeloid Leukemia Cells. *Blood* 83:2261-2267.

205. Newmark, H. L., J. R. Lupton, and C. W. Young. 1994. Butyrate as a differentiating agent: pharmacokinetics, analogues and current status. [Review] [28 refs]. *Cancer Letters* 78:1-5.

206. Ng, I. O. L., J. Ho, C. J. Pritchett, E. Y. T. Chan, and F. C. S. Ho. 1993. CEA Tissue Staining in Colorectal Cancer Patients - Correlation with Plasma CEA, Histology and Staging. *Pathology* 25:219-222.

207. O'Dwyer, P. J., C. Mojsizik, G. H. Hinkle, M. Rousseau, J. Olsen, S. E. Tuttle, R. F. Barth, M. O. Thurston, D. P. McCabe, W. B. Farrar, and E. W. Martin. 1986. Intraoperative Probe-directed Immunodetection Using a Monoclonal Antibody. *Archives of Surgery* 121:1391-1394.

208. Ofner, D., H. Maier, B. Riedmann, P. Holzberger, M. Nogler, M. Totsch, A. Bankalvi, G. Winde, W. Bocker, and K. W. Schmid. 1995. Immunohistochemically Detectable p53 and mdm-2 Oncoprotein Expression in Colorectal Carcinoma: Prognostic Significance. *Journal of Clinical Pathology* 48:12-16.

209. Olson, E. N. 1992. Interplay Between Proliferation and Differentiation within the Myogenic League. *Developmental Biology* 154:261-272.

210. Order, S. E. 1976. The History and Progress of Serologic Immunotherapy and Radiodiagnosis. *Radiology* 118:219-223.

211. Pedley, R. B., J. A. Boden, R. W. Boden, A. Green, G. M. Boxer, and K. D. Bagshawe. 1989. The Effect of Serum CEA on the Distribution and Clearance of Anti-CEA Antibody in a Pancreatic Tumour Xenograft Model. *British Journal of Cancer* 60:549-554.

212. Philben, V. J., J. G. Jakowatz, B. G. Beatty, W. G. Vlahos, R. J. Paxton, L. E. Williams, J. J. Shively, and J. D. Beatty. 1986. The Effect of Tumour CEA Content and Tumour Size on Tissue Uptake of Indium 111-labelled Anti-CEA Monoclonal Antibody. *Cancer* 57:571-576.

213. Pimm, M. V., S. J. Gribben, and T. M. Morris. 1991. Influence of Recombinant Tumour Necrosis Factor alpha on Blood Flow and Antibody Localisation in Human Tumour Xenografts in Nude Mice. *Journal of cancer Research and Clinical Oncology* 117:543-548.

214. Pimm, M. V., R. A. Robins, M. J. Embleton, E. Jacobs, A. J. Markham, A. Charleston, and R. W. Baldwin. 1990. A bispecific monoclonal antibody against methotrexate and a human tumour associated antigen augments cytotoxicity of methotrexate-carrier conjugate. *British Journal of Cancer* 61:508-513.

215. Pommier, G. J., F. L. Garrouste, F. el Atiq, M. Roccabianca, J. L. Marvaldi, and M. M. Remacle-Bonnet. 1992. Potential autocrine role of insulin-like growth factor II during suramin-induced differentiation of HT29-D4 human colonic adenocarcinoma cell line. *Cancer Res.* 52:3182-3188.

216. Porro, G., E. Tagliabu, S. Menard, and M. I. Colnaghi. 1986. Influence of In Vitro Culture Conditions on Tumour Cell Differentiation. *Tumori* 72:15-20.

217. Press, O. W., J. F. Eary, F. R. Appelbaum, P. J. Martin, C. C. Badger, W. B. Nelp, S. Glenn, G. Butchko, D. Fisher, B. Porter, and et al. 1993. Radiolabeled-antibody therapy of B-cell lymphoma with autologous bone marrow support [see comments]. *New England Journal of Medicine* 329:1219-1224.

218. Pressman, D. and G. Keighley. 1948. The Zone of Activity of Antibodies as Determined by the Use of Radioactive Tracers; The Zone of Activity of Nephritoxic Antikidney Serum. *Journal of Immunology* 59:141-146.

219. Pressman, D. and L. Korngold. 1953. The In-Vivo Localisation of Anti-Wagner-Osteogenic-Sarcoma Antibodies. *Cancer* 6:619-623.

220. Prete, S. P., A. Aquino, G. Masci, L. Orlando, A. Giuliani, S. De Santis, De, L. Vecchis, R. De Filippi, J. W. Greiner, E. Bonmassar, and G. Graziani. 1996. Drug-induced changes of carcinoembryonic antigen expression in human cancer cells: effect of 5-fluorouracil. *Journal of Pharmacology & Experimental Therapeutics* 279:1574-1581.

221. Primus, F. J., R. H. Pak, K. J. Richard-Dickson, J. L. J. Bolen, R. r. kane, and M. F. Hawthorne. 1996. Bispecific Antibody Targetting of Nido-carboranes to Human Colon Carcinoma Cells. *Biconjugate Chemistry* 7:532-535.

222. Pusztaszeri, G. and J. Mach. 1973. Carcinoembryonic antigen (CEA) in Nondigestive, Cancerous and Normal Tissues. *Immunochemistry* 10:197

223. Quinn, L. A., G. E. Moore, R. T. Morgan, and L. K. Woods. 1979. Cell Lines from Human Colon Carcinoma with Unusual Cell Products, Double Minutes and Homogeneously Staining Regions. *Cancer Res.* 39:4914-4924.

224. Rabson, A. S., R. Stern, T. S. Tralka, J. Costa, and J. Wilczek. 1977. Hexamethylene Bisacetamide Induces Morphological Changes and Increased Synthesis of Procollagen in Cell Line from Glioblastoma Multiforme. *proceedings of the National Academy of Sciences of the USA* 74:5060-5064.

225. Reithmuller, G., E. Schneider-Gadicke, G. Schlimok, W. Schmiegel, R. Raab, K. Hoffken, R. Gruber, H. Pichlmaier, H. Hirche, R. Pichlmayr, P. Buggisch, and J. Witte. 1994. Randomised Trial of Monoclonal Antibody for Adjuvant Therapy of Resected Duke's C Colorectal Carcinoma. *The Lancet* 343:1177-1183.

226. Remacle-Bonnet, M., F. Garrouste, F. el Atiq, M. Roccobianca, J. Marvaldi, and G. Pommier. 1992. DES-(1-3)-IGF-1, An Insulin-Like Growth Factor Analog Used to Mimic a Potential IGF-II Autocrine Loop,Promotes the Differentiation of Human Colon-carcinoma Cells. *International Journal of Cancer* 52:910-917.

227. Reuben, R. C., R. L. Wife, R. Breslow, R. A. Rifkind, and P. A. Marks. 1976. *proceedings of the National Academy of Sciences of the USA* 73:862-866.

228. Robert, B., J. P. Mach, J. C. Mani, M. Ychou, S. Folli, J. C. Artus, and A. Pelegrin. 1996. Cytokine targeting in tumors using a bispecific antibody directed against carcinoembryonic antigen and tumor necrosis factor alpha. *Cancer Res.* 56:4758-4765.

229. Rosenthal, K. L., W. A. F. Tompkins, and W. E. Rawls. 1980. Factors Affecting the Expression of Carcinoembryonic Antigen at the Surface of Cultured Human Colon Carcinoma Cells. *Cancer Res.* 40:4744-4750.

230. Rowlinson, G., F. Balkwill, D. Snook, G. Hooker, and A. A. Epenetos. 1986. Enhancement by gamma-interferon of in vivo tumor radiolocalization by a monoclonal antibody against HLA-DR antigen. *Cancer Res.* 46:6413-6417.

231. Sachs, L. 1987. The Molecular Control of Blood Cell Development. *Science* 238:1374-1379.

232. Sachs, L. 1987. Cell Differentiation and Bypassing of Genetic Defects in the Suppression of Malignancy. *Cancer Res.* 47:1981-1986.

233. Saini, K., G. Steele, and P. Thomas. 1990. Induction of carcinoembryonic-antigen-gene expression in human colorectal carcinoma by sodium butyrate. *Biochemical Journal* 272:541-544.

234. Saito, H., T. Morizane, T. Watanabe, T. Kagawa, S. Miyaguchi, N. Kumagai, and M. Tsuchiya. 1991. Differentiating Effect of Sodium Butyrate on Human Hepatoma Cell Lines PLC/PRF/5, HCC-M and HCC-T. *International Journal of Cancer* 48:291-299.

235. Sasaki, M., M. Okamoto, C. Sato, K. Sugio, T. Iwama, T. Ikeuchi, A. Tonomura, M. Miyaki, and T. Sasazuki. 1989. Loss of Constitutional Heterozygosity in Colorectal Tumours from Patients with Familial Polyposis and those with Nonpolyposis Colorectal Carcinoma. *Cancer Res.* 49:4402-4406.

236. Senter, P. D., M. G. Saulnier, G. J. Schreiber, D. L. Hirschberg, J. P. Brown, I. Hellstrom, and K. E. Hellstrom. 1988. Anti-tumour Effects of Antibody-Alkaline Phosphatase Conjugates in Combination with Etoposide Phosphate. *proceedings of the National Academy of Sciences of the USA* 85:4842-4846.

237. Shapiro, H. M. 1983. Multistation Multiparameter Flow Cytometry: A Critical Review and Rationale. *Cytometry* 3:227-243.

238. Sharkey, R. M., D. M. Goldenberg, S. Murthy, H. Pinsky, R. Vagg, D. Pawlyk, J. A. Siegel, G. Y. Wong, P. Gascon, D. O. Izon, M. Vezza, K. Burger, L. Swayne, C. M. Pinsky, and H. J. Hansen. 1993. Clinical Evaluation of Tumour Targetting with a High-affinity Anticarcinoembryonic-Antigen-Specific Murine Monoclonal Antibody,MN-14. *Cancer* 71:2082-2096.

239. Shi, Z. R., D. Tsao, and Y. S. Kim. 1983. Subcellular Distribution, Synthesis and Release of Carcinoembryonic Antigen in Cultured Human Colon Adenocarcinoma Cell Lines. *Cancer Res.* 43:4045-4049.

240. Shin, D. M., W. N. Hittelman, and W. K. Hong. 1994. Biomarkers in upper aerodigestive tract tumorigenesis: a review. [Review] [197 refs]. *Cancer Epidemiology, Biomarkers & Prevention* 3:697-709.

241. Siccardi, A. G., G. L. Buraggi, L. Callegaro, A. C. Colella, P. G. De Filippi, G. Galli, G. Mariani, R. Masi, R. Palumbo, P. Riva, and et al. 1989. Immunoscintigraphy of adenocarcinomas by means of radiolabeled F(ab')2 fragments of an anti-carcinoembryonic antigen monoclonal antibody: a multicenter study. *Cancer Res.* 49:3095-3103.

242. Sickle-Santanello, R., P. J. O'Dwyer, C. Mojsizik, S. E. Tuttle, G. H. Hinkle, M. Rousseau, J. Schlom, D. Colcher, M. O. Thurston, C. Nieroda, A. Sardi, W. B. Farrar, J. P. Minton, and E. W. J. Martin. 1987. Radioimmunoguided Surgery Using the Monoclonal Antibody B72.3 in Colorectal Tumours. *Diseases of the Colon and Rectum* 30:761-764.

243. Siddiqui, B. and Y. S. Kim. 1984. Effects of sodium butyrate, dimethyl sulfoxide, and retinoic acid on glycolipids of human rectal adenocarcinoma cells. *Cancer Res.* 44:1648-1652.

244. Silverberg, E., C. C. Boring, and P. H. Sugarbaker. 1990. Cancer Statistics. *CA-Cancer* 40:9-16.

245. Solomon, E., R. Voss, V. Hall, W. F. Bodmer, J. R. Jass, F. C. Lucibello, I. Patel, and S. H. Rider. 1987. Chromosome 5 Allele Loss in Human Colorectal Carcinomas. *Nature* 328:616-619.

246. Sorokin, J. J., P. H. Sugarbaker, N. Zamcheck, M. Pisick, H. Z. Zupchick, and F. D. Moore. 1974. Serial Carcinoembryonic Antigen Assays; Use in Detection of Cancer Recurrence. *Journal of the American Medical Association* 228:49-53.

247. Spar, I. L., W. F. Bale, D. Marrack, W. C. Dewey, R. J. McCardle, and P. V. Harper. 1967. 131-Iodine Labelled Antibodies to Human Fibrinogen. *Cancer* 20:865-870.

248. Staab, H. J., F. A. Anderer, T. Brummendorf, E. Stumpf, and R. Fischer. 1981. Prognostic Value of Preoperative Serum CEA Level Compared To Clinical Staging. *British Journal of Cancer* 44:652-662.

249. Staab, H. J., F. A. Anderer, E. Stumpf, and R. Fischer. 1978. Slope Analysis of the Postoperative CEA Time Course and Its Possible Application as an Aid in Diagnosis of Disease Progression in Gastrointestinal Cancer. *The American Journal of Surgery* 136:322-327.

250. Steinman, R. A., B. Hoffman, A. Iro, C. Guillouf, D. A. Liebermann, el-Houseini, and ME. 1994. Induction of p21 (WAF-1/CIP1) during differentiation. *Oncogene* 9:3389-3396.

251. Sugarbaker, P. H., F. J. Gianola, A. Dwyer, and N. R. Neuman. 1987. A Simplified Plan for Follow-up of Patients with Colon and Rectal Cancer Supported by Prospective Studies of Laboratory and Radiologic Test Results. *Surgery* 102:79-87.

252. Takahashi, H., Y. Okai, R. J. Paxton, L. J. Hefta, and J. E. Shively. 1993. Differential regulation of carcinoembryonic antigen and biliary glycoprotein by gamma-interferon. *Cancer Res.* 53:1612-1619.

253. Thompson, D. M. P., J. Krupey, S. O. Freedman, and P. Gold. 1969. The Radioimmunoassay of circulating carcinoembryonic antigen of the human digestive system. *proceedings of the National Academy of Sciences of the USA* 64:161-167.

254. Tjandra, J. J., G. A. Pietersz, J. G. Teh, A. M. Cuthbertson, J. R. Sullivan, C. Penfold, and I. F. C. McKenzie. 1989. Phase I Clinical Trial of Drug-Monoclonal Antibody Conjugates in Patients with Advanced Colorectal Carcinoma: A Preliminary Report. *Surgery* 106:533-546.

255. Toribara, N. W., T. L. Sack, J. R. Gum, S. B. Ho, J. E. Shively, J. K. Willson, and Y. S. Kim. 1989. Heterogeneity in the induction and expression of carcinoembryonic antigen-related antigens in human colon cancer cell lines. *Cancer Res.* 49:3321-3327.

256. Torosian, M. H. 1988. The Clinical Usefulness and Limitations of Tumour Markers. *Surgery, Gynaecology and Obstetrics* 166:567-576.

257. Trier, J. S. 1962. Morphological Alterations Induced By Methotrexate in the Mucose of Human Proximal Intestine. *Gastroenterology* 42:295-304.

258. Tsao, D., A. Morita, A. Bella,Jr., P. Luu, and Y. S. Kim. 1982. Differential effects of sodium butyrate, dimethyl sulfoxide, and retinoic acid on membrane-associated antigen, enzymes, and glycoproteins of human rectal adenocarcinoma cells. *Cancer Res.* 42:1052-1058.

259. Ullmann, C. D., J. Schlom, and J. W. Greiner. 1992. Interleukin-6 Increases Carcinoembryonic Antigen and Histocompatibility Leukocyte Antigen Expression on the Surface of Human Colorectal Carcinoma Cells. *Journal of*

Immunotherapy 12:231-241.

260. Umpleby, H. C., J. B. Bristol, J. B. Rainey, R. C. N. Williamson, and M. Chir. 1984. Survival of 727 Patients withSingle Carcinomas of the Large Bowel. *Diseases of the Colon and Rectum* 27:803-810.

261. Updyke, T. V. and G. L. Nicholson. 1986. Malignant Melanoma Cell Lines Selected in Vitro for Increased Homotypic Adhesion Properties have Increased Experimental Metastatic potential. *Clin. Exp. Met.* 4:273-284.

262. Vogelstein, B., E. R. Fearon, S. R. Hamilton, S. E. Kern, A. C. Preisinger, Y. Nakamura, R. White, A. M. M. Smits, and J. L. Bos. 1988. Genetic Alterations During Colorectal -Tumour Development. *The New England Journal of Medicine* 319:525-532.

263. Waldman, T., K. W. Kinzler, and B. Vogelstein. 1995. p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res.* 55:5187-5190.

264. Wanebo, H. J., B. Rao, C. M. Pinsky, R. G. Hoffman, M. Stearns, M. K. Schwartz, and H. F. Oettgen. 1978. Preoperative Carcinoembryonic Antigen Level as a Prognostic Indicator in Colorectal Cancer. *The New England Journal of Medicine* 299:448-451.

265. Weinberg, R. A. 1985. The Action of Oncogenes in the Cytoplasm and Nucleus. *Science* 230:770-776.

266. Weinert, T. and D. Lydall. 1993. Cell cycle checkpoints, genetic instability and cancer. [Review] [86 refs]. *Seminars in Cancer Biology* 4:129-140.

267. Wiernik, G. 1966. In-vivo Cell Kinetics of a Normal Human Tissue. *British Medical Journal* 2:385-387.

268. Williams, N. S., J. R. Jass, and J. D. Hardcastle. 1988. Clinicopathological assessment and staging of colorectal cancer. *British Journal of Surgery* 75:649-652.

269. Wittig, B. M., A. Hach, K. Hahn, K. H. Meyer zum Buschenfelde, and W. G. Dippold. 1993. CEA-immunoscintigraphy with 99m-technetium correlates with tumour cell differentiation in colorectal cancer. *European Journal of Cancer* 29A:1327-1329.

270. WOlf, B. C., R. R. Salem, H. F. Sears, D. A. Horst, P. T. Lavin, M. Herlyn, S. H. Itzkowitz, J. Schlom, and G. D. J. Steele. 1989. The Expression of Colorectal Carcinom-Associated Antigens in the Normal Colonic Mucosa. *American Journal of Pathology* 135:111-119.

271. Wong, J. Y. C. 1989. The Effects of Hyperthermia on Tumour Carcinoembryonic Antigens Expression. *International Journal of Radiation Oncology, Biology,Physics.* 17:803-808.

272. Woolf, N. 1986. Cell Proliferation and Neoplasia. In Cell Tissue and Disease. AnonymousBailliere Tindall, London. 361

273. Yan, X., J. Y. C. Wong, J. M. Esteban, J. Kuhn, B. G. Beatty, J. D. Beatty, and J. E. Shively. 1992. Effects of Recombinant Human gamma-Interferon on Carcinoembryonic Antigen Expression of Human colon Cancer Cells. *Journal of Immunotherapy* 11:77-84.

274. Yan, X. W., J. Y. C. Wong, J. M. Esteban, J. A. Kuhn, B. G. Beatty, J. D. Beatty, and J. E. Shively. 1991. Enhancement of Carcinoembryonic Antigen Expression on Human Colon Cancer Cells by Interferon and Hyperthermia. *Antibody Immun. Radiopharm.* 4:58

275. Zamcheck, N. 1974. Carcinoembryonic Antigen; Quantitative Variations in Circulating Levels in Benign and Malignant Digestive Tract Diseases. *Advances in Inter. Medicine* 19:413-433.

276. Zamcheck, N., W. G. Doos, R. Prudente, B. B. Lurie, and L. S. Gottlieb. 1997. Prognostic Factors in Colon Carcinoma; Correlation of Serum Carcinoembryonic Antigen Level and Tumour Histopathology. *Human Pathology* 6:31-45.

277. Ziegler-Heitbrock, H. W., R. Munker, J. Johnson, I. Petersmann, C. Schmoeckel, and G. Riethmuller. 1985. In vitro differentiation of human melanoma cells analyzed with monoclonal an bodies. *Cancer Res.* 45:1344-1350.

278. Zimmerman, W., R. Ortlieb, R. Friedrich, and S. von Kleist. 1987. Isolation and Characterisation of cDNA Clones Encoding the Human Carcinoembryonic Antigen Reveal a Highly Conerved Repeating Structure. *proceedings of the National Academy of*

Sciences of the USA 84:2960-2964.

279. Zupchik, H. Z., N. Zamcheck, and C. A. Saravis. 1973. Immunochemical Studies of Carcinoembryonic Antigens: Methodologic Considerations and Some Clinical Implications. *Journal of the National Cancer Institutes* 51:1741-1749.

280. Zweibaum, A. 1991. Use of Cultured Cell Lines in Studies of Intstinal Cell Differentiation and Function. In The Gastrointestinal System. S. Schultz, editor. American Physiology Society, 223-255.